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Ondřej Polák, Radim Cerkal, Natálie Březinová Belcredi, Pavel Horký, Patrik Vacek

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November 9 and 10, 2016
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PREFACE

This year´s 23rd International PhD Students Conference for undergraduate and postgraduate students is hosted by **the Faculty of AgriSciences**, Mendel University in Brno, the Czech Republic, in November 9–10, 2016. The conference has provided a platform to discuss new trends in plant and animal production, fisheries and hydrobiology, agroecology and rural development, food technology, plant and animal biology, techniques and technology, applied chemistry and biochemistry etc. with participants from European educational and research institutions.

Their success is reflected in the papers received, with participants coming from diverse backgrounds, allowing a real multinational and multicultural exchange of experiences and ideas.

The accepted papers of this conference are published in this full text that will be sent to international indexes.

Conferences such these can only succeed as a team effort, so the Editors want to thank the Committees and the Reviewers for their excellent work in reviewing the papers as well as their invaluable input and advice.

The Editors

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Section – Plant Production

MINERAL NITROGEN CONTENT IN THE SOIL AFTER DIFFERENT FERTILIZATION IN ORGANIC FARMING

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Abstract: In organic farming is not possible to rely on plants fertilizing during vegetation according to their actual needs. The right crop rotation and harmonic nutrition are necessary for good and quality products. This is realized mainly by cultivating green manure crop and fertilizing by organic fertilizers. The goal of this long-term experiment is to evaluate the effect of different intensity and fertilization in organic farming with and without breeding livestock on content of mineral nitrogen in soil. There are four variants in this experiment: 1. Unfertilized control, 2. Green manure, 3. Green manure + renewable external sources (27 t/ha of compost + 14 t/ha of digestate), 4. Green manure + farm fertilizers (27 t/ha of manure + 14 t/ha of liquid manure). The result obtained from the experimental year 2016 show, that the best variant providing good supply of nitrogen during vegetation is combination with green manure and renewable external resources (compost + digestate). Soil in this variant contained around 83 kg/ha of mineral nitrogen after emergence of potatoes. Variant with green manure and farm fertilizers contained 77 kg/ha and unfertilized soil contained around 46 kg/ha of mineral N. Content of mineral nitrogen in the flowering, around 55 kg/ha, was also the highest on variant 3. The remaining variants had a similar nitrogen content in the soil, around 44 kg/ha.

Key Words: organic farming, nitrogen, soil, green manure, farm fertilizers

INTRODUCTION

Organic agriculture, a production system with a particular attention to the environment and its individual components, is now a well-known concept among lots of people. Environmental protection is possible due to the restriction or prohibition of the use of certain burdensome substances, especially synthetic nitrogen fertilizers. However, content of nutrients from agro-ecosystem even in organic farming is decreasing because of production export and nutrient losses like leaching or volatilization. The precursor for higher yield and quality of products is good and fertile soil (Dvorský and Urban 2014). Organic farming, in comparison with conventional farming methods, cannot count on the fact, that plants can be fertilized directly to the roots according to actual needs in vegetation. The point of emphasis in organic farming is content of organic matter and quality of humus in the soil (Martin and MacRae 2014).

Today, the crucial nutrient is still nitrogen. However, balance of nitrogen in organic farming is relatively well solvable. The basis of nutrition in organic farming should be a good crop rotation (Urban et al. 2003). The supply of nitrogen from external environment is achieved primarily by growing legumes and plants for green manure. Another invaluable source of nutrients is organic fertilizers, especially manure and slurry, but also organic compost and in these days increasingly used digestate. The combination of well-chosen crop rotation with adequate dose of properly selected organic fertilizer is very interesting and for organic farming have irreplaceable role (Barker 2010).

The ultimate goal of this long-term experiment is to evaluate the effect of different intensity and fertilization in organic farming with and without breeding livestock on yield and quality of products, soil properties and nutrient balance. However, in this work, only the content of mineral nitrogen in soil in different vegetation stages of potatoes growth in year 2016 will be evaluated. There are also interesting questions that may be answered – is green manure alone good enough for good nitrogen supply or is the combination with some organic matter necessary? Will combination with green manure and renewable external source provide enough content of nitrogen in comparison with farm fertilizers?

MATERIAL AND METHODS

This work is a part of a long-term experiment established in 2014 by the Central Institute for Supervising and Testing in Agriculture. The experiment was established as small-plot field experiment ongoing at five different locations at the same time. These experimental stations are representing a different production area with a different soil and climatic conditions (Table 1). The variant of fertilization are described in Table 2. The experiment attempts to compare system of fertilization with (Variant 4) and without livestock (Variant 3) in organic farming. There is also an unfertilized variant (Variant 1) for control and variant based only on green manure (Variant 2). Every variant has three repetitions.

Before the start of the experiment, the land at every experimental station was left fallow in year 2014. The forecrop for potatoes was winter wheat in 2015. The combination of *Pisum sativum var. arvense*, *Vicia villosa* and *Brassica campestris var. sylvestris* was used as a green manure.

Potato fertilization in experimental year of 2016 is described in Table 2 and nitrogen content in fertilizers in Table 3. Potatoes planting were performed approximately 14 days after the incorporation of organic farming to the soil in early April. There is one average soil sample for each variant at every location. For this average sample, soil was collected from every repetition in every variant. The soil was collected before start of the experiment, after emergence and in flowering. The soil was collected by a manual soil probe in soil profile 0–30 cm. The nitrate nitrogen was determined by ion-selective electrode (ISE), the ammonia nitrogen was determined as Indolphenol spectrophotometry (Zbíral et al. 2004). Content of mineral nitrogen was determined by summing nitrate and ammonia nitrogen.

Table 1 Characteristics of experimental stations

Experimental station (district)	Characteristics					
	Main crop	MASL	Soil type	Soil texture	Average annual precipitation (mm)	Average annual temperature (°C)
Čáslav	Sugar beet	260	Black soil	Clay	555	8.9
Horažďovice	Potatoes	475	Cambisol	Sandy loam	585	7.8
Jaroměřice nad Rokytnou	Cereals	425	Brown soil	Clay loam	481	8.0
Lípa	Potatoes	505	Cambisol	Sandy loam	594	7.5
Věrovany	Sugar beet	207	Black soil	Clay	52	8.7

Table 2 Variants of fertilization used in the experiment

Variant	Application of organic fertilizers			
	Dose of fertilizer	Period	Dose of fertilizer	Period
1. Unfertilized	-	-	-	-
2. Green manure (GM)	-	-	-	-
3. GM + renewable external sources	27 t/ha of compost	Autumn	14 t/ha of digestate	April
4. GM + farm fertilizers	27 t/ha of manure	Autumn	14 t/ha of liquid manure	April

Table 3 Nitrogen content in organic fertilizers in original state (same fertilizers for every location)

Variant	Organic fertilizer	Nitrogen content in original state (%)
Renewable external sources	Compost	0.67
	Digestate	1.37
Farm fertilizers	Manure	0.43
	Liquid manure	0.06

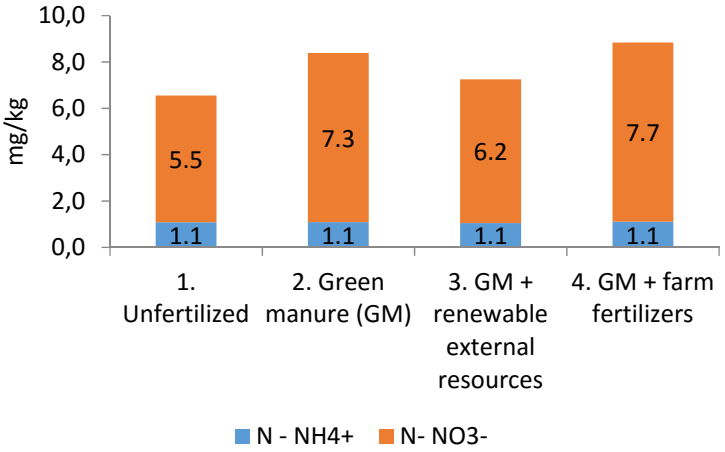
RESULTS AND DISCUSSION

Mineral nitrogen content before start of the experiment in early April

The initial state of the mineral nitrogen content in the soil before starting the experiment with potatoes is displayed in Figure 1. The lowest content of mineral nitrogen was located in unfertilized soil as expected. Most mineral nitrogen contained the soil on variant 4 after autumn application of manure. The nitrogen content on this variant was increased by 2.2 mg/kg compared to control variant. The current soil supply after recalculation (mg/kg x 4.5) makes nearly 40 kg of nitrogen per hectare on variant 4.

A similar amount of N, around 38 kg/ha, was contained in soil in variant 2 with just green manure crop. This result indicates a quick decomposing of green manure crop (Barker 2010). Green manure that decomposes rapidly is an excellent source of immediately available nutrients but is unlikely to contribute significantly to long-term development of soil organic matter (Martin and MacRae 2014). Content of mineral nitrogen in soil after turning over just green manure is therefore almost comparable with variant 4, where the mineralization does not fully starts at this period.

Figure 1 Average content of mineral nitrogen in soil before start of the experiment in early April



Legend: renewable external resources– compost + digestate; farm fertilizers – manure + liquid manure

There is an idea, that compost application is more efficient method compared to manure, because compost contained more concentrated nutrients due to significantly less C, less water and reduced potential for N loss through volatilization in time of application (Miller et al. 2009, Larney et al. 2006). In this experiment, however, a smaller decrease of mineral nitrogen content appeared on variant 3 following autumn application of compost. This may be caused by not entirely good C/N ratio in compost, which is quite common thing. A commonly used threshold suggests that amendments with C/N ratios less than 20 will cause net mineralization (Loecke et al. 2012). The ratio of compost in this case was 9 : 1, therefore a very thin ratio. This leads to very quick decomposition (Gale et al. 2006) and probably to loss of some nitrogen due leeching during winter.

There is also a different idea, that autumn application of compost does not have same effect on mineral nitrogen content in spring compared to application of quality manure (Hradil et al. 2007). This corresponds with result of soil analysis in April in this experiment.

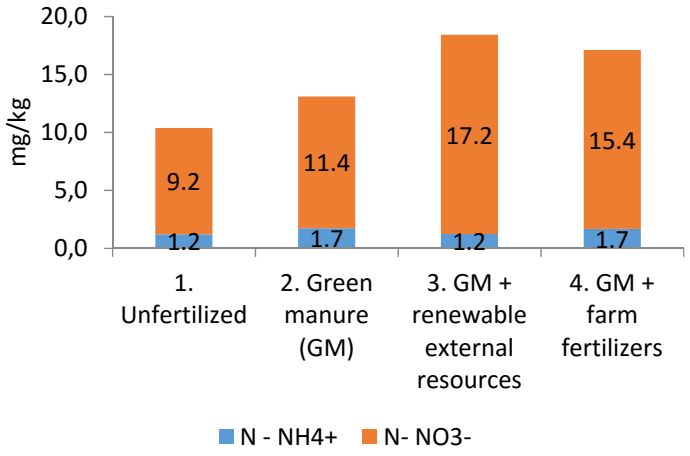
On the other hand, some authors (Gale et al. 2006, Sanchez et al. 2004) have observed immobilization of nitrogen for 60 or more days following incorporation of compost. They have also claim, that compost is therefore a generally contributing to the slow-release pool of nitrogen and other nutrients during vegetation. This idea is supported by result of soil analysis after emergence and in flowering (Figure 2 and 3).

Mineral nitrogen content after emergence in early June

State of mineral nitrogen content in soil after fertilization in the experimental year 2016 is reflected in figure 2. Soil samples were collected after emergence of potatoes around 6 June. At this point, mineralization and nitrification in soil should be slightly after spring maximum.

The largest content of nitrogen, around 83 kg/ha, was noticed in the combination of green manure and renewable external sources. This result support the idea of some authors, that digestate has higher total nitrogen content ranging from 0.2–1% compared to other organic fertilizers (Smatanová 2012). This fact is also evident from Table 3. Nitrogen content in applied digestate was higher compared to liquid manure. Digestate also have a large proportion of highly usable ammonia nitrogen (Moller and Muller 2012). This corresponds with low content of ammonia nitrogen in soil in the experiment.

Figure 2 Average content of mineral nitrogen in soil after emergence in early June



Legend: renewable external resources– compost + digestate; farm fertilizers – manure + liquid manure

Average nitrogen content in liquid manure is around 0.23%, but content of N in liquid manure used in this experiment was only 0.06%. The content is highly variable and dependent on the strong dilution with water. 90% of nitrogen in liquid manure is located in easily soluble form with the largest share of ammonia nitrogen (Urban et al. 2003). Application of liquid manure is also problematic due volatilization loss of ammonia (Mahimairaja et al. 1994). This also corresponds with low content of ammonia nitrogen in soil in the experiment. A smaller decrease of nitrogen content on variant 4, around 77 kg/ha, may be explained by all these facts.

Green manure is used in order to increase the content of rapidly decomposing organic matter, to promote fixation of atmospheric nitrogen and to increase the activity of microorganism in soil. The result obtained from the soil analysis in this stage of vegetation show, that mineral nitrogen content after turning under green manure crop alone (around 59 kg/ha) is not as good as combination of green manure and organic fertilizer. Green manure crop is ideal as a main crop on the field. This should be done every three years on the field for best result (Barker 2010). But this situation is rather impossible in common praxis. Green manure is often grown as a winter cover crop.

Green manure crop is not able to create enough organic matter during this short period (from autumn to early spring). As mentioned before, such green manure crop decomposes rapidly due their succulence. It is great on the start for early stages of vegetation, but may not provide the required supply of nitrogen at later stage of vegetation. This is also evident from Figure 2 and 3. Therefore, it is useful to combine green manure with further applications of organic matter, if possible (Kasal et al. 2010).

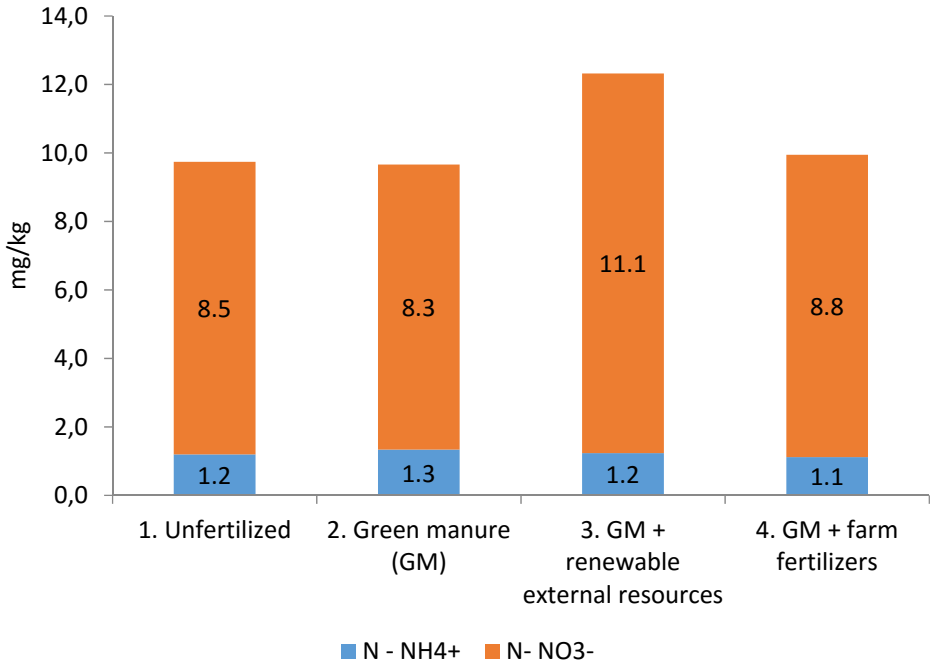
Mineral nitrogen content in flowering in early July

Higher uptake of nitrogen by potatoes is starting before crop cover is complete. The highest uptake starts in flowering (Kasal et al. 2010). Soil samples were collected at the begging of flowering around 1 July. The plants depleted a part of available nitrogen at this stage, which is evident from comparing Figure 3 and Figure 2.

The largest content of nitrogen in soil, around 55 kg/ha, remains after combination of green manure and renewable external resources. This variant had contained more nitrogen in applied fertilizers (compost + digestate) compared to variant 4 with farm fertilizers. As mentioned before, this result is supporting work of some authors (Miller et al. 2009, Larney et al. 2006) that compost is generally contributing to the slow-release pool of nitrogen and other nutrients during vegetation. On the other hand, the result from 9-year experiment by Miller et al. (2010) is quite opposite. In this experiment, the N availability of different fertilizers was also evaluated. Fresh manure increased the soil level of nitrogen the most in this experiment.

Variants 2 and 4 in this experiment with average content of nitrogen around 44 kg/ha are comparable, even with unfertilized variant. However, we can assume that nitrogen consumed by plants on variant with fertilization will result in higher yields. This hypothesis will be of course analysed right after harvest.

Figure 3 Average content of mineral nitrogen in soil in flowering in early July



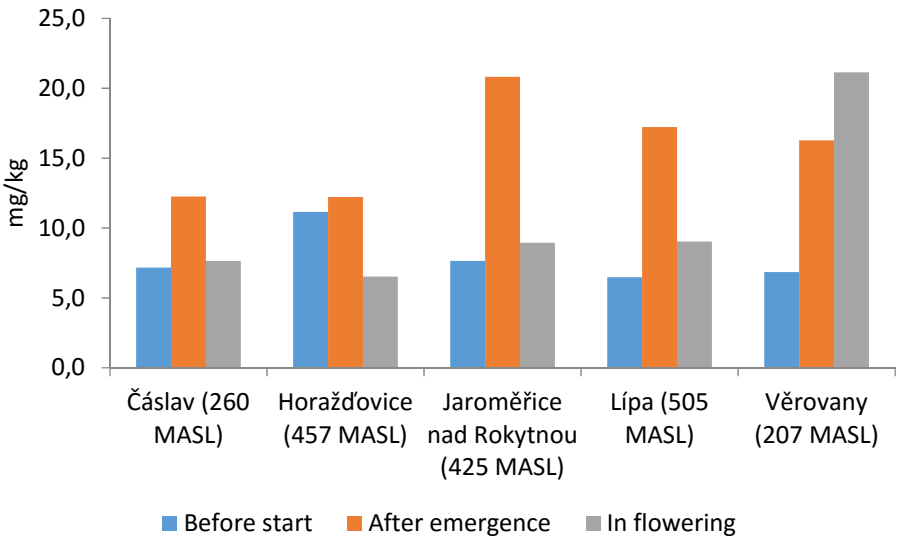
Legend: renewable external resources– compost + digestate; farm fertilizers – manure + liquid manure

Average content of mineral N in each experimental station at different stage of the experiment

As mentioned before, the experiment is ongoing on five different locations. The average content of nitrogen for each experimental station in different stage of vegetation is displayed in Figure 4. Content of nitrogen before start of the experiment is comparable on each location. Experimental station Jaroměřice nad Rokytnou had higher nitrogen content in soil after emergence and in flowering. There is a fertile brown soil at this station.

There is also a good combination of average annual precipitation and temperature as you can see in Table 2. The very fertile black soil is at station Věrovany, where the content of nitrogen was the highest in flowering. At this station were lower precipitation during early spring, therefore nitrogen content was not so high after emergence. The lowest content of nitrogen was observed at exp. station Čáslav, although there is a fertile black soil at this locality. Not enough precipitation and mostly drought caused a lower content of nitrogen at this station.

Figure 4 Average content of nitrogen in soil in each experimental station



CONCLUSION

The results obtained from soil analysis during the experiment shows that any application of organic matter either from renewable external resources or farm fertilizers is increasing mineral nitrogen content in the soil compared to unfertilized variant. However, in organic farming is not possible to rely on crop fertilizing during vegetation according current needs. Application of any organic matter has therefore a crucial role for plants.

Green manure in practice is often cultivated as a winter or stubble crop with a short growing period. Turning under such green manure alone do not provide enough organic matter and decomposes rapidly. Nearly 75% of such organic matter is rotted quickly in first season, leaving little residual effect afterward. There is also option to apply only organic fertilizer, but such option may also be not so tempting. We are losing erosion protection and competition against weeds and diseases in this case. Combination with green manure crop and organic fertilizer may be also more economic due possibility of lowering doses of organic fertilizer.

The result from this experiment show, that variant with combination of green manure and renewable external resources provide more supply of nitrogen compared to variant with green manure alone and variant with combination of green manure and farm fertilizers. In this variant with renewable external resources, compost and digestate were applied. These fertilizers had higher content of nitrogen in original state. There is also an idea, that compost provides more nutrients for plants in first year after incorporation compared to manure, which is supported by result of this experiment.

Every organic fertilizers is of course dependent of their quality, but result from this experiment is indicating, that farming without livestock can be as effective or even better as production with livestock. The influence of these combinations on content of mineral nitrogen in soil and also influence on yield needs to be of course further tested in this long term experiment.

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THE VARIABILITY OF CARAWAY (*CARUM CARVI* L.) ESSENTIAL OILS

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Abstract: The aim of this work was to highlight the great variability in the contained substances of caraway (*Carum carvi* L.) samples obtained from different types of cultivation and harvest years. The experiment included a total of 145 samples. At first, the isolation of essential oils by the means of steam distillation was performed, followed by the analysis of individual components using gas chromatography, where the main components carvone and limonene were determined. The presented results indicate that the coefficient of correlation between the essential oil content and the carvone content is relatively low statistically highly significant positive correlation (r = 0.32**) and for limonene there is even lower negative statistically highly significant correlation (r = -0.23**), suggesting a strong variability among individual samples regardless of their harvest year and origin.

Key Words: steam distillation, carvone, limonene, correlation, achenes

INTRODUCTION

The essential oils and other secondary plant metabolites have been used from the dawn of time in a wide range of industries from culinary to phytotherapy. One such specie rich with the essential oil is caraway (*Carum carvi* L.) (Kwiatkowski et al. 2015). It is a crop plant from the family *Apiaceae*, which has long tradition of growing in the Czech Republic a nowadays is still a highly demanded cash crop. Caraway is considered to be one of the oldest spice grown in Europe (Aćimović et al. 2015). According to the Situation and perspective reports (2014), caraway was cultivated in the area of 2.173 hectares with an average yield of 0.92 t/ha. The proof of the great importance of caraway cultivation and production in our country is the fact that caraway was awarded the Protected Designation of Origin "CZECH CARAWAY" according to Council Regulation (EC) no. 510/2006 on the protection of geographical indications and designations of origin for agricultural products and foodstuffs. The conditions to be fulfilled for obtaining this mark are following: definition of the growing area in the Czech Republic, the use of seeds of registered varieties of a biennial form and quality of achenes specified by the content of the essential oil, which is appointed to a minimum of 2.8% (Přibylová 2014).

Both biennial and perennial caraway can be found in fields in the Czech Republic. The amount and composition of the essential oil in the achenes is different according to the selected type of caraway. András et al. (2015) states that the essential oil content in the one-year varieties is about 2.5%, the essential oil content in biennial varieties can in exceptional cases even exceed 7%. However, the essential oil is not only contained in the achenes, it can be found in the whole plant, but only in a minimal amount (Aćimović et al. 2012).

The caraway essential oil is characterised as a clear, colourless liquid with a pleasant aroma and spicy flavor. It consists of a broad spectrum of components (up to 30), wherein the main components and the carrier of odour are carvone (50–80%) and limonene (up to 50%). Carvone and limonene create about 95% of the total essential oil content and the remaining ingredients are present only in trace

amounts. Terpenes, such as carvacrol, α-pinene, γ-terpinene, linalool, carvenone and p-cymene belong to these components (Seidler-Łożykowska et al. 2013, Agrahari and Singh 2014, Aćimović et al. 2015, Sachan et al. 2016). Both the use and effects of caraway essential oil and its quality depends mainly on the content of carvone or the ratio of carvone and limonene (Solberg et al. 2016). During maturation, the ratio of limonene and carvone is changing, there is an increase of carvone proportion and decrease of the limonene percentage. The achenes contain not only the essential oil but also oil (10–18%), proteins (20%), saccharides, flavonoids, beta carotene and minerals - calcium, potassium, magnesium and phosphorus (Raghavan 2007, Kozera et al. 2013).

The use of caraway is versatile, both of its achenes and the whole plant. Achenes are mainly used in gastronomy as the traditional spice and herbal medicine. Whole plants are treated as "green spice" in the culinary arts and are effective also as a natural plant protection, because their scent obscures adjacent cultivated plants that can have scents very alluring for pests and simultaneously it attracts predatory insects to these flowers (Agrahari and Singh 2014). Furthermore, the caraway extract, especially some of its ingredients, can be used as organic plant protection of stored vegetables against pests (András et al. 2015).

From the phytoterapeutic point of view it can be said that the caraway essential oil contains a wide spectrum of bioactive substances with carminative, anti-hypertensive, anti-diabetic, anti-cancer, antioxidant, analgesic and diuretic effects that promote digestion and appetite, soothe the bronchi and encourage the production of breast milk and lactation (András et al. 2015, Sachan et al. 2016). The caraway essential oil is also known to have medium antimicrobial activity and thus preventing the growth of many bacteria and fungi, including *Staphylococcus aureus, Escherichia coli, Salmonella typhi, Vibrio cholerae, Mycobacterium tuberculosis* (Seidler-Łożykowska et al. 2013).

MATERIAL AND METHODS

Plant material

Caraway samples were obtained from the plant breeding material from the company Agritec Plant Research Ltd. Šumperk (Czech Republic).

Determination of the essential oil content in caraway achenes

Isolation and determination of the essential oil in herbal drug was performed by the means of steam distillation using a distillation apparatus according to the Pharmacopea Bohemica (2009). Samples were ground using a laboratory mill and 2 × 10 g of ground caraway was weighed for the determination with n = 2. Each dose of the sample was immediately transferred into a round bottom flask and 200 ml of distilled water was added to the sample. Boiling chips were added to each round bottom flask to prevent hidden boiling. The flasks were placed in a heating mantle and connected to the distillation apparatus for essential oil distillation using ground joints. The distillation was carried out for 90 minutes at a speed of 2–3 mL per 60 seconds. The density of the caraway essential oil is 0.9090 g/cm^3.

Determination of compounds contained in the essential oil

The analysis of carvone and limonene in the caraway essential oil was carried out by the means of the gas chromatograph HP 4890D (Hewlett Packard) with a flame ionisation detector (GC-FID). The separation was performed using the HP-5MS column (5% diphenyl–95% dimethylpolysiloxane, 30 m × 0.25 mm, i.d. 0.25 μm). Following temperature program was used for the analysis: T1 = 100 °C, t1 = 0 min, 15 °C/min to T2 = 280 °C, t2 = 0 min. The injector temperature was 270 °C, the detector temperature 290 °C. 1 μl of the essential oil solution diluted in hexane (to the concentration 10 μl/ml) was injected into the column. The splitter ratio was set to 40:1. The flow-rate of the He carrier gas was 1 ml/min. For the calibration, five calibration solutions were prepared in the rate of concentrations 0.1–20 μl/ml of carvone and limonene. Resulting chromatograms were processed by the means of the CSW workstation (version 1.7, Data Apex, Praha).

Processing of results

The resulting values of the content and composition of the essential oils have been converted to 100% of caraway achenes dry weight. The moisture content was determined by drying 2 g

of samples at 130 °C for 120 min. For the evaluation of the results, statistical program STATISTICA (data analysis software system) by StatSoft, Inc. (2013), ver. 12 was used. The correlations between the characteristics were calculated as Pearson correlation coefficient (r). The results are presented in both numerical and graphic form in the paper.

RESULTS AND DISCUSSION

Table 1 The correlation coefficients of the relation between the essential oil content and investigated essential oil components in caraway achenes (n = 290)

	Average values	Standard deviation	Essential oil content [ml]	Carvone [mg/ml]	Limonene [mg/ml]
Limonene [mg/ml]	422.06	78.72	-0.23**	-0.66**	1.00
Carvone [mg/ml]	506.05	91.04	0.32**	1.00	
Essential oil volume [ml]	0.33	0.10	1.00		

*Legend: * - p = 0.05; ** - p = 0.01*

Mutual dependence of parameters and characteristics is referred to as a correlation. Correlation coefficient expresses the degree of closeness of relation between the essential oil content and its main components, carvone and limonene. For the 145 samples of caraway, analysed in two repetitions, a statistically highly significant negative relationship was found between the total content of the essential oil and the content of essential oil component limonene (r = -0.23**) (see Table 1). In contrast, a statistically highly significant positive relationship was found between the total content of the essential oil and the content of essential oil component carvone (r = 0.32**) (see Table 1). A highly significant negative relationship was also found between the content of investigated components of essential oils, carvone and limonene (r = -0.66**) (see Table 1). Thus, with the increasing total essential oil content, the amount of limonene component decreases and the amount of carvone component rises. The value of the correlation coefficient and the points in the figures show that the dependence can be influenced by many external factors (see Figure 1 and Figure 2). Since the determination of total essential oil content and consequently the analysis of essential oil composition was performed in various samples which came from different years of harvest (2009–2015), the relation of the total content of the essential oil and their major components was very likely affected also by the weather conditions during the harvest year and by storage time and conditions.

Figure 1 The relationship between the total essential oil content and the ratio of the carvone component in caraway achenes

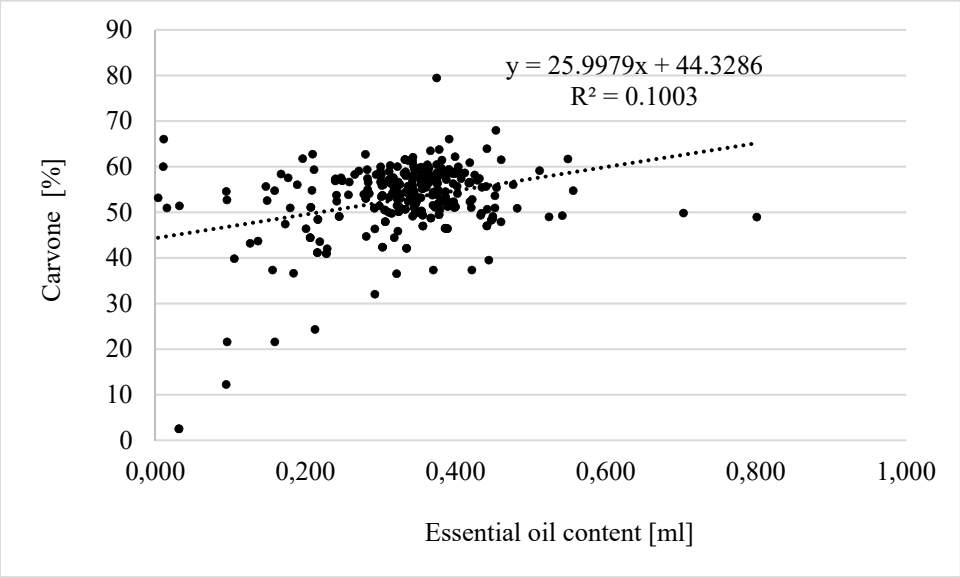

Figure 2 The relationship between the total essential oil content and the ratio of the limonene component in caraway achenes

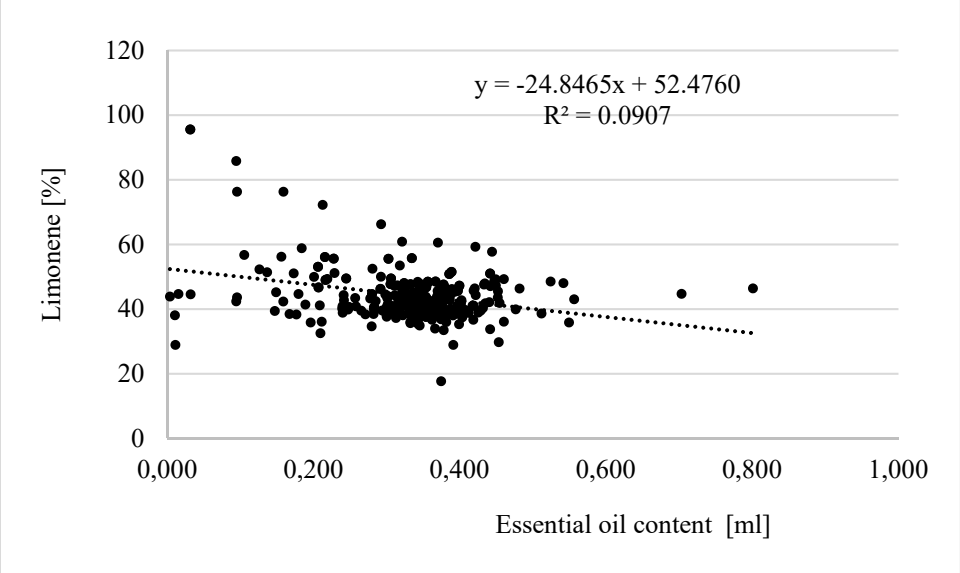

Table 2 The comparison of average total essential oil content values and the content of essential oil components described in this paper

Authors	Essential oil [%]	Carvone [%]	Limonene [%]
Bosko et al. 2016 (not published yet)	0.32–7.306	2.52–79.44	17.70–95.58
Sedláková et al. 2003	0.36–1.37	26.34–69.92	30.08–73.66
Seidler-Łożykowska et al. 2013	3.40–5.20	53.50–68.00	28.50–40.50
Aćimović et al. 2012	3.31–4.06	40.78–44.33	54.07–57.26
Solberg et al. 2016		13.57–14.65	69.93–71.49
Seo et al. 2015		48.70	24.20

In this work, the essential oils content was found to vary in the range of 0.32–7.31% (0.1–0.8 ml/10 g) (see Table 2). The wide range of both the total essential oils content and the content of carvone and limonene components was caused by the diversity of samples from breeding materials. The authors Seidler-Łożykowska et al. (2013), who observed the relationship between the total essential oil content and the representation of major components of the essential oils, obtained results similar to this work (-0.66**), a statistically highly significant negative relation between the essential oils components carvone and limonene (-0.93**).

CONCLUSION

The results obtained in this work indicate that the relationship between the total essential oil content and the content of main essential components oil were found to be statistically significant according to the Pearson correlation coefficient. It was found that with increasing total essential oils content, the relative content of the limonene component decreases. On the other hand, the percentage of carvone in the essential oil rises with increasing total essential oil content.

However, the reference method for the determination of the essential oil content and composition is time consuming and expensive. Results of these analyses will be further used for creating a calibration model for the FT-NIR spectroscopy, a promising method for rapid and non-destructive determination of the content of individual substances in caraway achenes. Because of the fact that the samples were obtained from plant breeding material there was very often a problem with insufficient amount of the samples for reference analysis and these analyses are also highly time consuming. The calibration model for FT-NIR spectroscopy would shorten the time of the determination significantly and since this method is non-destructive, the samples can be further

used, eg. for determination of germination ability etc. FT-NIR can be used easily also for the continuous monitoring of the content of substances after harvest and during the storage.

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EFFECT OF NITROGEN AND SULPHUR FERTILISATION ON MICROBIOLOGICAL PARAMETERS OF MILK THISTLE ACHENES

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Abstract: The achenes of milk thistle (*Silybum marianum* (L.) Gaertn.) are used in pharmaceutical and cosmetic products, as well as an ingredient of functional food and animal feed because they contain a variety of lipids, proteins and biologically active substances. The quality of seeds of milk thistle is not only given by contained substances but also by their microbiological purity. The objective of the vegetation experiment established in 2016 was to explore the effect of nitrogen (N) and sulphur (S) fertilization on microbiological quality of milk thistle seeds. Six treatments were established in the experiment: 1. N_2S_0, 2. N_3S_0, 3. N_2S_2, 4. N_3S_3, 5. N_1S_1+N_1S_1 and 6. N_2S_2+N_2S_2 (where: N_1 – 25 kg/ha, N_2 – 50 kg/ha, N_3 – 100 kg/ha, S_0 – without sulphur fertilization, S_1 – 12.5 kg/ha, S_2 – 25 kg/ha, S_3– 50 kg/ha). Variants 1–4 were treated with nitrogen and sulphur before sowing using one dose of fertilizer. Split doses application was applied at variants 5 and 6 fertilizing before sowing and at the beginning of extensive growth during elongation and branching of plants. In milk thistle achenes collected in the period of maturity of terminal flowerheads, these groups of microorganisms were determined: aerobic plate count, thermoresistant aerobic microorganisms and moulds. Protective effect of the applied sulphur against moulds and thermoresistant aerobic microorganisms on the seed was observed, but it was not significant. The aerobic plate count was not affected by fertilization.

Key Words: aerobic plate count, spore-forming microorganisms, moulds

INTRODUCTION

Milk thistle (*Silybum marianum* (L.) Gaertn.) is a popular herbal plant. Its seeds contain silymarin which is used as medicinal substance. Moreover, milk thistle achenes contain about 25% of oil, 25–30% of proteins, sterols (0.63%) with tocopherol (0.038%) and about 2% of flavonoids (Szczucinska et al. 2006).

Plant seeds may be contaminated by microorganisms in various ways. The main sources of microbial contamination can include contaminated water, soil or organic fertilizers, as stated by Bylund (2013). The high numbers of microorganisms on plant seeds may be also caused by inadequate hygiene of personnel, presence of rodents or birds, or by insufficient sanitation during processing and storage. Besides the above mentioned, microbiological purity of the seeds can be also affected by agricultural technology whose integral part is nutrition and fertilization. The objective of this study is to evaluate the effect of nitrogen and sulphur fertilization on the microbiological purity of milk thistle achenes.

MATERIAL AND METHODS

The effect of mineral fertilization with nitrogen (N) and sulphur (S) on the microbial contamination of seeds of milk thistle was observed in the small-plot field experiment on the land of Agritec Plant Research s.r.o. company in Šumperk. Milk thistle (variety Mirel) was seeded on April 22, 2016. Application of nutrients was carried out using single dose (before sowing on April 20, 2016) and split doses (before sowing and during vegetation - in the beginning of the elongation growth during elongation and branching of plants on June 24, 2016) according to the scheme in the following table (Table 1).

Table 1 Diagram of small-plot experiment

	Variant of fertilisation	Dose of N (kg/ha)	Dose of S (kg/ha)	Fertiliser	Term of application*
1	N_2S_0	50	0	LAV	1
2	N_3S_0	100	0	LAV	1
3	N_2S_2	50	25	DASA	1
4	N_3S_3	100	50	DASA	1
5	N_1S_1+N_1S_1	50	25	DASA	1+2
6	N_2S_2+N_2S_2	100	50	DASA	1+2

** Application terms: 1 - before sowing, 2 - in the beginning of the elongation growth*

Nitrogen was applied in the form of calcium ammonium nitrate (CAN) fertilizer containing 27% N. Ammonium sulphate nitrate (fertilizer DASA) which is composed of 26% N and 13% S was used on variants with combination of sulphur and nitrogen application. All variants were set up in four repetitions. Milk thistle achenes intended for microbiological analysis were taken in the period of maturity of terminal flowerheads (August 4, 2016).

For microbiological analysis of milk thistle achenes, 3 g samples were used. The samples with 27 ml of sterile saline solution were shaken (2300 rpm, Multi Speed Vortex Biosan) for 2 minutes. Subsequently, the following groups of microorganisms were determined in the samples of milk thistle using standard procedures:

- aerobic plate count (APC) on PCA (Biokar Diagnostic, France)) at 30 °C for 72 h,
- thermoresistant aerobic microorganisms (SPAE) on PCA (Biokar Diagnostic, France) at 30 °C for 72 h after previous pasteurization at 85 °C for 10 min, and
- moulds on GCHK (Biokar Diagnostic, France) at 25 °C for 120 h.

The data from microbiological analysis were subjected to statistical analysis in Statistica CZ 12. The influence of fertilization on monitored characteristics was described by mean values ± standard error (SE) and was evaluated by analysis of variance (Monofactorial ANOVA) followed by Fischer assaying at 95% ($p < 0.05$) significance level.

RESULTS AND DISCUSSION

Basic information about the extent to which the seeds are contaminated with microorganisms is represented by the aerobic plate count. These values can be give information on microbial contamination of the environment, where thistle was grown and also on the compliance with health guidelines in the production, transport and storage (Burdychová and Sládková 2007). Generally, plant seeds are usually significantly contaminated with microorganisms. Prokopowich and Blank (1991) found in commercially available seeds for germination (for edible sprouts) aerobic plate count from 3.0×10^3 to 4.0×10^6 CFU/g. In a study of Peles et al. (2012), the APC of organically grown wheat grains was 8.0×10^4 CFU/g. APC of *Silybum marianum* achenes was 1.7×10^7 CFU/g on average. Influence of sulphur and nitrogen nutrition on APC was not found to be significant ($p < 0.05$), consider the following table (Table 2).

Table 2 Average numbers of selected groups of microorganisms on seeds of Silybum marianum at different variants of cultivation (CFU/g)

	Variant of fertilization	APC (CFU/g ± SE)	SPAE (CFU/g ± SE)	Moulds (CFU/g ± SE)
1	N_2S_0	$1.9 \times 10^{7\,a} \pm 1.7 \times 10^6$	$1.1 \times 10^{3\,a} \pm 6.0 \times 10^2$	$2.0 \times 10^{4\,a} \pm 2.7 \times 10^3$
2	N_3S_0	$1.8 \times 10^{7\,a} \pm 5.4 \times 10^6$	$1.6 \times 10^{3\,a} \pm 7.8 \times 10^2$	$2.3 \times 10^{4\,a} \pm 4.0 \times 10^3$
3	N_2S_2	$1.8 \times 10^{7\,a} \pm 6.9 \times 10^6$	$1.2 \times 10^{2\,a} \pm 5.0 \times 10^1$	$2.4 \times 10^{4\,a} \pm 1.7 \times 10^3$
4	N_3S_3	$1.6 \times 10^{7\,a} \pm 7.0 \times 10^5$	$5.3 \times 10^{2\,a} \pm 2.1 \times 10^2$	$1.5 \times 10^{4\,a} \pm 2.9 \times 10^3$
5	$N_1S_1+N_1S_1$	$1.1 \times 10^{7\,a} \pm 4.9 \times 10^6$	$1.8 \times 10^{2\,a} \pm 1.7 \times 10^1$	$1.6 \times 10^{4\,a} \pm 2.5 \times 10^3$
6	$N_2S_2+N_2S_2$	$1.9 \times 10^{7\,a} \pm 4.6 \times 10^6$	$1.1 \times 10^{3\,a} \pm 7.0 \times 10^2$	$1.6 \times 10^{4\,a} \pm 4.4 \times 10^3$

Legend: APC – aerobic plate count; SPAE – thermoresistant aerobic microorganisms, values expressed as mean ± standard error of the mean. Means followed by the same letters are not significantly different ($p<0.05$).

Another significant group of microorganisms contaminating dry seeds of the milk thistle were micromycetes. Plant seeds contain low amount of water, therefore, they are prone to contamination, especially by moulds whose number was on average 1.9×10^4 CFU/g. One of the major health beneficial effects of milk thistle supplements is a positive effect on a liver, attributed to the high content of silymarin. Herbal supplements may be contaminated with potentially toxinogenic moulds which can be harmful to the liver due to their ability to produce mycotoxins (Tournas et al. 2013). In a study by Tournas et al. (2013), 60% of the samples of milk thistle supplements were contaminated with moulds. The whole seeds were the most contaminated (up 4.0×10^5 CFU/g), whereas moulds were not detected in tea bags, alcohol-based extract, oil-based extract, capsules, or gel. The predominant genera of moulds comprise *Eurotium*, *Aspergillus*, and *Alternaria*. *Aspergillus flavus* was detected in 29% of seeds. Tournas et al. (2012) found in dietary supplements of milk thistle (seed and oil-based extract) only low amounts of aflatoxins (0.04–2.00 ng/g). In ours samples, mainly *Mucor* and *Penicillium* genera were identified. The results of the experiment indicate a protective effect of the applied sulphur against moulds that are commonly reported in the literature (Williams and Cooper 2004, Rathi et al. 2015, Skwierawska et al. 2016). Also Ribas-Agustí et al. (2013) present sulphur as one of the most important natural fungicides used to protect plants against attack by molds. The number of colonies in variants fertilized by this nutrient decreased by 17.4% on average, in comparison with the variants fertilized only with nitrogen. The relatively low fungal infestation was recorded in the variant with the highest dose of sulphur fertilization variants and two-dose applications where sulphur was applied during the growing season.

In samples of milk thistle seeds, thermoresistant aerobic microorganisms were also detected. The main risk is that these bacteria are able to survive unfavourable conditions or heat treatment. Thus, these microorganisms may be subsequently originator of spoilage of products from a contaminated feedstock. The source of contamination is especially soil and dust. We found the number of thermoresistant aerobic microorganisms in the seeds of milk thistle was on average 7.2×10^2 CFU/g. Similarly, relative decrease in their numbers was found in variants fertilized with sulphur, but the difference was not significant ($p<0.05$).

CONCLUSION

Milk thistle is especially appreciated for its edible and medicinal seeds, which should be stored at the appropriate temperature-humidity conditions, especially to prevent mould growth, thereby the formation of mycotoxins. For proposing an appropriate technology for growing of this small-scale crop, it is important to focus on the optimization of plant nutrition, which commonly affects not only the yield but also the quality of production. Important parameters of milk thistle seeds quality is, inter alia, microbiological purity, whether the seeds are intend to be used to produce animal feed, human food, or pharmaceuticals. Development of microorganisms is an indicator of the plants health state, and is largely conditioned by optimal nutrition. Therefore, in a one-year experiment the effect of N and S fertilizers on the microbiological quality of milk thistle seeds was examined. Although the results of the field experiment showed no significant effect of fertilizers on the number of monitored microorganisms, from

the relative comparison, it can be seen some positive effect of the single and two doses applications of sulphur on the microbiological quality of seeds.

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GERMINATION OF PELLETIZED AND NATURAL *PETUNIA x HYBRIDA* SEEDS AFTER LONG TERM STORAGE

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Abstract: Pelleting is used by seed companies to improve the sowability of small and unevenly shaped seeds that could not be sown by a sowing machine. *Petunia x hybrida* is among the most popular annuals worldwide. The ´seeds are relatively small so young plants producers request pelleted seeds. The only drawback of pellets could be lowering the germination rate after 3 years of guaranteed shelf live. Natural seeds keep high germination longer, usually for 3–5 years. In this experiment was tested germination rate of 14 F1 Petunia varieties from 2000 till 2014. In 2006 the seeds were pelleted. According to this experiment the natural seeds kept high germination after 13 years if stored in optimal conditions. The germination rate after harvesting and in 2014 was not statistically different on p = 0.05. The germination of pelleted seeds and natural seeds stored in room temperature decreased over the years.

Key Words: seeds, Petunia, pelleting, long term storage, germination

INTRODUCTION

Pelleting is a technology nowadays widely used by seed companies. It does not influence the physiological attributes of the seeds, like priming or pregermination, but improves the sowability of the seeds (Halmer 1994). The layer of inert material significantly increases the size and weight of small seeds and also changes their irregular shape. This enables mechanical sowing with a sowing machine which is essential in cost-effective industrial production of young plants (McDonald and Kwong 2005).

Pellets differ in many attributes – size, shape, colour, material, but the pelletizing process is always the same. The seeds are placed in the rotation drum and sprayed with a glue so the pelleting material could stick to the seeds´ surface. Multiple layers of pelleting material (usually clay or silicates) and glue are applied until the specific size of the pellet is reached (Adkins et al. 2007, Baskin and Baskin 2014). Afterwards the pelleted seeds are slowly (2–3 hours) desiccated in a special drying unit where circulates hot air (24–26 °C). If not desiccated properly, shelf life of the seeds is very limited (Job et al. 1999).

If pelleting material as well as the glue are inert and the pellets are dried properly, then pelleting has limited impact on germination. The seed companies develop and constantly improve their own glues and materials and test if pellets have required characteristics. The pellet has to dissolve easily after sawing so the seed can start germinating. This technology also can´t dramatically lower the germination rate, right after the application as well as in longer term (Styer and Koranski 1997). Seed companies guarantee 3-years shelf life of pellets, if packed in three-layer bag (plastic foil, paper and aluminium layer) the germination remains high.

Petunia x hybrida is among the worlds´ top-selling annuals (Ball 1991). It is due to the big variability - different sizes and colours of flowers, upright or spreading habitus (Anderson 2007). The seeds are small, 0.6–0.7 mm in length and 0.5–0.6 mm in diameter (Gerats and Strommer 2008, Sink 1984) so young plants producers demand pelleted seeds. Petunia pellet is round, 1.0–1.2 mm in diameter (McDonald and Kwong 2005) so it can be sawn by a sowing machine.

Seeds remain the germination level for at least two years if held in dry and cool place (Sink 1984). Key role in germination rate of the seeds play conditions after harvesting, esp. temperature and relative moisture level (Sajjan et al. 2013).

The aim of this paper is to examine the declination of germination rate of *Petunia x hybrida* seeds if stored in optimal conditions and determine if the pelletization decreases the germination rate of seeds in short term (right after pelletization) and in long term if stored in room temperature. This simulates the conditions under which the seeds in pictorial packages are sold and stored in the shops.

MATERIAL AND METHODS

Characterization of material, experimental design and germination testing

In the experiment were tested seeds of 14 varieties of *Petunia x hybrida*. The seeds were harvested in 2000 and 2001, all varieties were F1 hybrid, represented original Czech breeding (company Černý-BioPro Ltd.), 6 varieties were grandiflora type (big flower) and 8 were multiflora type (small flower). The plants were planted in the greenhouse and throughout the whole cultivation had good growing conditions and were pest and disease free (except 1 component). Seed samples for the experiments were collected from the standard seed production of the company that was sold in the Czech Republic as well as exported. Every year is produced just a part of assortment due to extensive costs and space requirements. This is a reason why seeds were harvested in 2 years – some varieties in 2000, other in 2001.

After harvesting, the seeds were dried for 3 months (20 °C) until the constant balance of 35% of relative humidity was reached. Then the seeds were placed in the glass container with a metallic lid and rubber seal. The containers were stored in dark in air conditioned storage where was maintained constant temperature of 5 °C.

The germination of these 14 varieties was tested every year in March. The germination test of natural seeds was according to ISTA methodics - Jacobsen germinator, filtration paper, 4 x 50 seeds, 21 days, 22 °C.

Second part of the experiment was to determine if the pelletization decreases germination rate – right after pelletizing and in the longer term. In the seed shops are sold pelleted seeds wrapped in 3-layer bag. These are stored in the room temperature for 3 years. Seed companies guarantee the same germination for this time period. In order to do so, freshly harvested seeds with high germination are used for pelleting.

In 2006 the seed sample of the examined varieties was pelleted. The same seeds as in the first part of experiment were used. Seeds were harvested in year 2000 or 2001 and stored in dark and temperature 5 °C. Pelleting glue BioPro Coat SuperFine (starches and modified starches) and pelleting material BioPro Powder Sili (silicates) were used. Pellets were dried properly in the drying unit (26 °C, 3 hours). Samples of pelleted and natural seeds were stored in the 2 ml freezer Epruvet with seal lid and silicon gasket. Samples were stored in air conditioned room with maintained temperature 20 °C. The diameter of the pellet was 1.0–1.2 mm.

The germination of pellets was tested immediately after pelleting and then every 2 years in March. The germination of pellets was tested in the soil test according to ISTA methodics. The pellets samples (4 x 50 pellets) were tested in the germinating chamber (24 °C, relative humidity 90–100%, above the shelves are special fluorescent tubes with modified spectrum Osram Biolux, light intensity 2750 lux, 16 hours light, 8 hours dark). Natural seeds were tested also every 2 years but on the Jacobsen germinator.

Seed companies test the germination of seeds according to ISTA methodics. Natural seeds are tested on the filtration paper on the Jacobsen germinator placed on the Jacobsen table. Germination of pellets is tested in the soil. Jacobsen germinator is not optimal for this purpose because the pellets are not dissolved properly and the seed can´t start germinating. On the other hand, natural seeds tend to float to the lower levels of soil after irrigation and the germination rate is significantly influenced.

This experiment consists of 2 parts:

1) What is the decline of germination rate in a long term, if the seeds are dried properly and stored in optimal conditions in 5 °C.
2) Examine if the pelleting glue and material (BioPro Coat SuperFine and BioPro Powder Sili) are suitable also for older seeds (seeds harvested in 2000 and 2001) and if the pelleting don´t negatively influence germination for at least 3 years if stored in the room temperature.

The results were evaluated in program STATISTICS with the t-test.

RESULTS AND DISCUSSION

Long term storage of natural seeds – optimal conditions (5 °C)

If the natural seeds were stored in optimal conditions and the temperature 5 °C was maintained, the germination level of all 14 tested varieties remained high and did not statistically differ (on p = 0.05). Seed companies usually don´t sell seeds with germination lower than 85% because it is uneasy to guarantee the minimal germination 85% for the 3 years which is a usual shelf life of seeds packed in the 3-layer packages. Only one variety – Láska F1 had low germination level, right after harvesting and also during the whole duration of the experiment. The difference between the germination right after harvesting in 2001 (79%) and in 2014 (77%) is not statistically significant (p = 0.003839). Low germination of variety Láska F1 could be a result of worse health conditions of the maternal component due to Oidium spp. The germination of *Petunia x hybrida* seeds according to scientific research papers maintains for 3–5 years (Sink 1984, Gerats and Strommer 2008, McDonald and Kwong 2005). But according to our experiment the seeds remained their high germination rate much longer, more than 10 years (Table 1).

Table 1 Germination rate of natural seeds Petunia x hybrida – stored in optimal conditions

Variety	Grandiflora (G), Multiflora (M)	Germination rate (%)														
		2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014
Angelika F1	M	90	92	90	94	88	93	97	93	95	95	92	94	93	92	91
Belinda F1	M	94	95	92	92	92	94	98	95	95	95	97	97	93	94	94
Lucie F1	M	96	98	97	96	94	95	97	100	98	99	96	97	94	98	97
Marika F1	M	97	97	96	97	99	95	98	97	96	96	95	95	96	96	96
Rita F1	M	97	98	95	95	97	97	96	92	95	95	92	96	96	96	97
Simona F1	M	96	97	96	96	95	94	96	97	96	97	97	96	96	95	97
Brigitta F1	M		96	95	96	96	94	96	94	95	95	96	96	96	94	96
Sylvie F1	M		98	96	96	95	98	95	97	97	99	97	96	97	95	96
Přátelství F1	G	92	93	91	92	91	91	93	91	93	93	91	92	91	92	91
Radost F1	G	94	92	92	93	89	92	94	94	93	93	94	92	93	92	92
Láska F1	G		79	78	80	77	80	80	82	77	83	87	80	79	79	77
Půvab F1	G		94	93	94	93	94	94	93	93	92	94	94	93	93	93
Touha F1	G		93	94	93	92	94	92	93	93	94	93	94	94	93	94
Úsměv F1	G		97	96	93	92	93	93	96	96	98	94	94	94	98	98

Figure 1 Germination rate of natural seeds Petunia x hybrida – stored in 20 °C

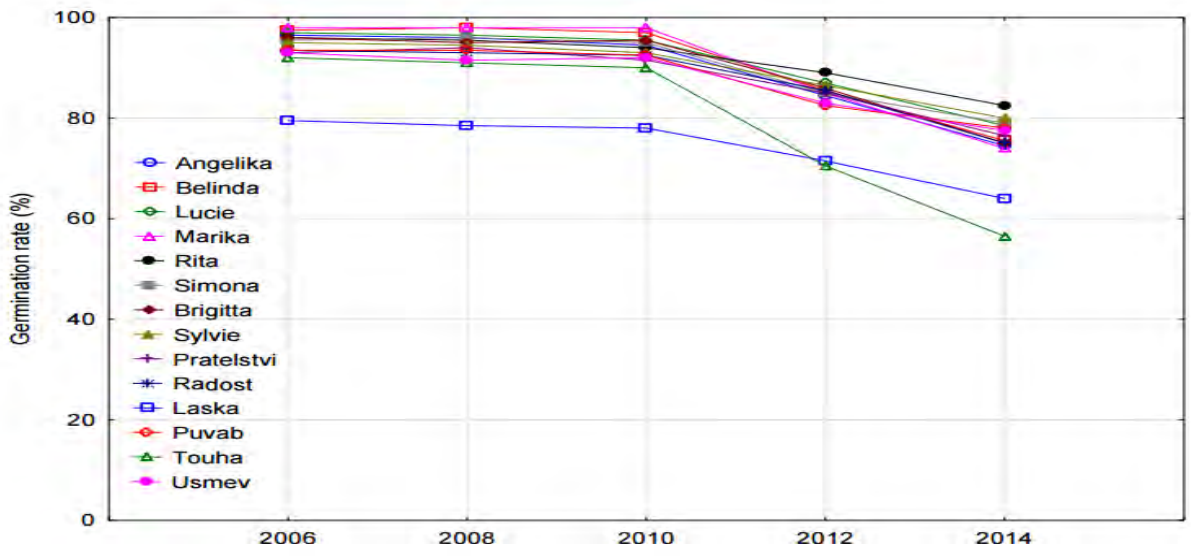
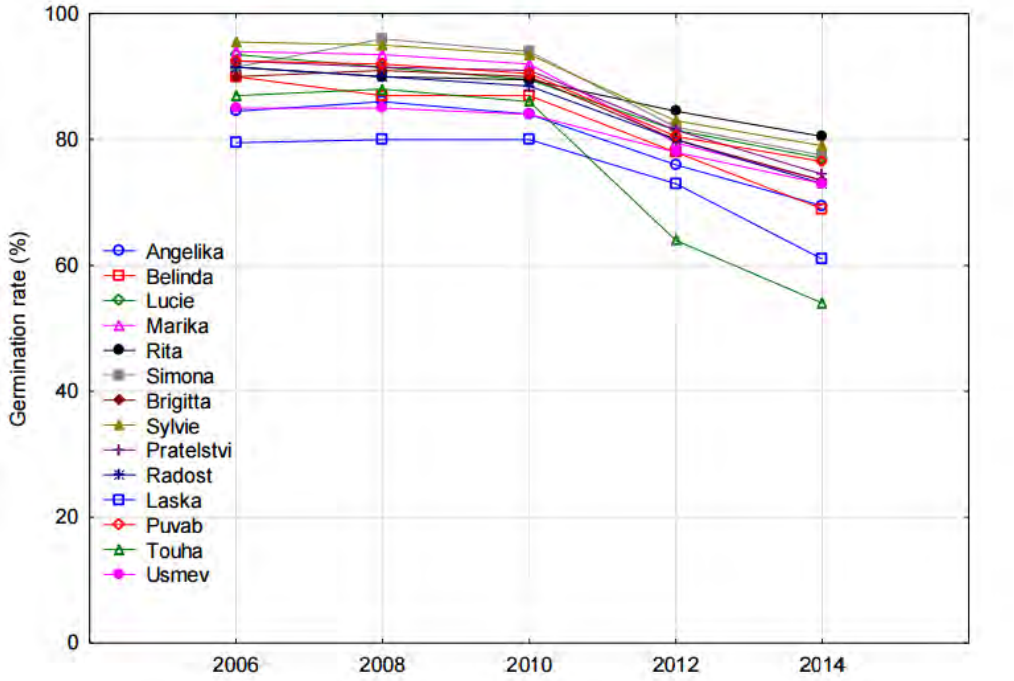


Figure 2 Germination rate of pelleted seeds Petunia x hybrida – stored in 20 °C



The pelleting process decreases the germination rate right after the process. This could be explained by existence of a barrier of an inert material (Figure 2). The seed has to have bigger vigour to start germinating. This is also the reason why the seed companies pellet only the seeds from new crop with high germination rate. In this experiment the seeds were pelleted 5 years after harvesting. T-test was not performed for the germination rate before and after pelleting due to different methodology of germination test. Natural seeds (before pelleting) are tested using Jacobsen germinator, pellets are tested in soil.

Table 2 T-test for natural and pelleted seeds – difference between 2006 and 2010

Variety	Results of t-test (p-value)	
	Pellets	Natural seeds
Angelika F1	0.620220	0.113532
Belinda F1	0.386704	0.670412
Lucie F1	0.087080	0.437237
Marika F1	0.266570	1.000000
Rita F1	0.382175	0.467994
Simona F1	0.270829	0.730358
Brigitta F1	1.000000	0.620220
Sylvie F1	0.190116	0.207031
Přátelství F1	0.320206	0.597620
Radost F1	0.142800	0.550415
Láska F1	0.779559	0.620220
Půvab F1	0.190116	0.390259
Touha F1	0.779559	0.133975
Úsměv F1	0.647967	0.670412

Four years after pelletization pellets remain the same germination rate as right after pelleting (Table 2). The same conclusions were obtained for natural seeds. The differences in germination rate are not statistically significant, all p-values are higher than 0.05.

Germination rate statistically significantly drops according to t-test on the significance level p = 0.05 after more than four years of storage in room temperature 20 °C. This drop is not only significant in pelleted seeds but also the same trend is by natural seeds (Figure 1). After 8 years of storage in these conditions natural seeds as well as pellets are not usable for professional purposes. The producers of young plants request the germination at least 90%, by Petunia seeds even above 95%. The results correspond with the conclusions in the literature (Sink 1984, McDonald and Kwong 2005, Sajjan et al. 2013). The storage conditions, especially the temperature play the main role in determination of germination rate over the storage time.

CONCLUSION

Seed pelleting has many positives. According to our experiment the pelleting glue BioPro Coat SuperFine (starches and modified starches) and pelleting material BioPro Powder Sili don´t influence the germination in long term. The drop in germination is significant right after the pelleting, because the pellet is a barrier in germination. The gradual decrease of germination over longer period of time is the same for pelleted seeds as well as natural seeds. If the natural seeds are dried properly and stored in optimal conditions where temperature 5 °C is maintained, the germination level does not change over more than 10 years. Pellets as well natural seeds remain the guaranteed germination level even if they are stored in room temperature.

ACKNOWLEDGEMENTS

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COMPARISON OF SELECTED PHYSICAL PARAMETERS IN SOILS ON SITES WITH DIFFERENT CULTURES

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Abstract: This paper deals with the evaluation of selected physical properties of soil in experimental plots of the Faculty of Horticulture, Mendel University in Brno. Measurements were carried out in spring and autumn 2015 in vineyards, fruit orchards, and vegetable plots. Individual measurements included sampling and analysis of intact soil samples, penetrometer measurements, measurements of infiltration capacity of soil, and determination of soil structure. The gained results confirm differences between the bulk density reduced in spring and autumn. The values of bulk density in the spring ranged between 1.43 and 2.30 g/cm^3 and in the autumn between 1.50 and 2.89 g/cm^3. Penetrometer measurement results confirm the higher values of soil penetration resistance in tyre tracks, compared with the centre between rows or areas near the planted rows. The highest values were measured in vineyards. The best values of infiltration measured by the Minidisc infiltrometer were measured in vegetable plots. The soil homogeneity in this variant facilitates good infiltration capability throughout the soil horizon. Soil structure was evaluated using structural coefficient, which expresses the relationship between agronomically valuable (0.25 to 10 mm) and less valuable structural elements (>10 and <0.25 mm). The results indicate that lower values of the structural coefficient are in Vineyard.

Key Words: soil, penetrometry, infiltration, soil structure

INTRODUCTION

The current status of soils is the result of long-term development affected by natural causes and the current ever increasing human activity. Therefore, careful management ought to seek to minimize the effects of degradation leading to water and wind erosion, soil compaction and acidification, as well as loss of organic matter in soil linked to edaphon or soil life (Cofie et al. 2000, Dexter 2004, Adam 2016).

Numerous studies such as Ferrero et al. (2005) and O'Green et al. (2006), increasingly point to the current critical state of agricultural soils, where soil compaction manifests itself in varying extents. This problem is most apparent in the case of permanent plantations, including vineyards.

Besides the intensification of vineyard production, which is one of the main causes of the degradation of agricultural land, the insufficient supply of organic fertilizers to soil has a great influence on soil compactness. This type of fertilizer not only improves the buffering (absorbing) capacity and soil structure, but also improves the holding capacity of soil for water (Borůvka 2001, Kalina 2004).

Soil structure is defined by balanced relationship between basic physical factors and has a decisive influence on the intake of water, as well as air and nutrients to the root system of plants. This situation may be in intensively managed soils disturbed by their pressing, especially by frequent use of mechanization (Arvidsson and Ristic 1996, Strauss 2006).

Compaction process, which is primarily induced by pressure, was dealt with by a number of authors (Fic 1983, Johnson and Bailey 2002, Pokorný et al. 2003). Compaction is a cumulative process, in which the cumulative adverse effects on the soil add up. For pressures with values lower than 0.1 MPa, in favourable moisture conditions, we can count on reversible changes. At higher pressures, irreversible changes may happen, in which also other factors can participate (Paglia et al. 2004).

Accurate assessment of bearing capacity of soil in the field is very problematic. It depends on many variables and constant factors, such as moisture, soil structure, chemical composition, grain size,

and so on. Widespread and rapid way to assess terrain passability is by using a penetrometer (Bengough 2001, Defossez et al. 2003).

The aim of this study was to evaluate the physical properties of soils on the property of the Faculty of Horticulture of the Mendel University in Brno.

MATERIALS AND METHODS

Experimental stations

Measurements were carried out in spring and autumn 2015 in vineyards (variant 1), orchards (variant 2), and vegetable plots (variant 3). Individual measurements consisted of sampling and analysis of intact soil samples, penetrometer measurements, measurements of infiltration capacity of soil, and assessment of soil structure. From the climatological point of view, the locality is a part of the T4 warm area (warm, dry climate with dry and mild winters). The average annual temperature is 8 to 9 °C while the average annual rainfall is 500 mm. The average relative humidity is 78%. From the pedological point of view, it is degraded chernozem on loess. In terms of soil typology, it is a modal pararendzina (PRM) with carbonates throughout the profile.

Evaluation of properties

Sampling and analysis of soil samples

Physical properties of the soil were monitored by means of rollers according to Kopecký. Monitoring provided the following determinations: reduced bulk density, total porosity, current contents of water and air, the maximum capillary water capacity, and minimum air capacity. Samples were taken from three depths of soil, namely 0 to 0.10 m, 0.10 to 0.20 m, and from 0.20 to 0.30 m in five repetitions.

Soil penetration resistance (penetrometry)

Penetrometer measurements were carried out crosswise between rows at a distance of 200 mm, in triplicate. Each measurement was performed to a depth of 520 mm. The device used for the actual measurement was a penetrometer type P1.52 made by the EIJKELKAMP Company. The device consists of measuring tip, tensiometric sensor, optical sensors for measuring depth, and evaluation electronics with a microprocessor and battery.

Measurement of infiltration properties of soil

Infiltration measurements were performed using MINIDISK infiltrometer. This infiltrometer permits to set and for the time of measurement to maintain a slight vacuum on its lower edge in the range of pressure height between -0.5 cm and -6 cm. The results represent a chronology of infiltrated water volumes. Measurements were recorded every 60 s for at least 0.45 hr in triplicate, at the beginning and at the end of the vegetation period. Infiltration rate was calculated from the amount of infiltrated water per unit area for each repetition, and subsequently averaged for each measurement.

Soil structure

Soil structure was determined by screening the dry soil on sieves with average openings of 0.25 mm, 0.5 mm, 2 mm, 5 mm, 10 mm, and 20 mm. Samples were collected from two depths, namely 0 to 0.15 m and 0.15 to 0.30 m in triplicate. Each structural fraction was weighed separately and converted to percentages. For the actual assessment, a structural coefficient was calculated. This coefficient expresses the relationship between agronomically valuable (0.25 to 10 mm) and less valuable structural elements (>10 and <0.25 mm).

RESULTS AND DISCUSSION

Table 1 shows the results of physical properties of soil. When measurements were taken, reduced bulk density averages corresponding to the soil conditions were assessed. There were significant differences between the reduced bulk density measured in spring and autumn seasons. The values of bulk density in the spring season ranged between 1.43 and 2.30 g/cm^3, while in the autumn season, they ranged between 1.50 and 2.89 g/cm^3.

The water content at the station was satisfactory and the air capacity was within limits. The total soil porosity at the experimental station corresponded to the values of soil compaction.

Table 1 The values of results of the physical properties of soil

Exp. variants	Replication	Depth of so il (m)	Bulk density (g/cm^3)	The total porosity (%)	Currently content (% vol.)		Max. cap. capacity	Min. air capacity
					Water	Air	(% vol.)	
Vineyard (var. 1)	Spring	0–0.1	1.43	48.78	20.13	28.73	36.14	12.64
		0.1–0.2	1.45	50.04	20.06	24.17	37.01	13.03
		0.2–0.3	1.52	47.21	25.87	25.19	34.69	12.52
		Average 0–0.3	1.47	48.68	22.02	26.03	35.95	12.73
	Autumn	0–0.1	1.39	47.67	22.41	25.36	36.46	11.21
		0.1–0.2	1.46	49.99	22.31	21.90	37.43	12.56
		0.2–0.3	1.51	48.58	28.09	24.31	36.02	12.38
		Average 0–0.3	1.45	48.75	24.27	23.86	36.64	12.05
Orchard (var. 2)	Spring	0–0.1	1.56	49.58	20.15	29.45	27.53	13.64
		0.1–0.2	1.86	50.98	20.68	24.86	38.21	13.98
		0.2–0.3	1.68	48.05	26.43	26.43	35.49	13.26
		Average 0–0.3	1.70	49.54	22.42	26.91	33.74	13.63
	Autumn	0–0.1	1.48	48.36	19.86	28.64	26.48	13.48
		0.1–0.2	1.68	50.42	20.03	24.13	37.89	13.46
		0.2–0.3	1.53	48.02	26.08	28.97	34.81	12.92
		Average 0–0.3	1.56	48.93	21.99	27.25	33.06	13.29
Vegetable area (var. 3)	Spring	0–0.1	1.71	50.16	20.95	31.45	29.42	14.56
		0.1–0.2	2.45	52.43	21.63	25.86	39.56	15.06
		0.2–0.3	1.98	50.12	27.84	27.83	37.09	15.24
		Average 0–0.3	2.05	50.90	23.47	28.38	35.36	14.95
	Autumn	0–0.1	1.67	49.84	20.75	31.01	29.16	14.21
		0.1–0.2	2.31	52.41	21.41	25.16	39.23	14.56
		0.2–0.3	1.73	49.79	26.98	27.64	36.84	14.95
		Average 0–0.3	1.90	50.68	23.05	27.94	35.08	14.57

Figures 1 to 3 show the values of soil penetration resistance measured at individual experimental plots. For perennial crops, measurements were taken crosswise between rows at a distance of 200 mm, in triplicate. The values in the figure show a significant difference between spring and autumn values. In the spring season, they ranged from 0.3 to 1.6 MPa and in the autumn season between from 1.3 to 3.4 MPa. The highest average values of soil penetration resistance were measured in permanent plantations along tyre tracks. When comparing the values obtained with the values reported by Arshad et al. (1992), the values in variant 1 and variant 2 exceeded the critical threshold for loamy soils.

Figure 1 Penetration resistance of soil – var.1

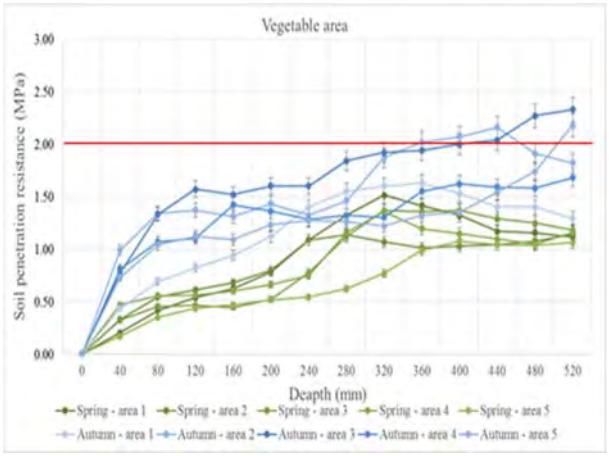
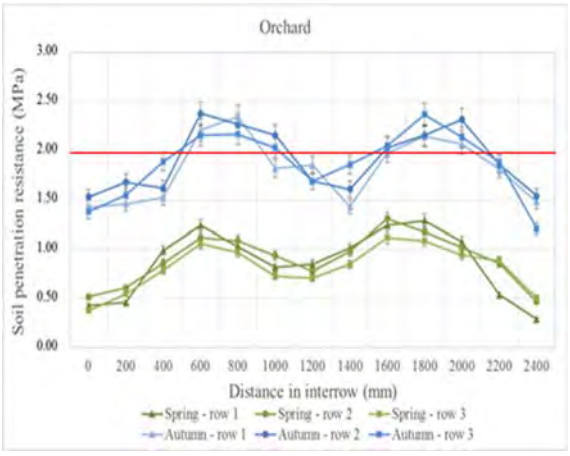
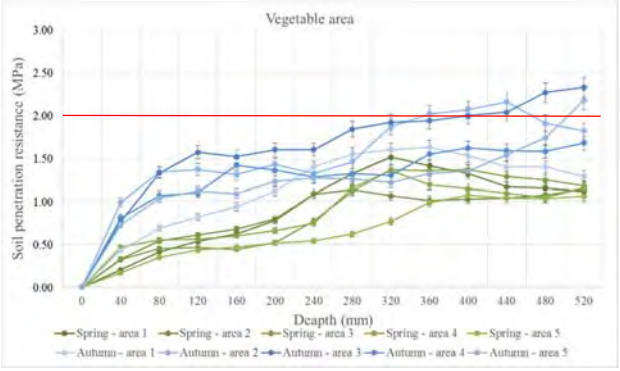


Figure 2 Penetration resistance of soil – var.2



In vegetable plots, individual measurements were carried out at five random locations. The results show that the values of the soil resistivity do not exceed the critical threshold, as shown in Figure 3.

Figure 3 Penetration resistance of soil – var.3



Figures 4 to 6 show infiltration of water into the soil in all monitored variants. Infiltration was measured from three sites in each variant. The course of the infiltration capacity of soil has been given by the shape of the curves that explain good or bad homogeneity of profile. The best infiltration characteristics of the soil were measured in variant 3 (Figure 4). The homogeneity of the soil in this variant facilitated good infiltration capability throughout the soil horizon. In the variant 2, the water infiltration was a little slower and smaller (Figure 5). The worst water infiltration capacity of the soil was in variant 1 (Figure 6), where the water infiltration, due to excessive compaction, was below the critical threshold. The figures indicate a better infiltration capacity in the spring compared to autumn. Badalíková et al. (2014) also examined the infiltration capacity of soil. Her published results confirmed a better infiltration capacity at the beginning of the growing season.

Figure 4 Infiltration of soil – var.1

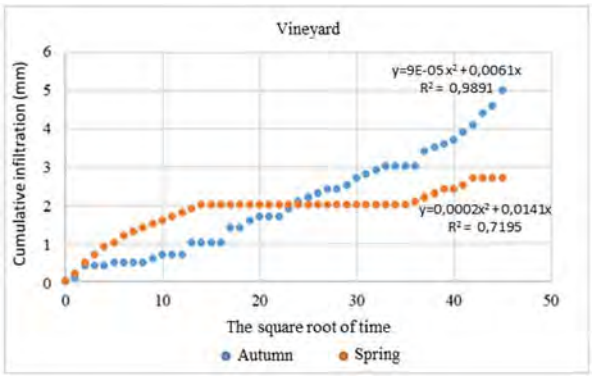


Figure 5 Infiltration of soil – var.2

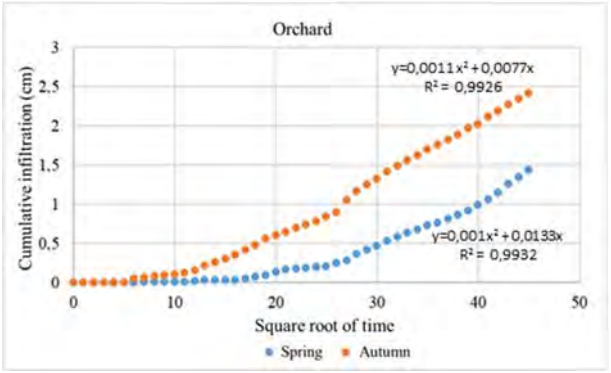


Figure 6 Infiltration of soil – var.3

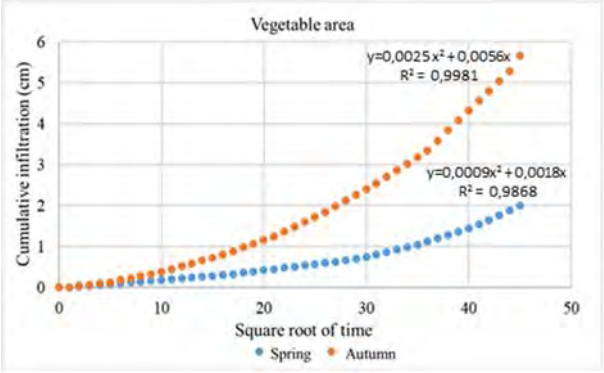


Table 2 shows the resulting values of structural coefficient in the three evaluated variants. The structural coefficient measures the degree of damage of the soil structure. At values higher than 1.0, the soil has a better structure and thus lower the risk of undesirable compaction, while values less than 1.0 are below the structural stability. This relates to the qualitative composition of soil humus, which forms agronomically valuable structure.

The results indicate that structural coefficient reaches lower values in variant 1, while at a depth from 0.00 m to 0.15 m the values of structural coefficient are around 1.3. This condition can lead to a reduction in the quality of soil environment in terms of other physical properties and consequently may negatively affect the chemical properties of soils.

Table 2 The average values of the structural coefficient

Exp. variants	Replication	Depth (m)	Structural elements (% weight)						Structural coefficient
			over 10	5–10	2–5	0.5–2	0.25–0.5	below 0.25	
Vineyard (var. 1)	Spring	0.00–0.15	41.62	17.72	22.14	16.31	0.92	1.57	1.36
		0.15–0.30	31.29	23.24	22.18	19.30	1.13	3.54	2.46
		Average	36.46	20.48	22.16	17.81	1.03	2.56	1.91
		St. deviation	7.30	3.90	0.03	2.11	0.15	1.39	0.78
	Autumn	0.00–0.15	42.01	17.89	22.65	16.89	1.06	1.98	1.39
		0.15–0.30	31.85	23.45	22.68	19.84	1.34	3.96	2.87
		Average	36.93	20.67	22.67	18.37	1.20	2.97	2.13
		St. deviation	7.18	3.93	0.02	2.09	0.20	1.40	1.05
Orchard (var. 2)	Spring	0.00–0.15	51.35	15.46	17.89	12.63	0.89	1.95	0.97
		0.15–0.30	32.12	18.32	23.57	19.32	1.45	5.51	1.89
		Average	41.74	16.89	20.73	15.98	1.17	3.73	1.43
		St. deviation	13.60	2.02	4.02	4.73	0.40	2.52	0.65
	Autumn	0.00–0.15	52.15	16.70	18.31	13.25	1.12	2.15	1.08
		0.15–0.30	32.54	18.76	23.61	20.06	1.76	5.89	1.92
		Average	42.35	17.73	20.96	16.66	1.44	4.02	1.50
		St. deviation	13.87	1.46	3.75	4.82	0.45	2.64	0.59
Vegetable area (var. 3)	Spring	0.00–0.15	52.16	17.23	19.98	15.16	2.06	2.87	1.91
		0.15–0.30	34.51	19.98	25.81	21.52	2.42	6.35	2.69
		Average	43.34	18.61	22.90	18.34	2.24	4.61	2.30
		St. deviation	12.48	1.94	4.12	4.50	0.25	2.46	0.55
	Autumn	0.00–0.15	54.12	18.65	21.41	16.42	2.24	3.02	1.98
		0.15–0.30	36.04	21.09	26.42	21.98	2.84	6.58	3.79
		Average	45.08	19.87	23.92	19.20	2.63	4.80	2.89
		St deviation	12.78	1.73	3.54	3.93	0.30	2.52	1.28

CONCLUSIONS

In 2015, selected physical parameters were evaluated at the experimental plots with different horticultural crops. Individual measurements consisted of sampling and analysis of intact soil samples, penetrometer measurements, measurements of infiltration capacity of soil, and determination of soil structure. Data from analyses focused on the evaluation of intact soil samples show that the highest values were reached in the autumn, when they ranged between 1.50 and 2.89 g/cm^3. Results of penetrometric measurements indicate that the greatest soil compaction occurs in areas around tyre tracks in vineyards and orchards. The values of soil penetration resistance here crossed a critical threshold and amounted from 0.3 to 1.6 MPa in the spring and from 1.3 to 2.4 MPa in the autumn. From the perspective of infiltration properties of soils, the best values were measured in the areas of vegetable plots, where $R^2 = 0.9981$. The soil homogeneity in this variant provides good absorption capacities throughout the soil horizon. In vineyards and orchards, the infiltration capacity was below normal values, ranging between 0.7195 and 0.9926. Results of soil structure evaluated by structural coefficient suggest that the best structural coefficient was measured in variant 3, where it reached 1.9. Depending on the depth, the structural coefficient in the depth from 0.15 m to 0.30 m was one times better than in the depth from 0.00 m to 0.15 m.

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GRAIN YIELD AND QUALITY OF SPRING BARLEY AFTER CATCH CROPS

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Abstract: Growing catch crops has many positive effects. The aim of this trial is to evaluate the effect of catch crops on the grain yield and quality of spring barley. The field trial was set up in Žabčice, one of the driest and warmest places in the Czech Republic. The trial examined the following crops: *Sinapis alba* and *Secale cereale v. multicaule*. The trial also included a control variant without catch crops. Catch crop growths were planted after winter wheat and left on the field until the spring. Spring barley was sown after the catch crops. *Sinapis alba* produced more biomass than *Secale cereale v. multicaule*. The grain yield of spring barley is mainly influenced by the species of catch crops and in combination with a particular year. *Sinapis alba*, except a drier beginning of the year, had no negative effect on the grain yield of spring barley. *Secale cereale v. multicaule* regularly reduced the grain yield of spring barley. The content of nitrogen compounds in grains in comparison with the control was lower after *Sinapis alba* and higher after *Secale cereale v. multicaule*, but with the exception of 2014, none of these results were statistically significant.

Key Words: grain yield, spring barley, quality, dry conditions, *Sinapis alba*, *Secale cereale v. multicaule*

INTRODUCTION

Catch crops are crops grown between two main crops. Catch crops enrich soil with biomass, reduce wind and water erosion, as well as leaching of nutrients and hold soil moisture. (Slepetienė and Kinderienė 2007, Rinnofner et al. 2008, Turmel et al. 2014, Scalise et al., 2015, Sparrow 2015) Catch crops suppress weeds and reduce the spread and incidence of diseases and pests (Murakami et al. 2000, Caner and Tuncer 2001, Romaneckas et al. 2012). In drier areas, such as Žabčice, catch crop water use may outweigh their positive effects. Sapkota et al. (2012) reported in their study that catch crops reduced the grain yield of barley probably because of competition between catch crops and barley for nitrogen, water, and light. However, Rinnofner et al. (2008), in their study found that catch crop water consumption has never affected the yield of subsequent crops (spring barley). Likewise Gaweda et al. (2012) found that *Sinapis alba* did not significantly change the grain yield of spring barley. However Gaweda (2011) stated that in a drier year, there is a risk of a decrease in the grain yield of spring barley after *Sinapis alba*. The aim of this trial is to evaluate the effect of catch crops on the grain yield and quality of spring barley.

MATERIAL AND METHODS

The field experiment was carried out on the experimental field station in Žabčice (South Moravia, Czech Republic). The experiment took place on a clay-loam fluvisols. Annual precipitation is 480 mm and the average annual temperature is 9.2 °C. Table 1 shows monthly precipitation and average monthly temperature in the years. The trial examined the following crops: *Sinapis alba* and *Secale cereale v. multicaule*. The experiment also included a control variant without catch crops. Catch crop growths were planted after harvesting winter wheat in mid-August. The harvest of aboveground mass of catch crops was conducted in late October. Traditional harvest of fresh plant mass of crops took place from the area of 0.25 m^2 with four replications for each variant of catch crops and subsequent drying to a constant value. Catch crops were left on the field until spring. In the spring, nitrogen fertilization with 60 kg/ha N was carried out. Spring barley was sown as the next crop after catch crops. After the harvest of spring

barley, grain yield and nitrogen compounds in the grain were evaluated. Catch crops results are for the period 2012 to 2015 while the results of spring barley after catch crops are for the years 2013 to 2016. The results were statistically processed by analysis of variance (ANOVA Statistica 12) and a subsequent use of Fisher's LSD post-hoc test at the significance level of 0.05.

Table 1 Monthly precipitation and average monthly temperature from Žabčice in the years 2012–2016

Year	Month											
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
	Precipitation (mm)											
2012	27.2	7.4	2.4	19.8	21.4	101.2	64.6	43.0	40.2	49.2	19.4	35.6
2013	20.2	42.1	40.8	20.2	109.0	147.4	4.7	43.6	63.2	35.2	20.4	6.2
2014	22.0	12.6	5.6	11.2	62.8	43.4	85.0	113.6	116.2	46.4	29.2	28.7
2015	20.0	7.4	28.0	9.4	33.8	22.4	22.4	106.0	23.8	48.0	24.8	17.2
2016	25.6	64.7	30.4	41.6	50.9	43.5	192.0	-	-	-	-	-
Norm. 61–90	24.8	24.9	23.9	33.2	62.8	68.6	57.1	54.3	35.5	31.8	36.8	26.3
	Temperature (°C)											
2012	1.0	-3.4	7.0	10.8	16.9	19.8	21.4	21.1	16.2	9.4	6.5	-1.2
2013	-1.0	0.7	1.8	10.6	14.7	18.3	21.9	20.3	13.9	10.1	5.3	2.1
2014	1.1	2.7	8.5	11.8	14.5	18.8	21.5	17.9	15.6	11.5	7.5	2.4
2015	1.8	1.6	5.5	10.1	14.7	19.1	22.9	23.6	15.9	9.6	6.2	2.9
2016	-1.2	5.1	5.5	9.9	15.7	19.8	21.3	-	-	-	-	-
Norm. 61–90	-2.0	0.2	4.3	9.6	14.6	17.7	19.3	18.6	14.7	9.5	4.1	0.0

RESULTS AND DISCUSSION

Results in Table 2 show the differences in the amount of dry mass from catch crops for the years 2012 to 2015 and among species of catch crops. Growth and development of catch crops is dependent on weather conditions in a given year. In all the tracked years, *Sinapis alba* reached the highest yields. Low yields of *Secale cereale v. multicaule* can be attributed to larger water need. Tables 3 and 4 also show the influence of these catch crops on the grain yield and quality of spring barley. For the years 2013 to 2016, the average highest grain yield of 7.02 t/ha was achieved for spring barley with the control variant without catch crops. Slightly lower grain yield of 6.88 t/ha was achieved after *Sinapis alba* while the lowest grain yield was recorded after the *Secale cereale v. multicaule*, namely 5.25 t/ha. In 2013, a statistically significant difference was in the grain yield of spring barley after catch crops. The grain yield of spring barley was even higher after *Sinapis alba* than in the variant without catch crops. In 2014, there was a statistically significant difference in the grain yield of spring barley between the variants after the *Secale cereale v. multicaule* (lower grain yield) and in control variant, after *Sinapis alba*. There was no statistically significant difference between the grain yield of spring barley after *Sinapis alba* and in the control variant. In 2015, there was a statistically significant difference in the grain yield between the spring barley in the control variant and after *Secale cereale v. Multicaule* or *Sinapis alba*. After the catch crops, there was a lower grain yield than in the control variant. In 2016, there was a higher grain yield of spring barley after *Sinapis alba* and in the control variant. There was no statistically significant difference between the grain yield of spring barley after *Sinapis alba* and control variant. With the exception of 2014, in each of the studied years, there was no statistically significant difference in the nitrogen content in grains of spring barley. When compared with the control variant, the lowest nitrogen content in grains of spring barley was, with the exception of 2015, reached after *Sinapis alba*. After *Secale cereale v. multicaule*, higher nitrogen content in grains of spring barley was recorded in almost all years. Nitrogen content in grains of spring barley after monitored catch crops was observed in the range of required values, but some risk can occur after *Secale cereale v. multicaule*. In years with

favourable precipitation, such as 2013 and 2016, after *Sinapis alba*, even higher grain yields of spring barley than in the control variant were recorded. Likewise, Rinnofner et al. (2008) and Gaweda (2012) in their studies found that catch crop water consumption has not significantly changed the yield of next crop. However, in case of water shortage during the winter and beginning of the growth season of spring barley, there is competition for water between the remnants of *Sinapis alba* and spring barley. It agrees with the claim of Gaweda (2011) that in a drier year, there is a risk of a decrease in yield of spring barley after *Sinapis alba*. The lowest yields of spring barley, even in a year with favourable precipitation, were recorded after *Secale cereale v. multicaule*, even though it forms a smaller amount of dry mass. A possible cause could be a reduced growth quality of the succeeding crop because *Secale cereale v. multicaule* is an overwintering crop.

Table 2 Dry mass yields of catch crops in the years 2012–2015

Dry mass yields of catch crops (t/ha)	2012	2013	2014	2015
Sinapis alba	4.28	3.67	1.60	1.13
Secale cereale v. multicaule	1.40	1.92	1.13	0.38
Average	2.84	2.80	1.37	0.76

Table 3 Grain yield of spring barley after catch crops in the years 2013–2016

Grain yield of spring barley (t/ha)	2013	2014	2015	2016	Average
Sinapis alba	6.73^{b}	6.82^{b}	7.59^{a}	6.37^{b}	6.88^{b}
Secale cereale v. multicaule	5.27^{a}	4.25^{a}	6.76^{a}	4.70^{a}	5.25^{a}
Control variant – without catch crops	6.43^{ab}	7.21^{b}	8.54^{b}	5.90^{b}	7.02^{b}

Legend: The results were statistically processed by analysis of variance (ANOVA Statistica 12) and a subsequent use of Fisher's LSD post-hoc test at the significance level of 0.05.

Table 4 Content of nitrogen compounds in grain of spring barley

Content of nitrogen compounds in grain of spring barley (%)	2013	2014	2015	2016	Average
Sinapis alba	10.00^{a}	10.85^{a}	10.80^{a}	10.75^{a}	10.65^{a}
Secale cereale v. multicaule	11.60^{a}	12.80^{b}	11.96^{a}	10.95^{a}	11.78^{b}
Control variant – without catch crops	11.35^{a}	11.40^{a}	10.56^{a}	11.25^{a}	11.14^{ab}

Legend: The results were statistically processed by analysis of variance (ANOVA Statistica 12) and a subsequent use of Fisher's LSD post-hoc test at the significance level of 0.05.

CONCLUSION

The trial studied two types of catch crops and their impact on the grain yield and quality of spring barley. *Sinapis alba* has produced more biomass than *Secale cereale v. multicaule*. The grain yield of spring barley is mainly influenced by the species of catch crops and in combination with a particular year. In the studied years, the *Sinapis alba*, except for a drier beginning of the year, had no negative effect on the grain yield of spring barley. The *Secale cereale v. multicaule* has regularly reduced the grain yield of spring barley. The content of nitrogen compounds in grains in comparison with the control was lower after *Sinapis alba* and higher after *Secale cereale v. multicaule*, but with the exception of 2014, none of these results were statistically significant. In one of the warmest and driest places in the Czech Republic, *Sinapis alba* seems to be the favourite. However, given the close cropping practices with a prevalence of *Brassica napus*, it is not appropriate to include *Sinapis alba*. The *Secale cereale v. multicaule* is a less suitable catch crop, which because of low yields cannot meet the goals for catch crop cultivation while it also reduces the grain yield of subsequent spring barley.

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GROWING OF VARIOUS SPECIES OF CATCH CROPS IN A MIXTURE

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Abstract: Importance of catch crops in crop production is multi-layered. However, not all catch crops provide the same benefits. The aim of the study was to monitor the yield of selected species of catch crops in a mixture, their biological differences, and the suitability of the mixture. The field trial was set up on a clay-loam fluvisol at a field experimental station in Žabčice (South Moravia, Czech Republic) in 2015. The trial included two mixtures of catch crops. The first mixture (Variant 1) is composed of *Sinapis alba*, *Phacelia tanacetifolia*, *Fagopyrum esculentum*, *Crambe abyssinica*, *Pisum sativum*, and *Vicia sativa*. The second mixture (Variant 2) includes no legumes and is composed of *Sinapis alba*, *Phacelia tanacetifolia*, *Fagopyrum esculentum*, and *Crambe abyssinica*. A statistically significant difference in dry matter yield was not observed among the mixtures of catch crops, but was recorded among the species of catch crops in the mixture. The highest yield and also rapid initial growth as well as good soil coverage was reached by *Phacelia tanacetifolia* and *Sinapis alba*. A similar growth dynamics also occurred in *Fagopyrum esculentum* that may be also incorporated into the mixture. Among *Pisum sativum* was the better choice. When deciding which species of catch crops to mix, it is necessary to respect biological differences among the different species of catch crops, especially similar growth dynamics, and their representation ratio.

Key Words: catch crops, yield, dry conditions

INTRODUCTION

Catch crops are crops grown between two main crops. Their importance in crop production is multi-layered. An important function of catch crops is enrichment of soil with organic matter and retention or binding of nitrogen. Significant application includes restoring of microbial life of the soil. Catch crops reduce the degree of compaction of our soils. Catch crops use rainfall in between growth periods to produce biomass and to reduce wind and water erosion as well as unproductive evaporation when compared with soils without vegetation cover. (Šlepetienė et al. 2007, Rinnofner et al. 2008, Chen et al. 2010, Scalise et al. 2015, Sparrow 2015) Catch crops act as breakers in crop rotation. They suppress weeds and reduce the spread and incidence of diseases and pests. (Murakami et al. 2000, Caner and Tuncer 2001, Romaneckas et al. 2012)

However, not all catch crops provide the same benefits. Individual species of catch crops often provide only one or two functions (White et al. 2015). Clark (2008) reported that mixtures of two or more catch crops are often more effective than planting a single species, for example to ensure the emergence of at least part of the crop under adverse conditions for germination, stabilizing the production of aboveground and belowground biomass, depending on weather conditions. However, determining the appropriate catch crops for the mixture and their seeding rates can be difficult (White et al. 2015). One of the fundamental factors influencing the successful establishment and subsequent development of crops is suitable choice of species in terms of growth dynamics and mixing ratio, such as predominance of less competitive species (Brant et al. 2015, White et al. 2015). Very important is the actual presentation of individual species at a site in relation to weather conditions and the manner of utilizing catch crops (Brant et al. 2015). Since 2015, there is a novelty in the Czech Republic called Greening (Payment for agricultural practices beneficial for climate and environment). To comply with the conditions of greening, a farmer can use catch crops cultivation, namely a mixture of catch crops.

The study aims to track the yields of selected species of catch crops in a mixture, their biological differences and suitability for growing in a mixture.

MATERIAL AND METHODS

The field experiment was set up on a clay-loam fluvisol at a field experimental station in Žabčice (South Moravia, Czech Republic) in 2015. The content of nutrients in the soil: P–140 mg/kg, K–336 mg/kg, Ca–5.513 mg/kg and pH/KCl–7.1 in soil depth 0–0.3 m. In layer 0.3–0.6 m the values are P–87 mg/kg, K–227 mg/kg, Ca–5.570 mg/kg and pH/KCl–7.3. Annual rainfall is 480 mm and the average annual temperature is 9.2 °C. This is one of the driest and warmest areas in the Czech Republic. Table 1 shows rainfall and average temperature during the reporting period, from sowing to sampling the aboveground mass of catch crops. The trial was set up as four replicates with the size of experimental plots of 10 m^2. It included two mixtures of catch crops. The first mixture (Variant 1) is composed of *Sinapis alba*, *Phacelia tanacetifolia*, *Fagopyrum esculentum*, *Crambe abyssinica*, *Pisum sativum*, and *Vicia sativa*. The second mixture (Variant 2) includes no legumes and is composed of *Sinapis alba*, *Phacelia tanacetifolia*, *Fagopyrum esculentum*, and *Crambe abyssinica*. The catch crop mixtures were planted after the harvest of winter wheat on August 17. The planting was done by sowing combination. After the catch crops, spring barley was grown. For determining the catch crop yield, a traditional sampling of fresh plant matter of the catch crops took place on October 22. The sampling of fresh plant matter of catch crops was conducted from 0.25 m^2 plot with four replications for each variant of catch crop mixture, and was followed by drying to a constant value. The results were statistically processed by analysis of variance (ANOVA Statistica 12) and a subsequent use of Fisher's LSD post-hoc test at the significance level of 0.05.

Figure 1 Rainfall and temperature from sowing after sampling of catch crops, Žabčice, CR, 2015

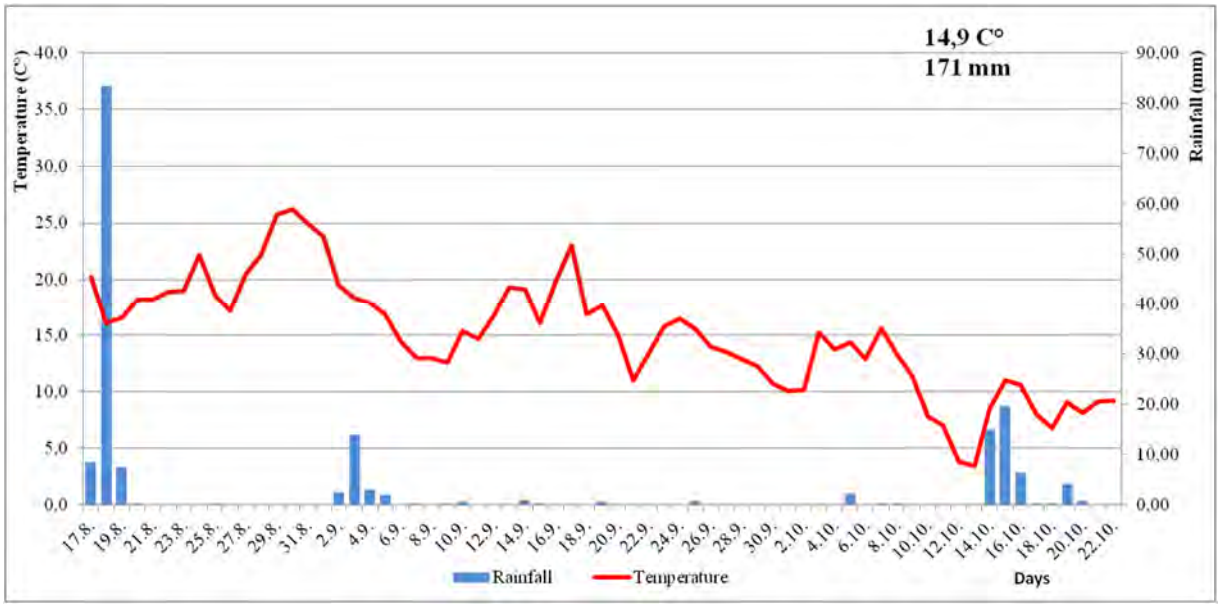

Table 1 Sowing individual species of catch crops in mixtures

Catch crops	Sowing species of catch crops in mixtures (kg/ha)	
	Variant 1	Variant 2
Sinapis alba	2	2
Phacelia tanacetifolia	7	7
Crambe abyssinica	3	3
Fagopyrum esculentum	20	20
Pisum sativum	20	-
Vicia sativa	20	-
Total	72	32

RESULTS AND DISCUSSION

In Table 2 shows the results of aboveground dry mass of the two mixtures of catch crops and individual species of catch crops in the mixture. A statistically significant difference in dry matter yield was not observed among the mixtures of catch crops, but was recorded among species of catch crops in the mixture. In the mixture of catch crops in Variant 1, *Phacelia tanacetifolia* and *Sinapis alba* achieved the highest yields. *Pisum sativum* and *Fagopyrum esculentum* achieved lower yields. Almost immeasurable yield was recorded for *Vicia sativa*. The Variant 2 mixture of catch crops included no legumes. The highest yield was recorded for *Sinapis alba*, *Phacelia tanacetifolia*, and *Fagopyrum esculentum*. *Crambe abyssinica* had the lowest yield. In general, catch crop yields are influenced mainly by the amount and distribution of rainfall and temperature. Chart number 1 indicates that in 2015, after sowing the catch crops, three days of rain brought nearly 90 mm of rain, which positively affected their initial growth and development. However, this was followed by unfavourable distribution of rainfall and this stress negatively affected all catch crops. As already mentioned, the highest yields in both mixtures were achieved by *Phacelia tanacetifolia*. Its seed quantity ratio in the mixture was the highest compared to the total amount of seed per hectare. *Phacelia tanacetifolia* and *Sinapis alba* in a mixture were characterized by rapid start of growth and good coverage as well as the highest biomass yield in a chosen seed rate. Despite the very small seed rate in the mixture compared to the total amount per hectare, *Sinapis alba* was very competitive and probably overcame some species in the mixture, as also presented by Bazzaz and Harper (1976). For *Sinapis alba*, we can choose a lower seed rate than the one tested. But in today's constricted rotations with predominance of *Brassica napus*, *Sinapis alba* cannot completely fulfil the function of protection against pests and diseases. It is possible to include *Sinapis alba* to the mixture for cereal crop rotation. *Crambe abyssinica*, a member of the Brassicaceae family just like the *Sinapis alba*, achieved very low yields of dry matter. It may be appropriate to include *Fagopyrum esculentum* in the mixture, as it was characterized by a very rapid start of growth and good soil coverage. Depending on the usage, it could be a disadvantage for *Fagopyrum esculentum* to be affected by the first autumn frosts, as some plants were brown at sampling, which led to loss of soil coverage. The more preferable legume than *Vicia sativa* in the mixture is *Pisum sativum*. The cause of low yield of *Vicia sativa* can be a poor growth due to lack of light from other competing catch crops as also stated by Clark (2008).

When deciding which species of catch crops to mix, it is necessary to respect the biological differences among the different species of catch crops, especially similar growth dynamics and their representation ratio. This agrees with Brant et al. (2015) and White et al. (2015). Cultivating properly blended mixtures of catch crops, we can combine more of their benefits together or eliminate their weaknesses even in conjunction with weather conditions.

Table 2 Yield of dry mass of the mixtures of catch crops

Catch crops	Yield of dry mass of catch crops (t/ha)	
	Variant 1	Variant 2
Sinapis alba	0.50^{bc}	0.65^{b}
Phacelia tanacetifolia	0.81^{c}	0.62^{b}
Crambe abyssinica	0.05^{a}	0.08^{a}
Fagopyrum esculentum	0.13^{a}	0.33^{ab}
Pisum sativum	0.21^{ab}	-
Vicia sativa	0.02^{a}	-
Total	1.71^{a}	1.68^{a}

Legend: The results were statistically processed by analysis of variance (ANOVA Statistica 12) and a subsequent use of Fisher's LSD post-hoc test at the significance level of 0.05.

CONCLUSION

The study aims to track the yields of selected species of catch crops in a mixture, their biological differences and suitability for growing in a mixture. Two mixtures of catch crops were monitored. The

first mixture was composed of *Phacelia tanacetifolia*, *Sinapis alba*, *Crambe abyssinica*, *Fagopyrum esculentum*, *Pisum sativum*, and *Vicia sativa*. The second mixture was composed of *Phacelia tanacetifolia*, *Sinapis alba*, *Crambe abyssinica*, and *Fagopyrum esculentum*. The yield results of dry matter of catch crops are from 2015. In one of the driest and warmest places in the Czech Republic, the results show that among the mixtures of catch crops, there was no statistically significant difference in their yields. Very important is the composition of the mixture depending on the biological differences among individual species of catch crops and especially on similar growth dynamics and their representation ratio. The highest yield and also rapid initial growth as well as good soil coverage was reached by *Phacelia tanacetifolia* and *Sinapis alba*. *Phacelia tanacetifolia* and *Sinapis alba* can be sown in a mixture at a lower dose than had been tested. It is not appropriate to include *Sinapis alba* in mixtures of catch crops in crop rotations with a predominance of *Brassica napus*. Similar growth dynamics was also experienced by *Fagopyrum esculentum*, which can be a good part of the catch crop mixture. Among the legumes, the most preferable is the *Pisum sativum* but to maximize the use of the fixation of atmospheric nitrogen, we need to increase the seed rate. Thus composed mixture of catch crops can then secure more necessary functions, such as a certainty of production of sufficient biomass, rapid initial growth, soil coverage, and fixation of atmospheric nitrogen.

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SPECIES COMPOSITION OF VASCULAR PLANTS IN SELECTED AGRI-ENVIRONMENTAL MEASURE

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Abstract: The contribution is focused on the evaluation of species composition of vascular plants of bio-belts and weed plants on the neighboring plots on the land blocks with the following crops: maize (*Zea mays*), soya (*Glycine max*), and spring barley (*Hordeum vulgare*). The monitored plots are located in the cadastral areas of Rostenice-Zvonovice and Hlubocany in South Moravian region. The total species composition and cover of weed plants were recorded in the field in July 2016. The total number of eleven weed species was found in the selected area. The most varied spectrum of species plants was found in bio-belts. The most frequently represented weed species was *Chenopodium album* which had also the highest mean cover in plots. Other weed species, *Avena fatua, Cirsium arvense* and *Convolvulus arvensis*, may represent menace for planted crops in the region.

Key Words: biodiversity, phytosociological plot, agriculture, environmentally friendly farming

INTRODUCTION

The problem of reducing biodiversity is considered as one of the major global problems (NATURE 2009). In many parts of Europe, agricultural landscapes are more than 2000 years old (Groppali 1993). The agricultural landscape in the Czech Republic, like in most of Europe, is the most common type of environment (occupies 54% of the state) (Marada et al. 2013). As part of agricultural landscape we can find significant landscape segments, for example fragments of native vegetation. Management systems that support the restoration and management of valuable habitats are usually expensive and therefore less viable (Demo et al. 2011).

Agri-environment schemes aim to counteract the negative-effects of modern agriculture on the environment by providing financial incentives to farmers for adopting environmentally friendly agricultural practices. Agri-environment schemes are considered the most important policy instruments to protect biodiversity in agricultural landscapes (EEA 2004). Donald and Evans (2006) argues that environmental measures can bring wider benefits. Agri-environment schemes could compensate some of the negative impacts on biodiversity agricultural habitats (Donald and Evans 2006).

In the Czech Republic, the agri-environmental measures are supported by the Rural Development Programme for years 2014–2020. One of them is to support the creation of bio-belts. A bio-belt is defined as a food line field of a width 6–24 meters. Seeds for the establishment of bio-belts comprise of spring cereals, buckwheat (*Fagopyrum esculentum*), millet (*Panicum miliaceum*), forage kale (*Brassica oleracea*) and from seeds of two other plants according to the choice of the bio-belt founder (Rural Development Programme for years 2014–2020). Bio-belts are among measures with the greatest benefits for biodiversity. They can provide food for a number of animal species, some bird species are even able to nest in bio-belts (Šarapatka 2008). The importance of bio-belts for the landscape is also great, because they increase the diversity and variety of landscapes, for example appropriately located on sloping land bio-belts can reduce soil erosion (Ministry of the Environment 2007).

MATERIAL AND METHODS

Characterization of selected area

The monitored areas are located in the cadastral areas of Rostenice-Zvonovice and Hlubocany in South Moravian region. Farmland in the cadastral area is farmed by Rostěnice Inc. and private

farmers. Rostenice Inc. founded monitored bio-belts. The lands are farmed in the conventional mode of agriculture.

The area is located in the climatic zone T2, which is characterized by long, hot and dry summer, a very short transition period to warm up slightly warm spring and autumn and short, moderately warm, dry to very dry winter with very short duration of snow cover (Quitt 1971). Geological bedrock is made up of loess and loess soil and is coverd by modal black soils on the surface.

Characterization of bio-belts

Bio-belts were sown in the period from 1. 4. to 31. 5. 2016. Their composition is as follows: *Fagopyrum esculentum*, *Triticum aestivum*, *Panicum miliaceum*, *Pisum sativum*, *Brassica oleracea* and *Phacelia tanacetifolia*. Three bio-belts were evaluated in July 2016. Bio-belts were located on land blocks with the following produced crops: maize (*Zea mays*), soya (*Glycine max*), and spring barley (*Hordeum vulgare*).

Evaluation of vegetation

Some preparatory works had to be conducted before the field research started. First, it was necessary to search for land blocks on which there are bio-belts. Subsequently, they were searched in LPIS (Public land register) and it was identified where to place phytosociological plot. Depending on the size of bio-belts, four or five phytosociological plots were recorded. Furthermore, phytosociological plots were recorded on the land block in the vicnicity of bio-belts and at the edge of the land block, where are no bio-belts, as a control. The plot size was 4 m^2. Total cover and list of plant species in plot with particular cover value were recorded in each of the phytosociological plots. Cover was estimated in percentage scale. Species composition of vascular plants and coverage were evaluated in Microsoft Excel. Plant names used in this text follow Kubát et al. (2002). Well-developed plants of *Amaranthus* genus were determined as *Amaranthus retroflexus*, but due to the difficult determination of *Amaranthus* species in young growth phases and due to the more species occurring in similar habitats, we present here only *Amaranthus* sp. on a genus level.

RESULTS AND DISCUSION

Land block with soya

The following tables (Table 1, Table 2 and Table 3) show the founded species on the land block with soya. 10 species of plants were found in bio-belts, including 5 species of weeds. 5 species of plants were found in the neighbouring land block, including 4 species of weeds. Weed species *Chenopodium album*, *Cirsium arvense*, *Galium aparine*, *Equisetum arvense*, *Amaranthus* sp. were found on the land block with soya in bio-belt. Weed species *Chenopodium album*, *Echinochloa crus-galli* and *Equisetum arvense* were found in the vicinity of bio-belt on the same land block. The same weed species were found on the edge of the land block with soya.

Table 1 Species in bio-belt on the land block with soya

Number of phytosociological plot	1	2	3	4	5
Species name	Cover within plot (%)				
*Fagopyrum esculentum**	15	15	15	25	25
*Triticum aestivum**	5	20	8	15	15
*Panicum miliaceum**	5	15	5	10	10
*Pisum sativum**	2	5	3	10	5
*Brassica oleracea**	-	1	1	2	-
Chenopodium album	10	20	5	15	3
Cirsium arvense	-	2	-	1	1
Galium aparine	-	-	-	3	-
Equisetum arvense	-	-	-	-	40
Amaranthus sp.	-	15	20	2	-

Legend: *the symbol * marks plants sown in bio-belts, the same in the following tables*

Table 2 Species on the land block in the vicinity of bio-belt on the land block with soya

Number of phytosociological plot	1	2	3	4	5
Species name	Cover within plot (%)				
Glycine max (crop)	60	60	55	65	70
Chenopodium album	5	10	8	8	20
Echinochloa crus-galli	2	3	2	10	15
Galium aparine	1	3	-	2	3

Table 3 Species on the edge of the land block (without bio-belt) on the land block with soya

Number of phytosociological plot	1	2	3	4	5
Species name	Cover within plot (%)				
Glycine max (crop)	50	60	90	70	70
Chenopodium album	3	2	14	15	5
Echinochloa crus-galli	1	3	2	3	1
Equisetum arvense	-	-	-	-	2

Land block with maize

The following tables (Table 4, Table 5 and Table 6) show the founded species on the land block with maize. 9 species of plants were found in bio-belts, including 4 species of weeds. 6 species of plants were found in the land block, including 5 species of weeds. Weed species *Chenopodium album*, *Cirsium arvense*, *Amaranthus* sp. were found in bio-belt on the land block with maize. *Echinochloa crus-galli* was the only species that was found in the vicinity of bio-belt on the same land block. Species *Lolium multiflorum*, *Convolvulus arvensis*, *Amaranthus* sp., *Calystegia sepium*, *Echinochloa crus-galli* were found on the edge of the land block (without bio-belt).

Table 4 Species in bio-belt on the land block with maize

Number of phytosociological plot	1	2	3	4
Species name	Cover within plot (%)			
Fagopyrum esculentum[*]	15	10	10	8
Triticum aestivum[*]	20	15	10	10
Phacelia tanacetifolia[*]	-	-	3	3
Panicum miliaceum[*]	10	10	5	5
Pisum sativum[*]	10	5	5	5
Chenopodium album	35	20	40	50
Cirsium arvense	-	-	3	0
Amaranthus sp.	5	3	2	3
Seedling of *Acer negundo*	-	-	2	-

Table 5 Species on the land block in the vicinity of bio-belt on the land block with maize

Number of phytosociological plot	1	2	3	4
Species name	Cover within plot (%)			
Zea mays (crop)	60	75	75	75
Echinochloa crus-galli	2	1	2	1

Table 6 Species on the edge of the land block (without bio-belt) on the land block with maize

Number of phytosociological plot	1	2	3	4
Species name	Cover within plot (%)			
Zea mays (crop)	60	70	75	75
Lolium multiflorum	1	1	-	0
Convolvulus arvensis	2	1	-	0
Amaranthus sp.	-	1	-	1
Calystegia sepium	-	1	-	-
Echinochloa crus-galli	-	-	1	1

Land block with spring barley

The following tables (Table 7, Table 8 and Table 9) show the founded species on the land block with spring barley. 7 species of plants were found in bio-belts, including 3 species of weeds. 6 species of plants were found in the land block, including 5 species of weeds. Species *Chenopodium album*, *Amaranthus* sp., *Equisetum arvense* were found in bio-belt on the land block with spring barley. Two species were found in the vicinity of bio-belt on the same land block, *Cirsium arvense* and *Avena fatua*. Species *Galium aparine*, *Chenopodium album*, *Convolvulus arvensis* and *Avena fatua* were found on the edge of the land block.

Table 7 Species in bio-belt on the land block with spring barley

Number of phytosociological plot	1	2	3	4	5
Species name	Cover within plot (%)				
Triticum aestivum[*]	10	15	10	5	10
Panicum miliaceum[*]	10	5	5	8	5
Pisum sativum[*]	5	2	5	4	5
Fagopyrum esculentum[*]	15	20	15	10	10
Chenopodium album	20	30	40	25	30
Amaranthus sp.	15	3	2	5	10
Equisetum arvense	-	-	-	-	1

Table 8 Species on the land block in the vicinity of bio-belt on the land block with spring barley

Number of phytosociological plot	1	2	3	4	5
Species name	Cover within plot (%)				
Hordeum vulgare (crop)	100	100	100	100	100
Cirsium arvense	-	1	2	-	-
Avena fatua	-	-	-	-	3

Table 9 Species on the edge of the land block (without bio-belt) on the land block with spring barley

Number of phytosociological plot	1	2	3	4	5
Species name	Cover within plot (%)				
Hordeum vulgare (crop)	100	100	100	100	100
Galium aparine	5	4	2	-	-
Chenopodium album	2	2	3	-	-
Convolvulus arvensis	1	1	-	-	-
Avena fatua	-	-	1	-	2

Some differences were observed among appearing weed species during the field survey. The smallest differences were observed among weed species in bio-belts. Weed species *Chenopodium album* and *Amaranthus* sp. were found in all three bio-belts, whereas *Cirsium arvense* was found only in bio-belts on the land blocks with soya and maize. *Equisetum arvense* occurred in bio-belts on the land blocks with soya and spring barley. *Acer negundo* is not a typical weed, but was found rarely as a seedling in bio-belts on the soil block with maize. It probably got in bio-belt from the nearby windbreak.

There is no distinguishable difference among weed species found in bio-belts, in the vicinity of bio-belts and at the edge on the land block. The exception can be perhaps the presence of *Echinochloa*

crus-galli which was found as a weed species in soya and maize, but it wasn't found in any bio-belts. *Echinochloa crus-galli* is a typical weed of wide-row crops, especially maize. *Echinochloa crus-galli* is also a late spring annual weed species, so there is a very low possibility to expand into full-grown vegetation of bio-belts during field observations. Further, *Avena fatua* is a typical weed of spring cereals. Our results confirm it by presence of *Avena fatua* only in spring barley and absence in all of the other plots. *Amaranthus* sp. was found only in bio-belts and it was not present neither in the vicinity of bio-belts or on the edge of the land block. Based on the one observation we can conclude that is very uncommon that this species does not spread in the nearby crops. However, further studies should be performed to prove the spreadability of *Amaranthus retroflexus* to the crops.

Weeds in bio-belts are considered as a source of weeds for the surrounding arable land. Spreading of weed species from bio-belts to the crop was not recorded. However, weeds can enrich the soil seed bank and it can cause a weed infestation of the area in the coming years. It may be problematic especially for the species which have fruits and seeds spread by wind (*Cirsium arvense*), or species that produce large amounts of seeds with long viability in the soil (*Chenopodium album*).

CONCLUSION

A total of 11 weed species was found during the field research. The most frequently represented weed species was *Chenopodium album* which had also the highest coverage. *Avena fatua, Cirsium arvense* and *Convolvulus arvensis* belongs to the weed species that can menace crop plants.

The most varied spectrum of species of plants was found in bio-belts. Bio-belts are designed to promote plant and animal biodiversity. With the founding of bio-belts are connected by certain rules that must be respected by the farmer. No plant protection products or fertilizers should be applied in bio-belts and the nearest vicinity . Farmers can be dissuaded these rules from establishing bio-belts. There is also the possibility of potential expansion of undesirable weed plants.

Problems of the influence of bio-belts to weed infestation of crops are not yet sufficiently explored. It would be appropriate to examine this issue more closely.

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EFFECT OF DROUGHT STRESS ON SELECTED WINTER WHEAT YIELD FORMATION COMPONENTS WITHIN POT AND FIELD EXPERIMENTAL DESIGN

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Abstract: The object of this study was to find out what is the behaviour of the same winter wheat variety (Bohemia) plants cultivated within pot and field experiment. Therefore, the main aim of this study was to verify (based on the pot experiment results) whether the pot experiment (that is limited by the soil area) does not substantially affect plant reactions. The pot experiment was carried out in growth chambers where daily temperature course, relative humidity (RH) and photosynthetically active radiation (PAR) were set via protocols. The pots were exposed to the drought stress for 14 days with the daily maximum temperature 26 °C from noon to 2 p.m. The pots were split into 2 groups: (1) *Dry* where the soil moisture within pots were maintained below 30% of the maximum water holding capacity, (2) *Wet* where the soil moisture did not decrease below 70% of the maximum water holding capacity. The plants within *Wet* variant were considered as a control group. The pots were placed onto the concrete floor of a vegetation hall (where the plants were exposed to the weather conditions) prior and after stress regime exposition. The field experiment was conducted within experimental station in Bystřice nad Pernštejnem belonging to the Bohemian-Moravian Highlands in the Czech Republic. The drought stress was established through the transparent roofs installed above plants' tops level in the field. The control experimental plot without roofs was nearby there as well. The plants were harvested when the full maturity was reached and the selected yield formation components were evaluated.

Key Words: grains number, growth chamber, Thousand Grain Weight (TGW), water shortage, winter wheat

INTRODUCTION

The Czech agricultural production is being increasingly faced with a problem of agricultural drought. The reason is an increase in the mean annual air temperature (the increase of 1.3 °C in the mean annual air temperature over the last 170 years), the total precipitation amount is still the same (Žalud 2016), but the precipitation distribution has been only changing over time (Trnka et al. 2014). However, there is increased tendency to lower soil moisture and the drought periods and higher temperatures are expected to be more frequent in the future too (Trnka et al. 2014). The risk of field crops yield decrease is supposed to increase as a consequence (Trnka et al. 2014, Žalud 2016). The wheat is the second most cultivated cereal all around the world (Reyer et al. 2013, Trnka et al. 2014), and it is the most grown cereal in the Czech Republic (Czech Statistical Office 2016). In order to understand better to the risk, we joint the experimental efforts to understand consequences of drought and increased temperatures. Most of our experiments has to be conducted as a pot experiments in order to use control climate of climate chambers but pot experiment can lead to bias compared to the field conditions. In order to explore this issue, we compared the results from 2 various experiments (pot vs. field experiment) with the same winter wheat variety (Bohemia). The main aim was to find out whether the winter wheat variety Bohemia cultivated in pots responses in the same way as Bohemia cultivated on fields, i.e. whether the results of pot experiments are applicable also on the field conditions. The plants were cultivated due to

the demands on nutrients and water that differed in the pots and in the field. The differences among the selected yield formation components (thousand grain weight – further abbreviated as TGW – and grains number – further abbreviated as GN) and percentage yields reductions were compared for these purposes.

MATERIAL AND METHODS

Experiment 1: Pot experiment design

Bohemia winter wheat variety belongs to the modern varieties reaching high yields within the conditions of the Czech Republic and also belonged to the winter wheat varieties with the largest seed production area in 2014 (CISTA 2015) and the second one in 2015 (CISTA 2016a). The Bohemia winter wheat variety seeds were sown (2 seeds per 1 pot) on 22 October 2014 into black plastic pots (inner dimensions: 10.5 × 10.5 × 21.5 cm). The soil used for pots filling came from the experimental station in Polkovice (altitude 199 m a.s.l.) belonging to Moravia in the Czech Republic. The soil type was qualified as a luvic chernozem with loess as a mother substrate. The pots were placed onto the concrete floor of a vegetation hall at Mendel University in Brno where the pots were exposed to ambient weather conditions until reaching the heading stage (Table 2). The data coming from the near climatological station (located at the arboretum of Mendel University in Brno: 239 m a.s.l.) was used for assessing of the weather conditions during the pots placement at Mendel University in Brno. This data included the daily temperature courses and daily precipitation amount. In relation to the precipitation and temperature course, the pots were irrigated if needed and the irrigation amounts were read from 2 garden rain gauges (the value was calculated as an arithmetic mean) placed directly within the experimental plot (Table 4). The pots were surrounded by the expanded clay to protect them from freezing. Fungicides and insecticides were also applied to protect plants against diseases and pests at doses recommended by the producers. The nitrogen doses were applied once (see Table 3)The pots were transported to Global Change Research Institute CAS on 15 May 2015 and put into one growth chamber FytoScope FS-SI 3400 model (Photon Systems Instruments LLC, www.psi.cz) for acclimation at the heading stage of development (18 May 2015, BBCH 51–52). The daily temperature course, PAR (photosynthetically active radiation) and RH (relative humidity) protocols, running within the chamber, are presented lower (see Table 1), and the values of individual environmental factors changed continuously between two time points. The drought stress regime was started on 21 May 2015 at BBCH 55–57 developmental stage. The pots were divided into 2 groups: well-watered (*Wet*) and drought stressed (*Dry*) with 7 replications (pots). The actual volumetric soil moisture was measured using ThetaProbe Soil Moisture Sensor (Delta-T Devices Ltd, http://www.delta-t.co.uk) for feedback control of irrigation of *Dry* variant. The soil moisture was maintained below 30% of the maximum water holding capacity within the pots of the drought stressed (*Dry*) variant, and it was maintained to not decrease approximately below 70% in the case of the well-watered (*Wet*) variant (the pots were still maintained wet, else irrigated). The pots were transported back to the vegetation hall of Mendel University in Brno after 14 days of stress conditions exposition within the growth chamber. The plants were harvested manually at the full maturity (July 2015).

Table 1 Protocols within the growth chamber – daily air temperature course (t, presented in °C), photosynthetically active radiation (PAR, presented in µmol/m^2/s), relative humidity (RH, presented in %)

Time	t [°C]	PAR [µmol/m^2/s]	RH [%]
0:00	20	0	85
4:00	18	0	90
6:00	18	0	90
12:00	26	1500	45
14:00	26	1500	45
20:00	22	0	75
0:00	20	0	85

Experiment 2: Field experiment design

The field experiment was carried out at the experimental station in Bystřice nad Pernštejnem (560 m a.s.l.). The soil type was qualified as a dystric cambisol and soil fraction was loamy. The Bohemia winter wheat variety seeds were sown on 30 September 2014 (see Table 2). Herbicides and fungicide were applied to protect plants against weed and diseases at doses recommended by the producers. The nitrogen doses were applied three times (see Table 3). The field experiment consisted of small plots (3.1 × 8 m) in two variants and three repetitions. The first variant *Wet plot* was uncovered, i.e. all ambient rainwater turned out there. The second variant *Dry plot* was covered through mobile rain-out shelters. Mobile rain-out shelters diverted all (100%) ambient rainwater away from coverage of the plots. The corrugated material polycarbonate Suntuf (PALRAM Ltd. – IL, UK) with clear colour was used for manufacture of the roofs. The thickness of polycarbonate Suntuf was 0.8 mm. The polycarbonate trapeze is impermeable to UV. The manufacturer states that the transmitted spectral composition of solar radiation, except UV, of this material is 90%. Mobile rain-out shelters were installed during part of the vegetation season (from 19 May 2015 to 6 August 2015), and crops were exposed to drought stress during that period. Harvested area was middle part of each plot (1.5 × 8 m) to avoid borderline effect to yield components. The sensors TDR (time domain reflectometry, CS 616, Campbell Scientific Inc., Shepshed, UK) were installed within *Wet* and *Dry plots* to measure soil moisture to the depth of 30 cm. Temperature and humidity were also measured – from 14 July 2015 to 6 August 2015 – both within *Wet* and *Dry plots*. The temperature within *Dry plot* was 0.37% higher in comparison to the *Wet plot* and the relative humidity was 5.97% higher within the *Dry plot.* Other meteorological parameters were monitored by meteorological station located near the field experiment. This data included daily temperature and daily precipitation amount as in the case of the pot experiment (see Table 4).

Yield formation components

Grains number (GN) per each main spike and numbers of tillers per 1 m^2 was counted for pot experiment. Grains weight per each main spike at actual moisture and as a dry matter was found out using balances with accuracy of 0.001 g. TGW was evaluated by the dry matter conversion into 14% moisture and grains weights were assessed by the actual-moisture weight also recalculated to 14% moisture where actual moisture (Act.m.) was calculated based on CISTA (2016b). The final TGW at 14% moisture ($TGW_{14\%}$) was calculated by the following equation (eq. 1):

$$TGW_{14\%} = \frac{TGW_{Act.m.} \times (100 - Act.m.)}{100 - 14} \quad (1)$$

Number of tillers and number of grains per spike (GN) were counted also within the field experiment. Thousand grain weights were also determined and recalculated to 14% moisture by the eq. 1. Grains weight per each main spike at actual moisture and as a dry matter was found out using balances with accuracy of 0.1 g.

The pot and field experiment sites comparison overview

The plants within pot and field experiment (i.e. in Brno and Bystřice) had various phenological stages onsets (see Table 2). Both experiments were fertilized by N fertilizers during vegetation season (see Table 3). The mean temperatures and precipitation amounts were also different across the experimental sites (see Table 4).

Table 2 The comparison of the actions related to the phenological stages onsets within pot and field experiment

Event	Brno	Bystřice
Sowing date	22 October 2014	30 September 2014
1st germination	10 November 2014	13 October 2014
Shooting	23 April 2015	27 April 2015
Heading	18 May 2015	2 June 2015
Harvesting	14 July 2015	6 August 2015

Table 3 Nitrogen doses treatments and application dates for A) pot experiment and B) field experiment that were different due to the conditions of cultivation

Application date	Fertilizer applied	N [kg/ha]
17 March 2015	NH_4NO_3	90
Application date	Fertilizer applied	N [kg/ha]
9 March 2015	DASA	35
19 March 2015	LAV	25
30 April 2015	LAV	60

Table 4 The total precipitation amounts, mean irrigations (pot experiment) and the mean air temperatures during the experiments durations (to be continued on the next page)

Month, year	Total precipitation [mm]		Total irrigation [mm]	Mean air temperature [°C]	
	Bystřice	Brno	Brno	Bystřice	Brno
October 2014	29.2	46.3	–	8.9	10.6
November 2014	32.6	19.8	19.9	5.6	7
December 2014	37.2	19.6	–	0.4	2.1
January 2015	42.4	16	–	-0.5	1.2
February 2015	7.0	4.9	–	-0.7	1.2
March 2015	46.0	24.4	–	3.5	5.1
April 2015	14.6	6.3	14.1	7.3	9.3
May 2015	38.2	40.8	19.3 *	11.5	13.5
June 2015	17.7	30.2	35.9 *	16.0	18.1
July 2015	56.4	29.5	53.3	19.9	21.8

*Legend: * 15 May 2015 – 17 June 2015: plants were put into the chambers where the actual soil moisture was controlled by the ThetaProbe Soil Moisture Sensor (Delta-T Devices Ltd, http://www.delta-t.co.uk) for feedback control of irrigation. The soil moisture was maintained below 30% of the maximum water-holding capacity within Dry variant, and to not decrease below 70% of water-holding capacity within Wet variant. The last irrigation of pots was carried out on 13 May 2015 before transportation to the chambers. The following irrigation after transportation back to the vegetation hall was carried out on 18 June 2015*

RESULTS AND DISCUSSION

Pot and field experiment results

The descriptive statistics data sets are presented for number of grains (GN) and thousand grain weights (TGW) values both for pot and field experiment (see Table 5 and 6). The data sets within tabular outputs are compared separately both for pot and field experiment.

Table 5 The descriptive statistics of GN data sets (from left to right): arithmetic mean, median, standard deviation, minimum and maximum value for A) pot and B) field experiment

A)	Mean	Me	σ_{GN}	GN_{min}	GN_{max}
Wet	34.4	34.0	4.2	27.0	43.0
Dry	32.8	34.0	5.2	23.0	41.0
B)	Mean	Me	σ_{GN}	GN_{min}	GN_{max}
Wet	31.3	31.0	7.8	14.0	49.0
Dry	31.4	31.0	10.6	13.0	64.0

Table 6 The descriptive statistics of TGW data sets (from left to right): arithmetic mean, median, standard deviation, minimum and maximum value for A) pot and B) field experiment

A)	Mean	Me	σ_{TGW}	TGW_{min}	TGW_{max}
Wet	50.5	51.3	6.0	39.5	59.1
Dry	46.5	51.2	12.1	25.2	58.9
B)	Mean	Me	σ_{TGW}	TGW_{min}	TGW_{max}
Wet	49.7	50.1	1.2	47.6	51.3
Dry	48.8	48.9	0.2	48.5	49.0

When the mean GN values from the presented experiments are compared, the higher value was found out for pot experiment both within *Dry plots* (1.4 pcs higher than in the field experiment) and *Wet plots* (3.1 pcs higher for pot experiment). If the GN mean values within *Dry plots* are related to the corresponding *Wet plots*, the mean GN value for pot experiment reached 95.3% of the *Wet plot* and it was 100.4% of the *Wet plot* within field experiment. Unlike the mean GN values for *Dry plots*, the mean TGW values for *Dry plots* evinced the higher mean value within field experiment than the pot experiment (2.3 g higher value for field experiment). On the other hand, the mean TGW values for *Wet plots* were recorded to be higher for pot experiment (0.9 g higher value). When the TGW mean values within *Dry plots* are related to the corresponding *Wet plots*, the mean TGW value for pot experiment reached 91.9% of the *Wet plot* and it was 98.2% of the *Wet plot* within field experiment. The total grain yield was reduced by 36.2% within *Dry plot* compared to *Wet plot* in the case of the pot as well as field experiment. The number of tillers per $1m^2$ was 181.4 within *Dry plot* and 298 within *Wet plot* of the pot experiment design and these mean values were 459 and 458 for field experiment, respectively. The yield of Bohemia winter wheat variety is based especially on a TGW value and less also on a number of grains per spike. It is the variety of a high A quality, with big sizes of grains and less number of tillers (CISTA 2016a). Based on the three-year results (2013–2015), the mean Bohemia TGW value was 50 g within CISTA experimental plots (CISTA 2016a) and it was even 52 g within the years 2011–2014 (CISTA 2015). The lower mean TGW value (48–50 g) was found out during long-term experiments of the company Agrotest fyto in Kroměříž (Palík et al. 2009). Dierauer and Stöppler–Zimmer (1994) obtained the mean wheat TGW ranging from 45.1 g (early sowing – 19 September) to 41.7 g (late sowing – 31 October) within ecological agriculture soil management. Horčička et al. (2012) recorded a value of the mean grain number per winter wheat spike from 41.46 (at number of 430 spikes per 1 m^2) to 37.32 (at number of 581 spikes per 1 m^2) in relation to the number of spikes per 1 m^2 within the experiments carried out in 2008–2011. They found out that the more spikes per 1 m^2 is, the smaller the number of grains per spike is. This fact corresponds to the obtained mean values within pot as well as field experiment.

CONCLUSION

The percentage yield reductions within pot and field experiments were approximately the same value as well as the mean GN and TGW values were very similar. The pot as well as field experiments are going to be established also in the following years.

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EFFECT OF HIGH TEMPERATURE AND WATER SHORTAGE STRESSES DURATION DURING ANTHESIS ON THE SELECTED WINTER WHEAT YIELD FORMATION COMPONENTS

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Abstract: The aim of this study was to assess the effect of drought and high temperatures on Tobak winter wheat variety during one of the most sensitive developmental stage (anthesis) from the viewpoint of harvest index (HI) and spike productivity (SP). The 5 growth chambers (where the plants were exposed to these stress factors) were used for these purposes. The various protocols consisting in photosynthetically active radiation (PAR) course, relative air humidity (RH) and daily temperature courses were run. The plants were divided into 2 groups within each growth chambers: (1) Drough-stressed (*Dry*) and (2) well-watered (*Wet*). Two lengths of stresses duration were tested: 3 and 7 days. The plants were exposed to ambient weather conditions up to the full maturity after stresses exposition within the growth chambers. Subsequently, the plants were harvested manually and HI and SP were evaluated. The statistical analyses showed that the effect of each stress factor separately was statistically significant both for HI and SP14, nevertheless, these two factors interaction was statistically significant only in the case of HI.

Key Words: growth chamber, harvest index (HI), spike productivity, Tobak, winter wheat

INTRODUCTION

The pilot study covering the whole Europe region (Trnka et al. 2014) showed that although the climatic estimations face with high uncertainty, the most of the significant agricultural regions will be affected by events related to great yields decreases (both the increase in frequency or drought intensity and also higher risk of the critical temperatures within sensitive phenological stages of wheat, especially during the time of anthesis and following stages). Recently, not a lot of studies dealing with systematic quantification of short periods of high temperatures impacts on winter wheat as the most important cereal within the middle Europe region. A wheat is known to be very sensitive to the extreme high temperatures during reproduction stage (Saini et al. 1983, Marcellos and Single 1984, Alghabari et al. 2014, Vara Prasad and Djanaguiraman 2014). The higher frequency of the high temperature at anthesis stage is expected in Europe (Semenov and Shewry 2011, Stratonovitch and Semenov 2015) in relation to the global climatic change. The temperatures above 30 °C may cause the complete grains sterility (Saini and Aspinall 1982). The temperature stress at the anthesis stage has also the serious impact on the number and grain size (Saini et al. 1983). A good indicator of field crops responses to the climatic changes is so called harvest index, further abbreviated as HI (Ludlow and Muchow 1993, Hay 1995), expressing the ratio of a grain yield mass and a rest of the above-ground biomass (Huehn 1993). Therefore, the objective of this study was to evaluate the effect of drought and high temperatures on Tobak winter wheat variety during anthesis, as one of the most sensitive developmental stages, from the viewpoint of harvest index (HI) and spike productivity (SP).

MATERIAL AND METHODS

The black plastic pots of the inner dimensions of 10.5 × 10.5 × 21.5 cm were used for the Tobak seeds sowing at the dose of 2 seeds per 1 pot on 10 October 2015. Prior the sowing, the pots were filled using the soil from the experimental station in Polkovice (altitude 199 m a.s.l.) belonging to Moravia in the Czech Republic. The soil type was qualified as a luvic chernozem with loess as a mother substrate. The pots were placed onto the concrete floor of a vegetation hall at Mendel University in Brno where the pots were exposed to ambient weather conditions until reaching the anthesis. The plants were irrigated if needed to support the growth and development. Subsequently, the Tobak plants were transported to the 5 growth chambers (FytoScope FS-SI 3400 model; Photon Systems Instruments LLC, www.psi.cz) of Global Change Research Institute CAS (Brno, Czech Republic) at BBCH 61 (beginning of flowering: 10% of flowers are opened), see details lower (Table 1a, b). The length of high temperature and water regime (*Wet* vs. *Dry*) exposition was also studied. The 130 plants in total were exposed to the stress conditions for the period of 3 days (designated as +3) and 7 days (designated as +7). The pots number was 9 per *Wet* and 9 per *Dry* variant within the control chamber (t_{max} = 26 °C), the pots numbers in other chambers were 7 within *Dry* and 7 within *Wet* per 1 stresses exposition length (i.e. the same number per stresses length exposition 3 days and 7 days). *Wet* variant plants were irrigated on the soil layer of the pots and to small bowls placed under the pots using garden hose until the pots' soil seemed to be sufficiently wet. *Dry* variant plants were irrigated only on the soil layer of the pots with water dose of 100 ml per 1 pot. The actual volumetric soil moisture was controlled using ThetaProbe Soil Moisture Sensor (Delta-T Devices Ltd, http://www.delta-t.co.uk) within *Dry* variants to maintain the volumetric soil moisture about 15%. The particular plants were gradually moved from their actual growth chamber to the chamber with the controlled environmental condition (t_{max} = 26 °C) after stresses exposition finishing within the regime +3 and subsequently +7. After ending of the stress regimes, the plants were transported to the vegetation hall when were placed up to their manually harvest at the full maturity stage.

Table 1a Growth chambers protocols: air temperature protocols – the t_{max} represents maximum temperature within particular growth chamber; the particular environmental factors changed continuously between two time points

Time	t_{max} = 26 °C	t_{max} = 32 °C	t_{max} = 35 °C	t_{max} = 38 °C
0:00–4:00	20–18	20–18	20–18	20–18
4:00–6:00	18	18	18	18
6:00–12:00	18–26	18–32	18–35	18–38
12:00–14:00	26	32	35	38
14:00–20:00	26–22	32–22	35–22	38–22
20:00–24:00	22–20	22–20	22–20	22–20

Table 1b Growth chambers protocols: photosynthetically active radiation (PAR) and relative air humidity (RH); the particular environmental factors changed continuously between two time points

Time	PAR [µmol/m^2/s] *	RH [%] **	RH [%] ***
0:00–4:00	0	85–90	85–90
4:00–6:00	0	90	90
6:00–12:00	0–1500	90–50	90–75
12:00–14:00	1500	50	75
14:00–20:00	1500–0	50–75	75
20:00–24:00	0	75–85	75–85

*Legend: * valid for the chambers with t_{max} = 26, 32, 35, 38 °C; ** valid for the chambers with t_{max} = 26, 32, 35, 38 °C; *** valid for the chamber designated as 38K with t_{max} = 38 °C*

The harvest indices (HI) and spike productivities at 14% moisture (SP14) were assessed for the main spikes within pots. At first, the weights of the above-ground biomass parts were found out on the scales with accuracy of 0.001 g. The grains weights were also assessed. The harvest index was calculated

as the ratio of the grains weight (X) and the sum of the weights of grains, straws, leaves and rest of spikes without grains (S) by the following equation – eq. 1 (Huehn 1993, Pennington 2013):

$$HI = X/(X + S) \quad (1)$$

Subsequently, the grains were dried out at 70 °C for 48 hours (to avoid the grains fertility ability). Then, their weights were found out again and the moisture at the harvesting (*act.m.*) was calculated (see Hellevang 1995 or CISTA 2013). The final spike productivity was assessed at 14% moisture (*SP14*) was established by the recalculation of the grains weights with moisture at the harvesting (*SPact.m.*) by the recalculation to the standardized moisture (CISTA 2013) by the following equation (eq. 2):

$$SP14 = (SPact.m. \times (100 - act.m.))/(100 - 14) \quad (2)$$

RESULTS AND DISCUSSION

Arithmetic means and standard deviations (SD) are presented, subsequently, the results of statistical evaluations are shown both for harvest indices (HI) and spike productivity at 14% moisture (SP14). The statistical analyses (two-way ANOVA summary and subsequent Tukey's HSD test that was also calculated for one-way ANOVA analyses) were performed separately for HI and SP14 data sets. At first, these results for HI are presented (see Table 2 and Table 3a, b). The mean minimal HI value was reached within the chamber with t_{max} = 38 °C within *Wet* variant plants exposed to the stress regimes for the period of 7 days, while mean maximum was recognized within the chamber with t_{max} = 35 °C within *Dry* variant plants exposed to the stress regimes for the period of 7 days.

Table 2 Arithmetic means and standard deviations of HI values

Temperature	D+3		D+7		W+3		W+7	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
26	0.458	0.042	0.458	0.042	0.482	0.036	0.482	0.036
32	0.404	0.057	0.459	0.069	0.496	0.017	0.458	0.062
35	0.473	0.043	0.517	0.016	0.494	0.028	0.493	0.013
38	0.420	0.054	0.415	0.061	0.469	0.033	0.382	0.087
38 ****	0.446	0.055	0.441	0.084	0.495	0.037	0.384	0.070

*Legend: **** valid for the chamber designated as 38K with t_{max} = 38 °C but with the different daily RH course*

When field crops are exposed to serious stress conditions (water, temperature, floods, diseases), the very low HI values may be expected (Comeau and Barnett 1979, Hay 1995). The pot experiment carried out in Australia can be mentioned as an example. The HI values for wheat cultivars exposed to serious water stress ranged between 0.45 to the value less than 0.1 in the mentioned experiment (Passioura 1977), while in another experiment (also from Australia), the decrease in HI values of plants grown inside greenhouse was noticed to be significant but milder with HI values between 0.49 and 0.36 (Davidson and Birch 1978). The HI values of a common wheat (*Triticum aestivum* L.) within the Czech Republic mostly range close to 0.5 (Konvalina et al. 2010).

The statistical analysis of HI data set was also performed (see Table 3a, b lower). The summary results of the two-way ANOVA (Table 3a) showed statistically significant effect both temperature and water regime (*Wet* versus *Dry*) factor as well as these stress factors interaction.

Table 3a A summary of two-way interactions in ANOVA for HI data set

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Temperature	4	0.167	0.042	14.543	< 0.001
WaterRegime	3	0.078	0.026	9.025	< 0.001
Temperature:WaterRegime	12	0.104	0.009	3.015	< 0.001
Residuals	232	0.667	0.003		

Since the two-way ANOVA proved the statistical significance of the factors effects studied, the subsequent Tukey's HSD test was performed to show how the differences are statistically significant (see Table 3b). Tukey's HSD tests for one-way ANOVA analyses are also presented to show the interactions among particular temperatures as well as water regimes.

*Table 3b Tukey's HSD tests of one-way and two-way ANOVA analyses for HI data set showing differences among stress variants; the values denoted by *, ** and *** mean the significance level for p-values p < 0.05, p < 0.01 and p < 0.001, respectively; only statistically significant differences are presented; the most significant statistical difference is highlighted as bold*

Interaction	*p*-value	Interaction	*p*-value
38-26	< 0.001***	38:W+7-35:D+7	< 0.001***
35-32	0.001*	38K:W+7-35:D+7	< 0.001***
38-32	0.016*	32:W+3-38:D+7	0.041*
38-35	**< 0.001*****	35:W+3-38:D+7	0.032*
38K-35	< 0.001***	38K:W+3-38:D+7	0.036*
W+3-D+3	< 0.001***	35:W+7-38:D+7	0.026*
W+3-D+7	0.026*	38:W+7-26:W+3	< 0.001
W+7-W+3	< 0.001***	38K:W+7-26:W+3	0.001**
38:W+7-26:D+3	0.03*	38:W+7-32:W+3	< 0.001***
35:D+7-32:D+3	< 0.001	38K:W+7-32:W+3	< 0.001***
26:W+3-32:D+3	0.031*	38:W+7-35:W+3	< 0.001***
32:W+3-32:D+3	0.013*	38K:W+7-35:W+3	< 0.001***
35:W+3-32:D+3	0.009**	38:W+7-38:W+3	0.011*
38K:W+3-32:D+3	0.011*	38K:W+7-38:W+3	0.032*
26:W+7-32:D+3	0.031*	38:W+7-38K:W+3	< 0.001***
35:W+7-32:D+3	0.008**	38K:W+7-38K:W+3	< 0.001***
38:W+7-35:D+3	0.005**	38:W+7-26:W+7	< 0.001***
38K:W+7-35:D+3	0.017*	38K:W+7-26:W+7	< 0.001***
35:D+7-38:D+3	0.002**	38:W+7-32:W+7	0.04*
38:W+7-26:D+7	0.03*	38:W+7-35:W+7	< 0.001***
38:W+7-32:D+7	0.031*	38K:W+7-35:W+7	< 0.001***
38:D+7-35:D+7	< 0.001***		

If the temperature factor is assessed separately, the most significant statistical difference was found out among the chambers with t_{max} = 35 and 38 °C. When the water regime factor is evaluated separately, the most significant statistical difference was recorded among the *Wet* regimes with the exposition lengths for 3 and 7 days, the less significant statistical difference was found out among the regimes *Wet* and *Dry* with the exposition lengths of 3 days. The most significant statistical difference was found out among the *Wet* variant in the chamber with t_{max} = 38 °C and *Dry* variant in the chamber with t_{max} = 35 °C – both with exposition to the stress factors for 7 days.

At first, the arithmetic means and standard deviations of SP14 are presented (see Table 4).

Table 4 Arithmetic means and standard deviations of SP14 values

Temperature	D+3		D+7		W+3		W+7	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
26	1.274	0.645	1.274	0.645	1.604	0.356	1.604	0.356
32	1.041	0.305	1.361	0.398	1.701	0.372	1.336	0.555
35	1.615	0.469	1.756	0.165	1.721	0.177	1.657	0.293
38	1.160	0.382	1.104	0.305	1.359	0.489	1.078	0.479
38K	1.343	0.514	1.239	0.178	1.626	0.422	1.082	0.562

The mean minimal SP14 value was reached within the chamber with t_{max} = 32 °C within *Dry* variant plants exposed to the stress regimes for the period of 3 days, while mean maximum was recognized within the chamber with t_{max} = 35 °C within *Dry* variant plants exposed to the stress regimes for the period of 7 days.

The statistical analysis of SP14 data set was also performed (see Table 5a, b lower). It is obvious that when the stress factors are tested separately, the effect is statistically significant, whereas their interaction is statistically insignificant (Table 5a). Therefore, the separate effects of the particular temperatures, particular stress regimes and also interactions of these two factors can show the effects by the better way (Table 5b). Tukey's HSD tests of one-way ANOVA (separate effect of temperature as well as water regime was evaluated) and also Tukey's HSD test of two-way ANOVA (interaction of these two stress factors evaluation) are presented for these purposes (see summary in Table 5b).

Table 5a A summary of two-way interactions in ANOVA for SP14 data set

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Temperature	4	7.103	1.776	8.465	< 0.001
WaterRegime	3	3.700	1.233	5.879	< 0.001
Temperature:WaterRegime	12	3.229	0.269	1.283	0.229
Residuals	244	51.184	0.210		

*Table 5b Tukey's HSD tests of one-way and two-way ANOVA analyses for SP14 data set showing differences among stress variants; the values denoted by *, ** and *** mean the significance level for p-values p < 0.05, p < 0.01 and p < 0.001, respectively; only statistically significant differences are presented; the most significant statistical difference is highlighted as bold*

Interaction	*p*-value
35-26	0.029*
38-26	0.025*
35-32	0.003**
38-35	**< 0.001***
38K-35	0.002**
W+3-D+3	< 0.001
W+3-D+7	0.009**
W+7-W+3	0.015*
35:D+7-32:D+3	0.023*
38:W+7-35:D+7	0.028*
38K:W+7-35:D+7	0.047*

When the temperature stress factor effect is evaluated separately, the most significant statistical difference was recorded among the chambers with t_{max} = 35 and 38 °C. When only water regime effect is evaluated, the most significant statistical difference was among the regimes *Wet* and *Dry* with the exposition to this factor for 3 days. If the interaction of both stress factors is evaluated, the most significant statistical difference was found out among the varieties within *Dry* variants at the chambers with tmax = 32 and 35 °C with exposition lengths to these factors for 3 and 7 days, respectively.

CONCLUSION

When the factors were evaluated separately, the most significant statistical difference was recorded among the chambers with t_{max} = 35 and 38 °C and almost the same significance level was recorded among *Wet* variants with exposition lengths of 3 days for HI as well as SP14 data sets. The most significant statistical difference was found out among the *Wet* variant in the chamber with maximum temperature 38 °C and *Dry* variant in the chamber with t_{max} = 35 °C – both with exposition to the stress factors for 7 days – for HI data, and it was among the varieties within *Dry* variants at the chambers with t_{max} = 32 and 35 °C with exposition lengths to these factors for 3 and 7 days, respectively, for SP14 data set.

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INFLUENCE OF SELENIUM NANOPARTICLES AND SODIUM SELENITE ON THE ANTIOXIDANT POTENTIAL AND YIELDS OF RED CLOVER

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Abstract: In this study, we examined the application of selenium in various forms and doses on the growth of red clover. The first experimental factor was the form of selenium – sodium selenite and selenium nanoparticles modified pork gelatin. The second experimental factor was dose selenium – 0, 2 and 20 mg/m^2. Sampling was conducted at an interval of 14 days. It has been shown that high doses of sodium selenite and selenium nanoparticles decrease the yield of green clover. Conversely used form of selenium had no effect on the yield and other indicators.

Key Words: Trifolium pratense L., selenium, yield, GSH, forage

INTRODUCTION

Selenium (Se) is a widely studied a trace element and its role in plant growth and physiology are well documented. At low concentrations plays a protective role in abiotic stress tolerance, while higher concentrations show phytotoxicity. Plant species differ markedly ability to scavenge and accumulate selenium. Insufficient supply of the organism that element leads to many diseases (Hasanuzzaman et al. 2014, Kaur et al. 2014, Wu et al. 2015). It is part of selenoproteins (e.g. glutathione) prevents oxidative destruction the biological diaphragms. Its deficiency causes therefore weakening the overall health status (Horký et al. 2016, Skaličková et al. 2016).

Selenium nanoparticles exhibit excellent biological activity and low toxicity (Zhang et al. 2001, Wang et al. 2007).

The tripeptide glutathione in animal and plant cells is represented in a high concentration and contributes to the elimination of free radicals. The reduced form of glutathione (GSH) in cells involved in protective and detoxification processes (Wünschiers 2012, Fajt et al. 2009).

The perennial red clover belong to the family *Fabacae* and this is one of the most commonly used clover for feed purposes. It is very suitable for grazing use, because it contains high-quality and digestible protein. Deep roots allow it to grow even during the summer and drought (Graves et al. 2012).

The aim of this study was to determine the effect of foliar application of selenium in various forms and doses on antioxidant status and forage yield of red clover.

MATERIALS AND METHODS

The experiment was established as a pot experiment in climate chamber CLF PlantMaster – CLF Plant Climatics (Wertingen, Germany). Mode was set to 24 °C day temperature, 20 °C night temperature, humidity of 65% throughout the day, duration of sunshine 12 hours, light intensity of 300 µm/m/s. To provide the experiment was chosen species red clover (*Trifolium pratense* L.), two forms of selenium (sodium selenite and selenium nanoparticles modified pork gelatin). It was used as substrate grass substrate with silica sand. To each container was weighed 500 g substrate, 0.05 g of red clover

seed and supplemented by additional 50 g of substrate. The experiment was maintained only watering demineralized water as needed, until the cover is fully engaged.

37 days after the establishment of the experiment was performed application of solutions of various concentrations outside climate chamber (Table 1). On the day of application were prepared solutions and with the help of hand sprayer applied to individual groups of homogeneous test plants. The plants were then left for 24 hours after application in standard conditions (22 °C, 70% relative humidity) and then displaced back to climate chamber.

Table 1 Forms and dose applied solutions (mg/m^2)

Factor	Dose of Se
Control	0
A – Sodium selenite	2
B – Sodium selenite	20
C – Selenium nanoparticles modified pork gelatin	2
D – Selenium nanoparticles modified pork gelatin	20

The first samples were taken on the day of application of selenium. Other sampling took place 14 and 28 days after application. Overhead phytomass from each container was trimmed to 1 cm stubble height, weighed and immediately after sampling deep-frozen. The vegetation was nursed only watering demineralized water evenly in all groups. Continuously throughout the duration of the experiment was weeding vegetation..

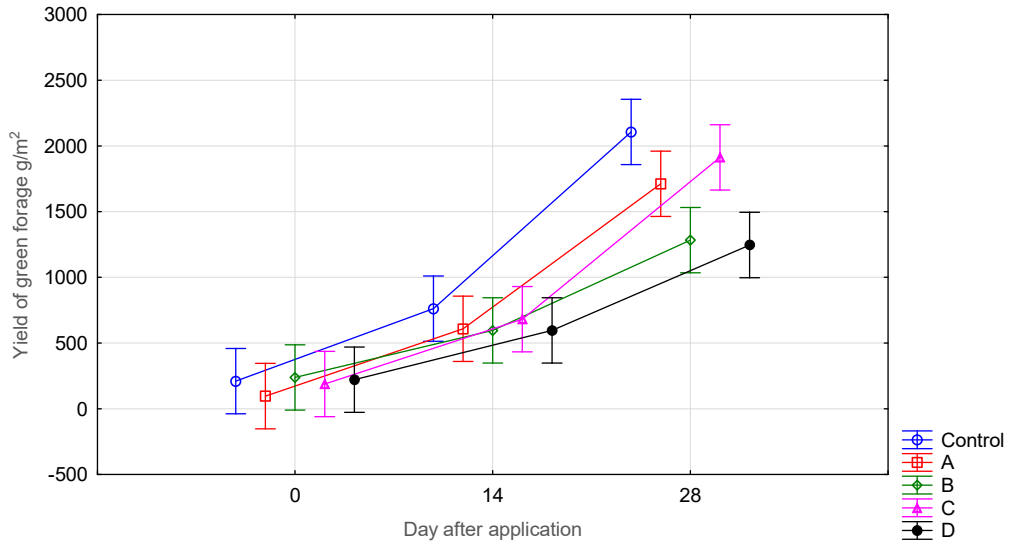
To optimize the determination of the reduced form of glutathione (GSH) was used flow injection analysis with electrochemical detection system (FIA-ED). GSH was determined by HPLC-ED (Potěšil et al. 2005).

The results were processed in the STATISTICA 10 CZ (Czech Republic) using a multifactor analysis of variance ANOVA. Differences were considered significant at a P <0.05.

RESULTS AND DISCUSSION

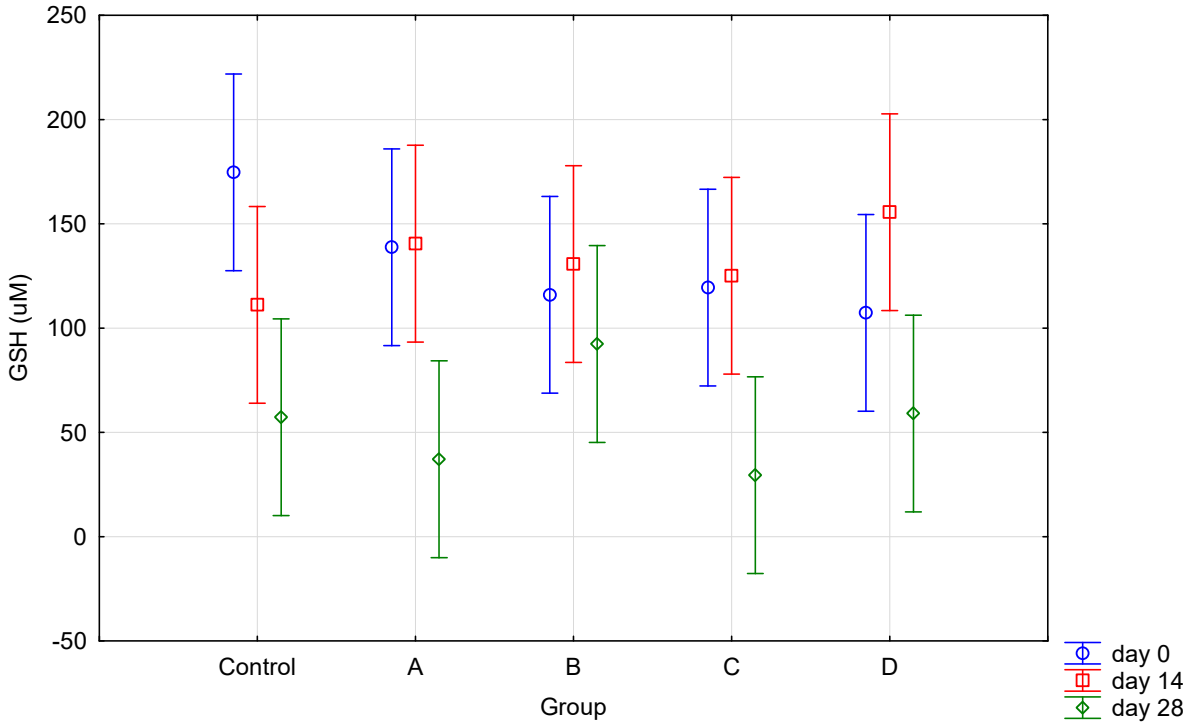
Yield of green forage clover was the day of application and the first collection of all experimental groups balanced. 14 days after application of selenium yield increased independent of dose and form of selenium. 28 days after application is observed a lower yield of green forage in groups B and D with selenium dose of 20 mg/m^2, compared to the control group. Conversely application selenite and selenium particles at a dose of 2 mg/m^2 had no significant effect on the yield of red clover (Figure 1).

Figure 1 Effect of selenium in various forms and dose on the yield of red clover (g/m^2)



Legend: A – selenium as selenite 2 mg/m^2; B – selenium as selenite 20 mg/m^2; C – selenium as selenium nanoparticles 2 mg/m^2; D – selenium as selenium nanoparticles 20 mg/m^2. Error bars indicate 95% confidence interval.

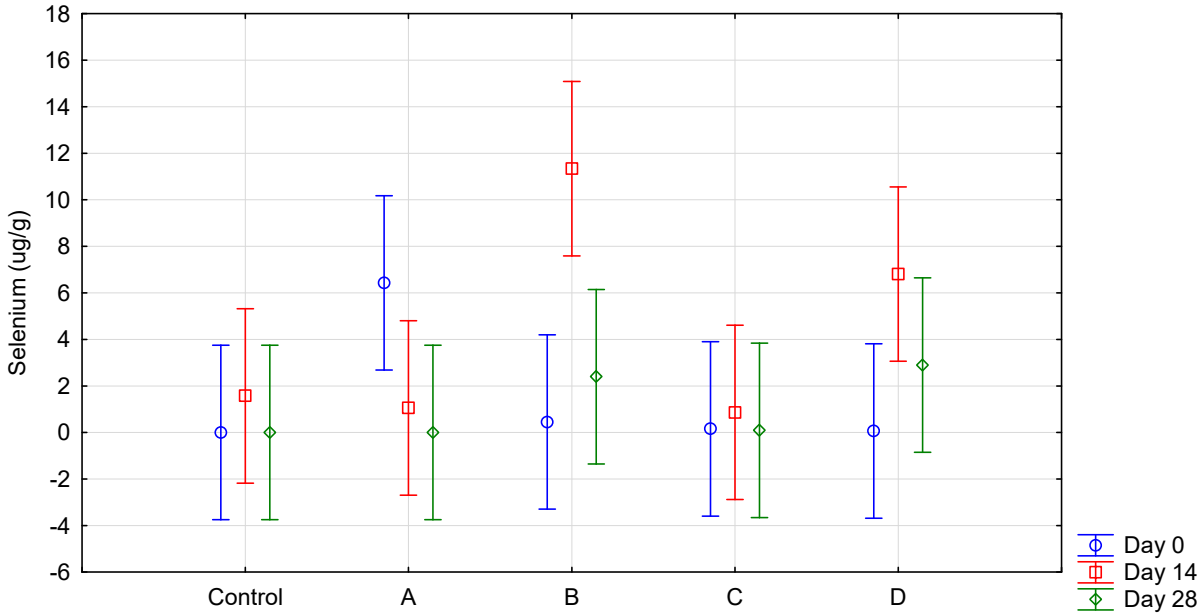
Figure 2 Effect of selenium in various forms and dose on the GSH of red clover (µM)



Legend: A – selenium as selenite 2 mg/m²; B – selenium as selenite 20 mg/m²; C – selenium as selenium nanoparticles 2 mg/m²; D – selenium as selenium nanoparticles 20 mg/m². Error bars indicate 95% confidence interval.

GSH content as indicators of antioxidant potential of the organism is illustrated in Figure 2. For the control group is the marked reduction in value between the first and third collection. Generally observed in all other groups, reduction of GSH values in time. It does not depend on the dose used or the form of selenium, after application temporarily GSH values slightly increase (P> 0.05), then there is a reduction values. A GSH level varies depending on the selenium content in forage (Figure 3). After application increases the value of selenium in the phytomass and over time these values decrease as selenium in the organism metabolized and stored in the root system.

Figure 3 Effect of selenium in various forms and dose on the selenium contain of red clover (µg/g)



Legend: A – selenium as selenite 2 mg/m²; B – selenium as selenite 20 mg/m²; C – selenium as selenium nanoparticles 2 mg/m²; D – selenium as selenium nanoparticles 20 mg/m². Error bars indicate 95% confidence interval.

CONCLUSION

Our results show that selenium affects the selenium content in the biomass and the value of GSH in the first two weeks after application. It was also observed decrease of the yield on treated crops of selenium. Used form of selenium had no significant impact on the evaluation indicators (yield, GSH and selenium content). It can therefore be understood selenium nanoparticles as an alternative form of selenium in nutrition of plants.

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EFFECT OF SOWN PASTURES ON NITROGENOUS SUBSTANCE CONTENT IN THE FORAGE

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Abstract: The aim of the diploma thesis was to analyse the effect of seeding on forage quality as well as the participation of added varieties of the clover meadow and other hybrids. Sowing machines with different intensity of the original turf were chosen for seeding. The types of sowing machines were included: GP TP–300, SE 2–024 and PP–2. The crop yield of dry basis, the content of nitrogenous substances, a fibre and NEL were evaluated. The values were specified using the method of Spektroscopy in the near infrared area (NIR Systems 6500). Participation of added species was evaluated using the projective dominance method. Significance difference in the content of growth nitrogenous substances was not proved in the added varieties but the content of CP was different with regard to the sequence of mowings ($P<0.05$). The seeding of clover meadow had a positive influence on the content of NEL in the growth phase ($P<0.05$). It is recommended to add Felina variety to a pasture for horses because of its suitability for seeding and providing advantages such as resistance to trampling or appropriate hibernation. It also provides high crop yield and due to its structure and nutrient content can be used for horse feeding.

Key Words: seeding, pasture for horses, pasture renewal

INTRODUCTION

The pastures are communities of plants that were formed during a long historical development. Since the beginning, the basis for livelihoods especially domesticated animals have been created, but in addition to them, for example, forest wildlife. Typical grazing animals include horses characterized by specific requirements for grassland (Skládanka et al. 2010).

We often call pastures the semi-natural growths. They are a colourful herb-grass communities. Pastures formed from the feed of valuable species, their composition, and especially the quality, maintain long years, they are very valuable (Šrámek 2001).

The quality of pasture growth is a crucial factor in the security of animal nutrition. Pasture quality is influenced by many factors. Habitat conditions, the composition of the grassland, growth stage, the use of meadows and pastures, and many others belong to them. Forage quality can also affect many pratotechnic hits. The grassland and forests with a high proportion of valuable species can regenerate through seeding (Skládanka et al. 2010).

The grassland can be supplemented with a number of valuable species, and thereby increase its nutritional value using seeding method. Different technologies are used for seeding. In practice, we were mainly interested in these parametres the stand – pulp, nitrogenous substances, NEL. The contents of folders are most affected by the composition of the vegetation, thus seeding success, mows rank and utilitarian year (Šrámek 2001).

The nitrogenous substances content in the crop should be viewed in terms of the type of farm animal and its needs. Other composition of grassland can be useful for cattle and other horses for

example. It should be considered the category of animals for which the pasture is intended. In the case of meadow vegetation, the vegetation used to produce hay and the difference in use, for example clovergrass or so meadow vegetation.

Therefore, the aim is to create more productive and qualitative area with long-term effect. In the case of plant introduction it concerns of the increase of grassland species diversity in the selected locations (Kohoutek 2007).

MATERIALS AND METHODS

The experimental area

The experimental station is located in the Vatín (CZE), which is a part of the forage research station of the Institute of Animal Nutrition and Forage Mendel University. The Vatín village is located at the link between Ždár nad Sázavou (the distance of 5 km) and Velké Meziříčí (the distance of 24 km) and belongs to Vysočina region. The complex of research station is located on the Czech-Moravian highlands at an altitude of 430–450 m above sea level (Hrabě and Buchgraber 2009). The optimal annual rainfall for grassland is between 700–800 mm and 400–500 mm, which is usual during the growing season (Skládanka et al. 2009). Monthly precipitation of Vatín stations are shown in Table 1.

Table 1 Monthly precipitation for Vatín stations in 2012 and 2013

Year	m	1	2	3	4	5	6	7	8	9	10	11	12	Summary
2012	S	102.6	40.6	21.2	27.4	42.4	49.7	131.8	67.8	50	50.4	30.8	70.2	657.9
	N	45.4	30.6	41.6	38	66.5	75	79.5	62.5	53.2	38.4	40.6	46.2	617.5
2013	S	84.1	55	36.5	19.9	102.2	119	32.3	77.7	79.7	46.6	24.9	27.3	705.2
	N	45.4	30.6	41.6	38	66.5	75	79.5	62.5	53.2	38.4	40.6	46.2	617.5

Legend: m – month, S – rainfall in mm, N – Long-term rainfall normal for the years 1961–1990 mm

Temperature is important for plant growth affecting vegetation throughout the year. The temperature of 17–20 °C can be regarded for the optimum growht. The average monthly temperature of stations Vatín is shown in Table 2.

Table 2 Average monthly temperatures for Vatín stations in 2012 and 2013

Year	m	1	2	3	4	5	6	7	8	9	10	11	12	Average
2012	T	-1.6	-6.8	-0.3	6.9	13.3	16.1	17.4	17.1	12.1	6.5	4.3	-3.1	6.8
	N	-3.3	-1.7	2.1	6.6	12.2	14.9	16.4	16.3	12	7.2	1.8	-1.5	6.9
2013	T	-2.3	-0.2	-1.1	6.9	10.9	14.7	18.1	17.3	11.1	8.8	3.5	0.4	7.34
	N	-3.3	-1.7	2.1	6.6	12.2	14.9	16.4	16.3	12	7.2	1.8	-1.5	6.9

Legend: m – Month, T– temperature in °C, N – long-term average air temperature for the years 1961–1990

Species

Individual varieties were double-seeded into a semi-natural permanent grassland (dominant species were *Taraxacum officinale, Phleum pratense, Dactylis glomerata* L.) in seed rate 35 kg/ha. Seeding was implemeted in two Term: 16 June 2012 (Term of double-seeding 1) and 18 July 2012 (Term of double-seeding 2).

Amos variety is tetraploid, medium-early variety of red clover. It is used as a component in clover stands and also suitable for seeding into unploughed meadows and pastures. Amos proves a high yield of green mass and even in drought conditions. The variety is resistant to *Erysiphe polygoni*. It is a perennial in the second year of the utility, the rate of regrowth after mowing shows to be high. Amos also produces high-quality material with a lower fiber content but higher content of soluble sugars and very high in protein and slow aging.

Suez variety is diploid early to mid-early variety of red clover. It has medium rapid growth in the spring. Suez is used in the classic crop rotation system as a biennial. Suez is suitable for clover and meadow vegetation. Suez is moderately resistant to diseases and lodging.

Felina is variety suitable for meadow usage. In the spring, it quickly develops. It is intergeneric hybrid ryegrass and fescue and it is festucoid type of festulolium. It is appropriate for preparation of grass-clover mixture, which is resistant to disease, drought and high water table.

Hostyn is tetraploid variety for meadow use. Rapid grow in the spring, after mows it grows moderately fast. Medium length of the flag leaf, wide, medium green leaf color. Resistant variety against snow mold, less resistant to rusts. High forage yield. It is intergeneric hybrid fescue and perennial ryegrass and it is loloid type of festulolium.

Machines for seeding

Land intendent to double-seeding is mowed on the low stable and afterward, all the mass is taken out. Before seeding, fertilization should be applied to increase their competitiveness of the original grassland. In the case of a large growth of the weeds, we can treate with Roundup applied on a rate of 0.5–1.0 liters per 200 liters of water. Seeding can be performed three weeks after the application of Roundup (Skládanka et al. 2010).

Seeding machine SE 2–024

It is a belt seeder, which partially distorts the original sward. The machine is used to slit sowing grass and clover mixtures for regeneration and seeding of meadows and pastures. Grass mixture is sown into treated tape rotation coulter with a width of 4–6 cm. The depth can be modified with regard to the sown seed but usually of 2–4 cm. Seeds are stored at the bottom of the groove and covered with flying soil.

Seeding machine GP TP 300

The sod is not disturbed during the seedbed preparation of full width thus reducing side effects of erosion. The preparation is proved by cutting the land because the intrusion is not into mulch seedbed. Thrust washers in the rear of the machine provide a complete closed seeding slots and thus create the optimal conditions for germination. The machine designe allow seeding in slope without any difficulties. The machine is equipped with a fine nap seed wheels. Starting dose of mineral fertilizer can also be applied at sowing customer.

Seeder PP-8 (PP-2 for use in small plot trials)

Drill PP-8 can perform seeding in the same range as shallow surface seeding. The exceptions are stony soils and soils with bedrock protruding from the profile, finely dispersed backbone is no barrier to the use of PP-2 machine. The machine rotary mills of 8 slots in the spacing of 45 cm. Width of milled row is 15−17 cm, depth loosening could be set to 5−15 cm. Seeds are sown on the surface milling line or disc coulters and subsequently incorporated in the profile milling line. The machine is equipped with a pneumatic drill device.

Sampling and sample evaluation, forage quality evaluation using NIRS

The methods of near infrared spectroscopy (NIR Systems 6500) were used to evaluate the quality of the forage. We found out the content of crude protein.

The samples, coming from Vatin small plot experiments, were used for evaluating the quality of forage taken immediately after harvest, and dried at 60 °C and then homogenized to a particle size of 1 mm.

NIR Systems 6500 is machine for samples evaluating without need of degradation or depletion. The sample should contain the C-H, N-H, S-H and O-H, measured at a concentration of more than 1 $g.kg^{-1}$. You can measure samples of all three states at normal temperature. It is a secondary method and must be calibrated. The evaluation of the sample is very fast; it does not take more than 2 minutes.

RESULTS AND DISCUSSION

We are focused on effect of double-seeded variety of red clover and grasses intergeneric hybrid during the monitoring of effect of CP (g/kg) content in harvested forage in the first and second crop year. We have taken into consideration the influence of the used drill and order cuts. The experiment was established in two sowing dates. The higher differences in the content of CP in forage have been

reached in the order of cuts. Supreme crude protein content (P>0.05), identically in both years, was evaluated in the third mowing, to 195.03 g/kg crude protein.

Hrabě and Buchgraber (2009) found out that the concentration of essential nutrients decreases with increasing fiber content. For example crude protein decreases from 200 g/kg up to 70 g/kg. Aging of forage is important for the forage digestibility, availability and hygiene.

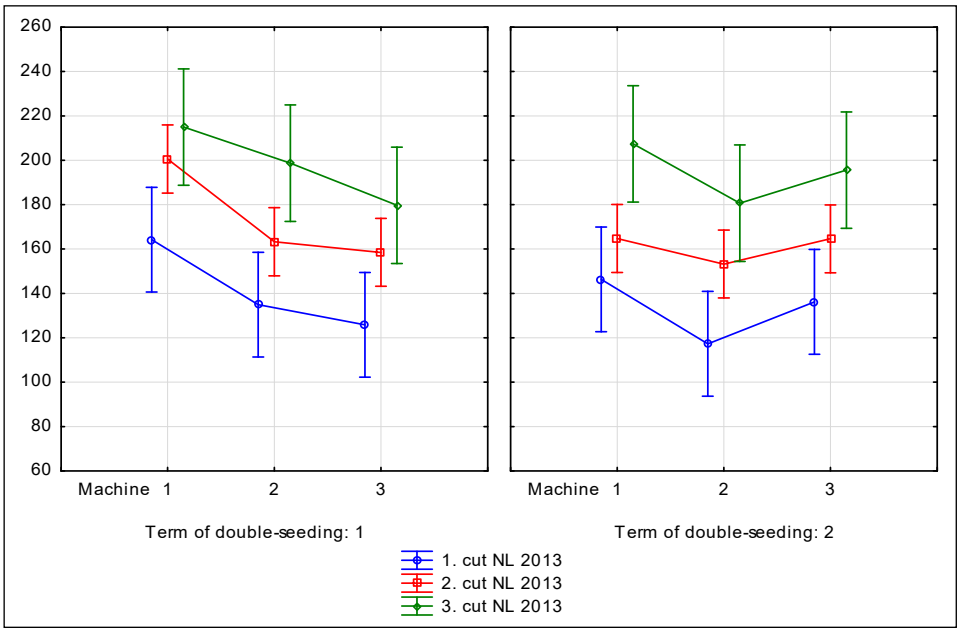
The relatively high nitrate content can be monitored in Amos variety, which in the third mowing reaches values above of 200 g/kg (Figure 1). An interesting result is very similar to Hostýn variety (Figure 2).

Table 3 Effect of variety, seed technology, rank and date of establishment cuts to crude protein content (g/kg) in the stand in 2012 and 2013

Factor	Content of Crude protein (g/kg)	
	2012	2013
Variety		
Amos	167.34 [a]	167.04 [a]
Suez	159.43 [a]	162.32 [ab]
Felina	161.91 [a]	171.13 [a]
Hostyn	150.34 [b]	156.98 [b]
Technology – Machine		
SE 2–024	155.69	159.49 [a]
GP TP 300	162.42	158.73 [b]
PP – 2	161.16	164.89 [ab]
Order cuts		
1.	131.53 [a]	128.71 [a]
2.	166.36 [b]	169.37 [b]
3.	181.37 [c]	195.03 [c]
Term of double-seeding		
1/ 2012	159.33	164.05
2/ 2012	160.18	164.68

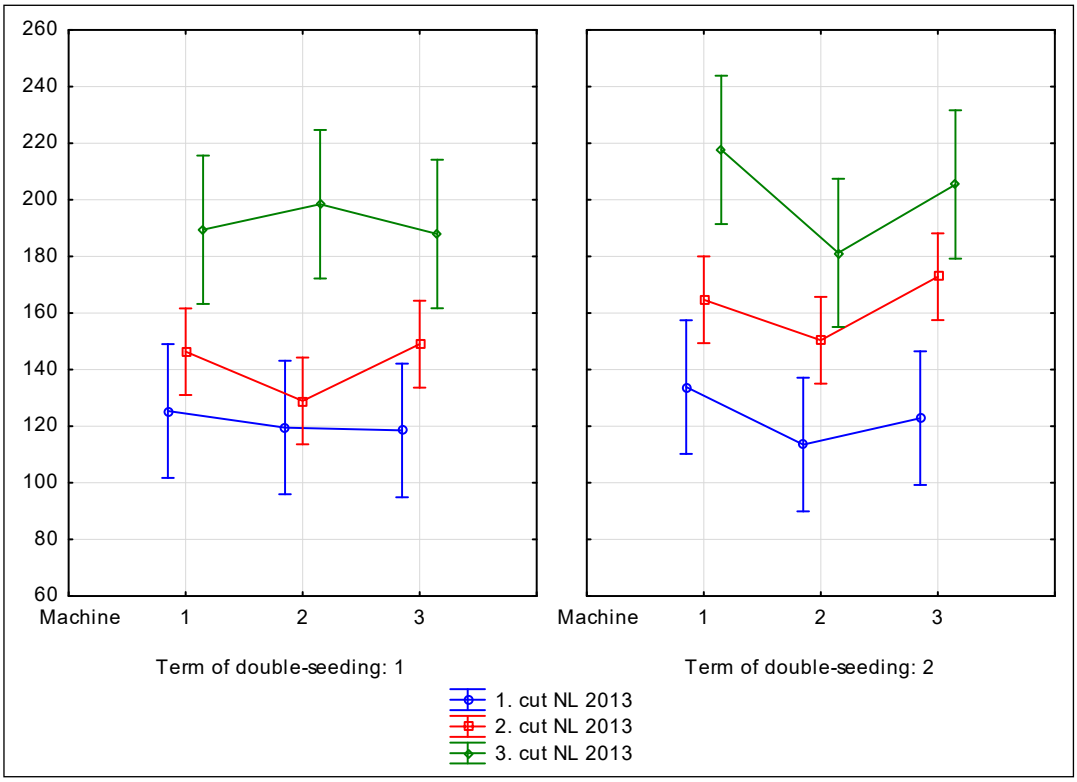
Legend: Among the average values of various indexes (a, b, c) in the columns is significant difference at p <0.05

Figure 1 Contents of nitrogenous substances (g/kg) in dry matter in the stand with more cut variety AMOS (Trifolium pratense L.), depending on the drill from 1 to 3 mowing, 2013, in the first and second term foundation.



Legend: Machine 1 - SE 2–024, Machine 2 - GP TP 300, Machine 3 - PP-2.

Figure 2 Crude protein content (g/kg) in dry matter in the stand with more cut variety HOSTYN (loloid type of festulolium), depending on the drill from 1 to 3 mowing, 2013, in the first and second term foundation.



Legend: Machine 1 - SE 2–024, Machine 2 - GP TP 300, Machine 3 - PP-2.

CONCLUSION

More sawn type (variety) had effect on the crude protein content of forage grass. Crude protein content affect also the order of cuts. Crude protein content in the year 2013 was the lowest ($P<0.05$) (128.71 g/kg) in the first mowing, while in the third mowing high - up to 195.03 g/kg.

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INSECTICIDAL ACTIVITY OF NEEM, PYRETHRUM AND QUASSIA EXTRACTS AND THEIR MIXTURES AGAINST DIAMONDBACK MOTH LARVAE (*Plutella xylostella* L.)

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Abstract: The diamondback moth is known globally by many as the most destructive and economically important insect pest of cruciferous crops. It is also known to have developed resistance to numerous synthetic insecticides including those with newer active ingredients (Shelton et al. 2008); and this has triggered the development of alternative measures, including botanical insecticides (Oyedokun et al. 2011). The aim of this study was to evaluate the toxic effect of *Azadirachta indica*, *Chrysanthemum cinerariaefolium* and *Quassia amara* extracts and their mixtures on the diamondback moth larvae; in an attempt to find a superior mixture with more than one major active ingredient for the control of diamondback moth. The study consisted of two separate experiments; a feeding test, and a tarsal contact test. Mortality was recorded at 24, 48 and 72-h intervals from the start of the study. Varying levels of mortalities was recorded in both tests; mortalities ranged between 2–58% and 8% to 76% for the feeding test and the tarsal contact experiment respectively. In both experiments, the highest mortality was recorded in the first 24h and in formulations with the highest concentration. For the feeding test, Pyrethrins, Azadirachtin + Pyrethrins and Azadirachtin + Pyrethrins + Quassin extract/extract mixtures, produced the best effect; with Pyrethrins recording a 54% total mortality count, Azadirachtin + Pyrethrins combination producing a 48% mortality count and a 58% mortality count from Azadirachtin + Pyrethrins + Quassin extract combination. In the contact experiment, the highest mortality was observed in Pyrethrins, and Pyrethrins + Azadirachtin mixture; recording 76% and 64% mortality count respectively.

Key Words: botanical insecticides, feeding test, contact test, larval mortality, brassica pests

INTRODUCTION

The diamondback moth (*Plutella xylostella)* is considered the most destructive and economically important insect pest of cruciferous crops in the world (Sarfraz et al. 2006, You and Wei 2007). The economic loss due to this pest has been estimated worldwide to be US$4-US$5 billion (Zalucki et al. 2012). In addition to crop losses, the annual management costs for controlling this pest were estimated to be more than US$1.0 billion globally (Grzywaez et al. 2010). As a result, a lot of effort has been devoted to find alternative control measures for this pest because of the negative impact of pesticides and the problems encountered in controlling diamondback moth populations (Isman 2006). Having observed that some plants protect themselves better than others, humans developed the use of plants as pesticides. Historically, botanicals were used before other kinds of pesticides. They are mentioned in Hieroglyph, Chinese, Greek, and Roman antiquity and also in India where the use of the neem tree (*Azadirachta indica* Juss.; Meliaceae) was reported in the Veda, a body of manuscripts written in archaic Sanskrit dated at least 4,000 years ago (Philogène et al. 2005).

Botanical pesticides are naturally occurring chemicals extracted from plants. The plant kingdom is the most efficient producer of chemical compounds (primary and secondary metabolites), synthesizing many products having wide array of functions that are used in defence against herbivores (Croteau et al. 2000). Extracts from *Azadirachta indica*, *Chrysanthemum cinerariifolium* and *Quassia amara* have all been reported individually to possess insecticidal effects against the diamondback moth larvae. Grainge et al. 1984, and others; stated the neem plant possessed Insecticidal, Contact Poison, Stomach Poison,

Growth Inhibitory, Antifeedant and Repellant activity against the diamondback moth. They also reported that the whole plant, bark, stem, leaves, fruits and seeds, were among plant parts from which extracts could be made.

Pyrethrum (active component being pyrethrins) is the powdered, dried flower head of the daisy; *Chrysanthemum cinerariaefolium* (Asteraceae). The flowers are ground to a powder and then extracted with hexane or a similar nonpolar solvent (Casida and Quistad 1995, Glynne-Jones 2001). The insecticidal action of the pyrethrins is characterized by a rapid knockdown effect, particularly in flying insects, and hyperactivity and convulsions in most insects. These symptoms are a result of the neurotoxic action of the pyrethrins, which block voltage-gated sodium channels in nerve axons. In purity, pyrethrins are moderately toxic to mammals (rat oral acute LD^{50} values range from 350 to 500 mg/kg), but technical grade pyrethrum is considerably less toxic (ca. 1,500 mg/kg) (Casida and Quistad 1995). Technical grade pyrethrum, the resin used in formulating commercial pesticides, typically contains from 20–25% pyrethrins (Casida and Quistad 1995, Isman 2006).

Two types of botanical pesticides can be obtained from seeds of the Indian neem tree; *Azadirachta indica* (Schmutterer 1990, 2002); at the physiological level, azadirachtin blocks the synthesis and release of molting hormones from the prothoracic gland, leading to incomplete ecdysis in immature insects. And in adult female insects, a similar mechanism of action leads to sterility. In addition, azadirachtin is a potent antifeedant to many insects. Neem oil, obtained by cold-pressing seeds, can be effective against soft-bodied insects and mites but is also useful in the management of phytopathogens. Apart from the physical effects of neem oil on pests and fungi, disulfides in the oil likely contribute to the bioactivity of this material (Dimetry 2012).

Quassia amara is a neotropical forest shrub or small tree, whose range extends from Mexico to Ecuador, including the Caribbean basin, where it normally grows in the forest understory, but it also grows easily in disturbed areas (Villalobos 1995). Its wood contains several quassinoids with insecticidal properties (Polonsky 1973). Daido et al. (1995), also noted that many cytotoxic quassinoids have been shown to have insect antifeedant activity.

It is assumed that a binary mixture (having two components, A and B) can elicit at least three types of responses on an insect (Bitterman 1996, Caouvillon and Bitterman 1982). Two of these correspond to each of the components, where the response of one of the components dominates over the response to the other, and would be similar to the effects produced when presented separately. These elemental qualities might be diminished or enhanced in intensity via mixture suppression or synergism (Cromarty and Derby 1997). A mixture of A and B could also give rise to a third element (AB) that is activated only when both elemental stimuli are present. And it is AB that is associated with reinforcement instead of A and B themselves (Rudy and Sutherland, 1992).

Based on the many available evidence on the insecticidal activities of the Neem, Pyrethrum and the *Quassia amara* extracts individually, we decided to further investigate the effects of extracts from those plant; individually and in a 1:1 and 1:1:1 mixture on DBM larvae under laboratory conditions.

MATERIALS AND METHODS

This research work was conducted in the laboratories of the Faculty of Horticulture of the Mendel University in Brno (Czech Republic) between September and December 2015. All botanical pesticides (NeemAzal T/S, Spruzit®, and Quassia wood chips) used for this experiment were purchased from local authorized dealers in the Czech Republic.

Cabbage, *Brassica oleracea* var. *capitata* L. (HORNET F1) was planted in plastic pots (100 mm diameter) in a mixture of 70% peat substrate and 20% perlite. Plants were maintained under normal greenhouse conditions, and six weeks-old plants were used for the experiments.

Plutella xylostella larvae used in this study were obtained from the laboratory Crop Research Institute; Czech Republic. The colony was reared on cabbage seedlings (*Brassica oleracea* var *capitata* L.) and maintained at room temperature (20–25 °C), RH of 60–65% and a 16:8 h light: dark regime.

Bio-pesticides Used

A test solution of Spruzit, [with 4.59 g/l Pyrethrins; equivalent to 18.36 g/l natural pyrethrum and 825.3 g/l rapeseed oil]; with different concentrations (2.0%, 1.0%, and 0.50% v/ v) was prepared with

PYRETHRIN being the active ingredient. For the Neem extract/Azadirachtin (NeemAzal-T/S), with azadirachtin 10.6 g/l (Azadirachtin A 1%); a test solution with different concentrations of NeemAzal-T/S (2.0%, 1.0%, and 0.5% v/ v) were prepared with AZADIRACHTIN being the active ingredient. A test solution of 3% w/v *which was* latter diluted to 1.5% and 0.75%, was also prepared from *Quassia amara* (Quassia wood chips) with QUASSIN being the active component. The aqueous extract of *Quassia amara* was prepared by soaking Quassia wood chips overnight in water and boiled for 30 minutes, following procedures similar to those described by Dodia et al. (2008) and Zijp and Blommers (2002). A brown-coloured solution which was subsequently obtained was decanted and used immediately. The supplier did however not specify the amount of quassins in the wood chips, but it is known that the source of quassin and neoquassin is the wood of *Quassia amara* L. (Sapindales: Simaroubaceae), containing; depending on the age, 0.14–0.28% of quassinoids (quassin and neoquassin) (Villalobos et al. 1999). In all, there were eight formulations/treatments, including a control and a 1:1 mixture and 1:1:1 mixture of the above bio-pesticides. Each treatment consisted of 3 concentrations; designated C1, C2 and C3; for high, medium and low treatments concentration respectively.

Feeding Bioassays

Diamondback moth larvae collected from the rearing chamber were starved for two hours. Fresh un-infested cabbage leaf disks produced under greenhouse conditions were used for this experiment. Leaf disks were treated with the respective doses of the botanical preparations by dipping the leaf disks in the solutions for 20 second and then air-drying for one hour on a paper at room temperature before being fed to larvae. The fresh treated un-infested leaves were then placed in Petri dishes with moistened Whatman filter paper placed beneath the leaves to avoid desiccation of the leaf disc in the Petri dishes. Ten starved third instar larvae were transferred into each Petri dish and allowed to feed on the treated leaf disks. There were eight (8) formulations in all, including a control and each treatment replicated five (5) times. The experiment was carried out at 16:8 h light/darkness regime, 20–25 °C and 65% relative humidity. Treated leaf disks were replaced with untreated leaf disks after 24 h after exposure of the larvae to the treated leaf disks, and mortality recorded. Mortality was determined 24, 48, and 72-h after treatment.

Tarsal Contact Bioassays

Before transferring Diamondback moth larvae into petri dishes with the various treatments, the larvae were fed on untreated cabbage leaves to reduce the amount of feeding on the labelled solution. A 125mm Whatman filter paper soaked with 2ml of the various labelled solutions, was placed in a 150mm petri dish. Ten third instar Diamondback moth larvae were transferred onto the wetted filter paper in the petri dish and then covered. After an hour of exposure of the larvae on the wetted filter paper, the larvae were transferred into a clean petri dish and fed ad libitum with untreated cabbage leaves. Each Petri dish was kept under ambient laboratory conditions (20–25 °C, 60–65% RH and a 16:8 h light: dark regime). Mortality was determined 24, 48, and 72-h after treatment. There were eight (8) formulations in all, including a control and each treatment replicated five (5) times.

Data Collection and analysis

Data analysis was done with STATISTICA 12; statistical differences among the treatments were determined by a t-test analysis; and all treatments were compared to the controlled treatment. Significance to the corresponding control is designated as follows: '*' $P<0.05$, '**' $P<0.01$, '***'$P<0.001$.

In both the feeding and tarsal contact bioassays, larval mortality was determined and recorded at 24, 48 and 72 hours after feeding starved larvae on treated leaf disks in petri dishes and exposure by walking on the wetted filter paper respectively. Larvae were considered dead if they failed to respond to stimulation by touch.

RESULTS AND DISCUSSION

All formulations; individually or mixtures, revealed varying level of efficacy against the diamondback moth larvae. The highest mortalities were recorded in the first 24-h (less than 10% mortality was recorded after 48-h and 72-h after exposure of larvae to treatments) and in preparations with the highest concentration. Mortalities decreased with treatment concentration and time in both bioassays. It decreased from C1 treatment concentrations to C3 treatment concentration, and from 24-h

to 72-h in both tests; very little or no mortality was recorded after 48 and 72-h of feeding and exposure to treatments in both bioassays. Results also revealed that the highest level of diamondback moth larval mortality was recorded in the feeding test as seen in Table 1. With the exception of the treatment with Quassin as the only active ingredient, all other treatments in the feeding bioassay, produced a significant effect, especially in C1 treatment concentrations.

In the tarsal contact bioassay, only treatment with Pyrethrin as the active ingredient (C1 and C2 treatment concentration), and Azadirachtin + Pyrethrin active ingredient combination (C1 treatment concentration) produced a significant effect. All other treatments in the tarsal contact bioassays did not produce a significant effect, as seen in Table 2. Also, in both bioassays, only Pyrethrin, and Azadirachtin + Pyrethrin combination produced the most consistent and highly significant results.

Table 1 Mean Plutella xylostella larval mortality, 72-h after larval exposure to the treated leaves in the Feeding Test (% ± SD).

Conc.	C	A	P	Q	A+P	A+Q	P+Q	A+P+Q
C1	0	18 ± 8.4**	54 ± 11.4***	14 ± 5.5 **	48 ± 13.0**	16 ± 8.9*	20 ± 7.1**	58 ± 8.4***
C2	0	8 ± 8.4	24 ± 16.7*	4 ± 5.5	30 ± 7.1***	6 ± 5.5	8 ± 8.4	56 ± 15.2 **
C3	0	2 ± 4.5	16 ± 11.4*	4 ± 5.5	16 ± 13.4*	4 ± 5.5	4 ± 5.5	16 ± 15.2

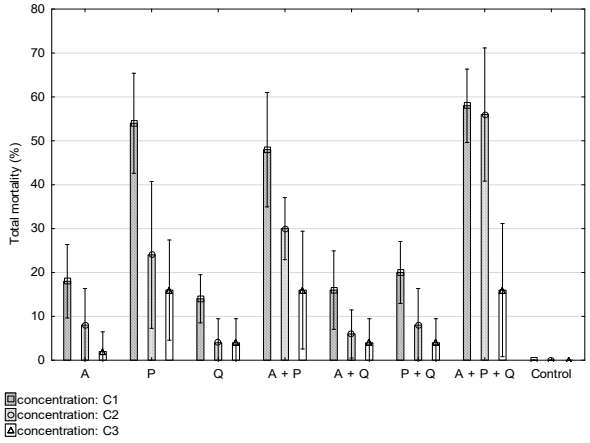
Legend: In both Table 1 and 2, and in Figure 1 and 2; A - Azadirachtin, P - Pyrethrin, Q - Quassin, A+P - Azadirachtin + Pyrethrin, A+Q - Azadirachtin + Quassin, P+Q - Pyrethrin + Quassin, A+P+Q - Azadirachtin + Pyrethrin + Quassin.

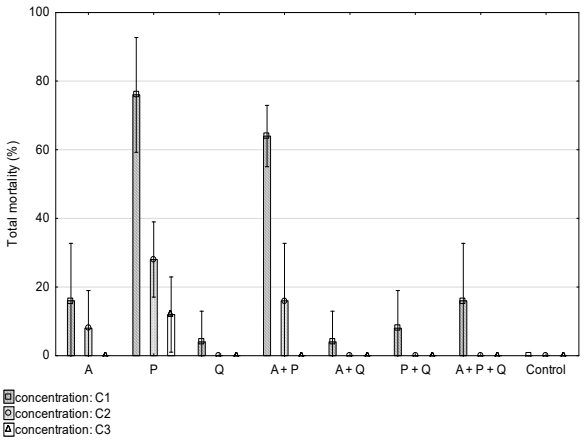
Table 2 Mean Plutella xylostella larval mortality, 72-h after larval exposure to wetted filter paper in the Tarsal Contact Test (% ± SD).

Conc.	C	A	P	Q	A+P	A+Q	P+Q	A+P+Q
C1	0	16 ± 16.7	76 ± 16.7***	4 ± 8.9	64 ± 8.9***	4 ± 8.9	8 ± 11.0	16 ± 16.7
C2	0	8 ± 11.0	28 ± 11.0**	0	16 ± 16.7	0	0	0
C3	0	0	12 ± 11.0	0	0	0	0	0

In the feeding bioassays, the highest percentage mortality was recorded by Pyrethrin, Azadirachtin + Pyrethrin combination, and Azadirachtin + Pyrethrin + Quassin active ingredient combinations, producing 54%, 48% and 58% total mortalities respectively, as against Pyrethrin (76%) and Azadirachtin + Pyrethrin combination (64%) in the tarsal contact bioassay. Figure 1 and 2 summarizes treatments effects for all the bioassays.

Figure 1 Total larval mortality, 72-h after larval exposure to the treated leaves in the Feeding Test. Figure 2 (right) Total larval mortality, 72-h after larval exposure to the wetted filter paper in the Tarsal Contact Test.





The study also reveals that, formulations from all individual species (Azadirachtin, Pyrethrin and Quassin) resulted in varying level of larval mortalities in both tests, and these preparations could be a better option for the control of *Plutella xylostella*. All the individual formulations confirm previous studies which states their efficacies against the *Plutella xylostella* larvae; and in line with many other studies that also stated that extracts from these plant species resulted in *Plutella xylostella* larval mortality. With respect to the mixtures, even though our results are in line with Cromarty and Derby's (1997) revelation that intensities of elemental qualities in mixtures might be diminished or enhanced through mixture suppression or synergism, observed synergism or depression was in both tests. We however did not determine whether the effects shown by the mixtures was as a result of an interaction between the individual components, as stated by Rudy and Sutherland (1992).

Among all the formulations; Pyrethrin alone, Pyrethrin + Azadirachtin mixture, and Pyrethrin + Azadirachtin + Quassin combination, proved much effective than the rest. As seen in figure 1 and 2, these extracts show very strong feeding and contact effects on the diamondback moth larvae; and could have a strong collective effect for the control of the diamondback moth larvae.

CONCLUSION

The results proved that *Azadirachta indica*, *Chrysanthemum cinerariaefolium* and *Quassia amara* extracts are effective as botanical insecticides for the control of *Plutella xylostella* larvae, and also exhibiting mutual effects in a 1:1 or 1:1:1 mixture. Performance however corresponded to treatment concentrations; the higher the concentration, the higher the performance.

With the exception of Pyrethrin alone as the active ingredient, formulations made from a combination of active ingredients were much effective than all other formulations. All in all, Azadirachtin + Pyrethrin, and Azadirachtin + Pyrethrin + Quassin combination produced the best performance in both the feeding test and the tarsal contact experiment.

Azadirachtin + Pyrethrin formulation applied at 2% v/v Neem and 2% v/v Pyrethrin in a 1:1 ratio, exhibited better insecticidal activity in both the feeding test and the tarsal contact experiment than all other combined formulations. Pyrethrin, and Azadirachtin + Pyrethrin formulation could also be easily prepared and economically, and thus strongly recommended for the control of *Plutella xylostella*.

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CROP YIELD ESTIMATION IN THE FIELD LEVEL USING VEGETATION INDICES

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Abstract: Remote sensing can be very useful tool for agriculture management. In this study, remote sensing methods were applied for yield estimation in the field level. There were compared remote sensing data together with yield data obtained from the field. The study area is located in Polkovice in Olomoucký region and a crop planted there in the year 2016 was spring barley as one of most important crops grown in the region. The study area in Polkovice is located at lower elevations with intensive crop production and is climatologically warmer and drier than other areas of the Czech Republic. Year 2016 was the first year when the harvest device has been used for yield analysis in this study area. The output of this method is the yield map displaying the amount of crop harvested in the particular place in the field. The yield data from the field were then compared with remote sensing data in the form of vegetation indices. Two of them were used for comparison – Normalized Difference Vegetation Index (NDVI) and a two-band Enhanced Vegetation Index (EVI2). These indices have been often used for yield estimation in different studies but mostly in larger scales. This study investigates use of NDVI and EVI2 at more detailed scale while using various remote sensing methods. Comparisons show that remote sensing data can provide accurate estimation and can be used for yield forecasting or supplement traditional ways of yield estimation. Results of the study show that yield-index correlations are stronger for satellite data than for the drone data. NDVI showed slightly stronger correlations than EVI2. Strongest correlations between vegetation indices and yields were found for NDVI from Sentinel 2.

Key Words: Yield, drone, spring barley, NDVI, EVI2

INTRODUCTION

Remote sensing can be very useful tool in the area of drought monitoring, providing valuable spatiotemporal information about yield-limiting moisture conditions and crop response under current climate conditions (Anderson et al. 2015). Remote sensing indicators are now widely used in agriculture for monitoring crop condition and forecasting yield (Brown et al. 2008). Indicators commonly used in agriculture include vegetation indices, such as the Normalized Difference Vegetation Index (NDVI) and the Enhanced Vegetation Index (EVI), that track crop progress and evolution in green biomass amount (Becker-Reshef et al. 2010, Esquerdo et al. 2011). There are also other more physically based vegetation indices that describe light-harvesting capacity or photosynthetic rates, including the Leaf Area Index (LAI) or the fraction of Absorbed Photosynthetically Active Radiation (fAPAR) (Doraiswamy et al. 2005, Guan et al. 2015). Another class of remote sensing indicators is connected with various aspects of the surface moisture status, e.g. plant water use via satellite-based estimates of evapotranspiration (ET) (Anderson et al. 2007).

Many studies have investigated correlation between various satellite indices and crop yields (Anderson et al. 2016). Conclusions of these studies showed that no single indicator is valuable always and everywhere, and its performance depends on many factors, e.g. climate, soils, management, crop type, growing season or limitation of given device or sensor (Johnson 2014).

There is clearly a benefit to integrate information from multiple satellites (Anderson et al. 2016) or even compare satellite data and ground observed data. This paper describes a strategy for combining satellite remotely sensed data with other modern technologies to support water use, drought monitoring and yield analysis.

The goal of this work is to identify remote sensing data and yield monitoring tools that can be used at more detailed scale – at the field level in the certain regions of CR and neighboring countries.

MATERIAL AND METHODS

The important part of the study was yield estimation done by NDVI that has been widely used to monitor vegetation at regional to global scales (Tucker et al. 1985). The NDVI (Tucker 1979) is a numerical transform of the visible red (RED) and near infrared (NIR) spectral bands that takes following form (Tucker 1979):

$$NDVI = \frac{NIR - RED}{NIR + RED}$$

NDVI represents a dimensionless, radiometric measure that obtain different response from the incident visible red (absorbed by chlorophyll) and NIR (that is reflected by the mesophyll layer of leaves) radiation with the vegetation canopy. NDVI correlates with the relative abundance and condition of green vegetation, therefore NDVI time series have been widely used in research connected to drought and agriculture, as e.g. quantification of seasonal events, classification of land cover types or vegetation conditions (Brown et al. 2008).

The second vegetation index used in the study is EVI2 which is two-band EVI – without a blue band. EVI2 was developed due to limitation of EVI to sensor systems designed with a blue band, in addition to the red and near-infrared bands. This fact made it difficult generate long-term time series as we know e.g. from NDVI. EVI2 has the best similarity with the 3-band EVI, particularly when atmospheric effects are insignificant and data quality is good (Jiang et al. 2008). 3-band EVI is calculated as showing following form:

$$EVI = G\frac{NIR - RED}{NIR + C_1 RED - C_2 BLUE + L}$$

NIR, RED and BLUE are the reflectances in near infrared, red and blue bands respectively. G is a gain factor; C_1 and C_2 are the coefficients of the aerosol resistance term which uses the blue band to correct for aerosol influence in the red band. L functions as the soil-adjustment factor (Jiang et al. 2008). EVI2 is calculated in the following way:

$$EVI2 = G\frac{NIR - RED}{NIR + (6 - 7.5/c)R + 1}$$

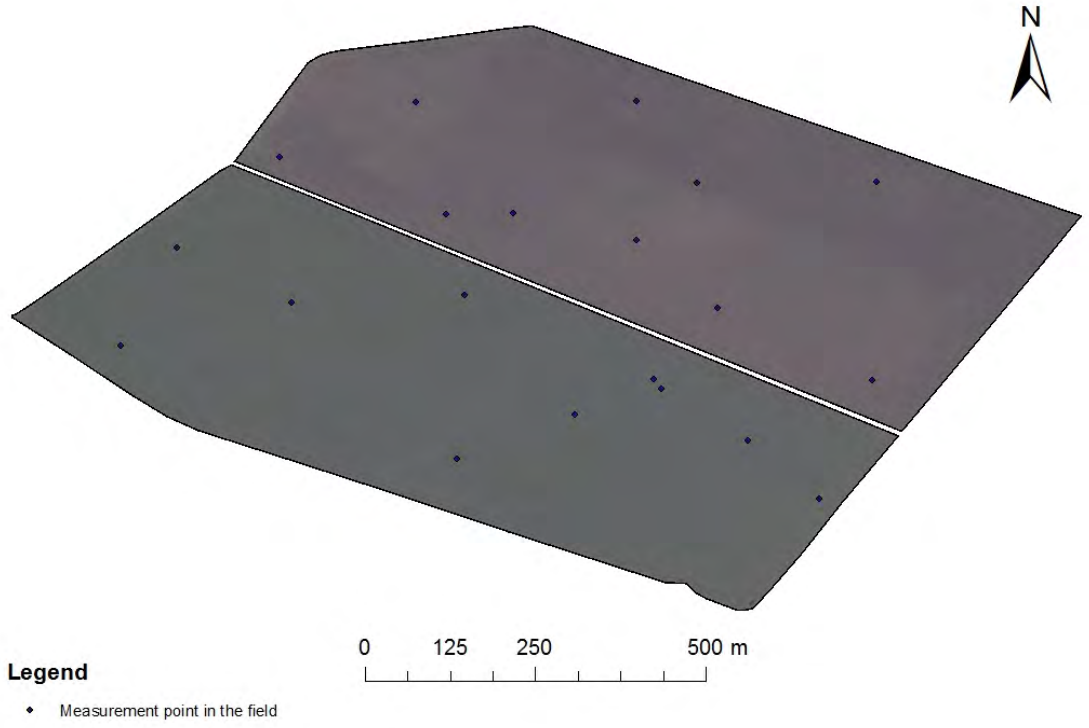
Parameter G is to be determined according to the c value. Parameter c comes from the relationship RED = c x BLUE. It should be noted that c derived by fitting the blue reflectance to the red reflectance might not be the same as that derived by fitting EVI2 to EVI. The reason is that NIR reflectances are involved in fitting EVI2 to EVI but they aren't used to relate the blue reflectance to the red reflectance (Jiang et al. 2008).

In this study, data for computing NDVI and EVI2 for Polkovice site were obtained from more sources. As mentioned above, study area is located in Olomoucký region in the eastern part of the country. Coordinates (GPS position) of locality is following: 49°23'50.05"N, 17°14'52.25"E. One of sources was a drone overpassing the study area. Another important set of data were satellite data. Data for NDVI and EVI2 were obtained from Landsat 8 with the spatial resolution 30 m. NDVI was also computed from another satellite – Sentinel 2 with the spatial resolution 10 m. Values of vegetation indices from drone and satellite data were extracted from 20 places located in the field. NDVI measured in the field was used for yield-index correlation, too. NDVI was measured by the GreenSeeker handheld crop sensor. There were 20 measurement points in the field (Figure 1).

All available remote sensing data were used for correlation with yield data obtained in the field. There were two data sets available. Firstly, yield was calculated from harvest sampling done shortly before harvest. Sampling was done in the same points where NDVI were measured during the growing

season. The result yield was calculated in grams per m^2. Secondly, yield data were obtained from harvest device located in the harvester. The result of this yield analysis is the yield map demonstrating variability in field level. From this yield map, yield data were extracted for the same 20 points as harvest sampling was done. Result yield data are in tons per hectare. Then index-yield correlations were quantified by use of the correlation coefficient.

Figure 1 A map showing measurement points in the study area in Polkovice



RESULTS AND DISCUSSION

Exploration of dependencies between vegetation indices and crop yield data was undertaken by use of the correlation coefficient. Results are shown in the Table 1 that shows values of correlation coefficient between NDVI and EVI2 and yield data. Two yield data sets are highlighted in bolt for easier orientation.

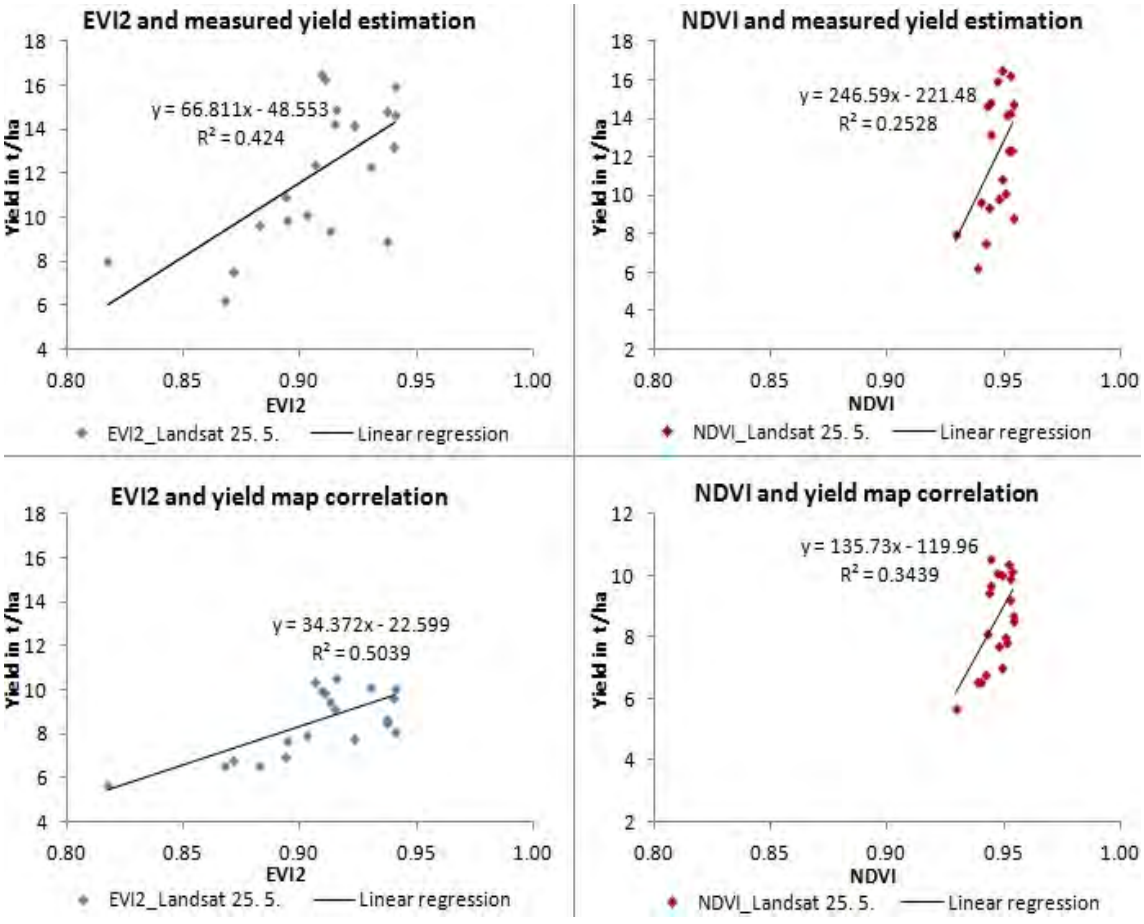
Table 1 Correlation coefficients between vegetation indices and yields

	1	2	3	4	5	6	7	8	9	10	11	12	13
1	1												
2	0.66	1											
3	0.54	0.77	1										
4	0.40	0.64	0.42	1									
5	0.56	0.59	0.33	0.59	1								
6	0.32	0.68	0.59	0.47	0.44	1							
7	0.40	0.73	0.68	0.46	0.45	0.96	1						
8	0.49	0.81	0.68	0.53	0.48	0.89	0.93	1					
9	0.24	0.70	0.63	0.40	0.45	0.74	0.70	0.71	1				
10	0.43	0.59	0.50	0.65	0.55	0.79	0.78	0.72	0.43	1			
11	0.55	0.86	0.64	0.77	0.66	0.66	0.70	0.75	0.69	0.67	1		
12	**0.47**	**0.64**	**0.64**	**0.72**	**0.61**	**0.66**	**0.66**	**0.65**	**0.50**	**0.82**	**0.78**	1	
13	**0.46**	**0.71**	**0.61**	**0.58**	**0.69**	**0.55**	**0.67**	**0.71**	**0.59**	**0.55**	**0.83**	**0.70**	1

1 – EVI2 obtained from the drone on 7. 6. 2016
2 – NDVI obtained from the drone on 7. 6. 2016
3 – NDVI measured in the field by GreenSeeker on 19. 5. 2016
4 – NDVI measured in the field by GreenSeeker on 23. 6. 2016
5 – NDVI measured in the field by GreenSeeker on 4. 7. 2016
6 – EVI2 obtained from Landsat 8 on 9. 5. 2016
7 – NDVI obtained from Landsat 8 on 9. 5. 2016
8 – EVI2 obtained from Landsat 8 on 25. 5. 2016
9 – NDVI obtained from Landsat 8 on 25. 5. 2016
10 – NDVI obtained from Sentinel 2 on 6. 5. 2016
11 – NDVI obtained from Sentinel 2 on 25. 6. 2016
12 – yield obtained from harvest sampling (g/m^2)
13 – yield obtained from the harvest device during the harvest (t/ha)

The strongest correlations between NDVI and yield were indentified in data from Sentinel 2. Correlation coefficient was 0.82 for the scene from 6. 5. 2016 and 0.78 for the scene from 25. 6. 2016. These correlations are between NDVI and yield data coming from harvest sampling. The strongest correlation between EVI2 and yield was found for the Landsat 8 scene from 25. 5. 2015. Correlation is 0.71 and compared yield data comes from the harvest device. NDVI measured by GreenSeeker crop sensor show also good results. Correlation between GreenSeeker NDVI and yield from harvest sampling are between 0.61 and 0.72 and are a bit higher than in the case of yield data from harvest device. Figure 2 shows relationships between vegetation indices and yield data (both from crop sampling and harvest device) for the Landsat 8 scene from 25. 5. 2016 that showed also strong correlations – from 0.5 to 0.71.

Figure 2 Comparison of vegetation indices and yields



CONCLUSION

According to rather strong correlations between vegetation indices and yield data, it seems to be obvious that vegetation indices can be used for pre-harvest yield estimation and calibration of yield analysis coming from both traditional and modern methods. Yield-index dependence should be investigated in bigger detail especially for the field level analysis that is quite new subject of study.

Following study should include other index or indices, e.g. Evaporative Stress Index (ESI) – an indicator of agricultural drought expressed as standardized anomalies in the ratio of actual-to-potential ET (Anderson et al. 2011, 2013, 2015). It would be also very useful to compare yield-index correlation for longer time scale – to include remote sensing and yield data for more than one growing season.

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STABILISATION OF THE YOUNG BARLEY JUICE USING ESSENTIAL OILS OF SELECTED PLANT SPECIES

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Abstract: The aim of this work was to investigate the possibilities of stabilisation of the juice freshly pressed from young barley by the means of the essential oils of selected plant species. Barley was grown in the laboratory into the development phase DC 29. Spring barley variety Francin was used for the experiment. Essential oils of fennel (*Foeniculum*), cinnamon (*Cinnamonum*), lemon balm (*Melissa*) and mint (*Mentha*) were used in various concentrations for the stabilisation of the young barley juice. Sensory evaluation was performed for individual samples of young barley juice after the essential oil addition. The results indicate that cinnamon and mint essential oils proved to have the best preservation effects, but the best in terms of taste the use of fennel and lemon balm essential oils.

Key Words: barley, essential oils, fennel, cinnamon, lemon balm, mint, preservation

INTRODUCTION

The many of essential oil have been proven to have significant antimicrobial, antifungal, antihelmitic and antiseptic effects against a wide range of microorganisms. Significant antioxidant properties of the essential oils were also demonstrated and thus they can be used in food industry as active preservation agents (Ghabraje et al. 2016).

Food preservation is a procedure which is able to prolong the shelf time of raw materials and foodstuff. The treated products are more sustainable in the short or long term. It is important to keep the organoleptic properties unchanged during the preservation, as well as the nutritional components of the food (Ingr 2007).

A significant characteristic of young barley is the content of active enzymes that constitute about 40% of its weight. Young barley contains cytochrome oxidase, which is responsible for cellular respiration, peroxidase, which decomposes hydrogen peroxide, catalase, oxidase of fatty acids and transhydrogenase (Dallen et al. 2010, Takano et al. 2013, Yamura et al. 2013, Lahouar et al. 2015).

Cinnamon is a plant from the *Lauraceae* family. Both wood and leaves, seeds, bark and roots of the plant are used. The main active agent is cinnamon essential oil, consisting mostly of cinnamaldehyde, eugenol, cinnamyl acetate, cinnamyl alcohol, o-methoxycinnamaldehyde and cinnamic acid. Other metabolites such as diterpenes, hydroxy alcohols, mucilage and tannins are also present. It is recommended to use cinnamon against anorexia, indigestion, to improve the immunity and in the case of hormonal disorders (Jahodář 2010).

Fennel belongs to the *Apiaceae* family. It is a biennial perennial herb desired for its fruits, elongateddiachenia (Wenzel 2014). The fruits contain mostly the essential oil with anethole, fenchone and estragole as major constituents. Epoxy-p-menthane, hydroxycoumarins, furanocoumarins, pyranocoumarins, flavonoids, beta–carotene and phytosterols are also present (Grešík 2013). It is used against flatulence and to stimulate bowel movements, the release of mucus, to increase the production of breast milk, where it also influences the course of digestion in infants, to inflamed eyes washing and for cosmetic coverings (Erdelská et al. 2008).

Lemon balm is a perennial herb from the *Lamiaceae* family with typical lemon scent (Erdelská et al. 2008). The plant contains hydroxycinnamic acids - rosmarinic, coumaric, caffeic and chlorogenic acid. The compounds responsible for the properties of the essential oils are citral, citronellal, geraniol, nerol, linalool, humulene, farnesyl acetate, beta-caryophyllene and eremophilene. In contains also

flavonoids, tannins and pentacyclictriterpenic acids (Jahodář 2010). Lemon balm soothes the nervous system in the case of exhaustion, insomnia, headaches, dizziness and also is used for painful menstruation, treatment of heart activity and to lower blood pressure, digestive disorders and problems with gallbladder function (Wenzel 2014).

Mint is a perennial herb belonging to the *Lamiaceae* family. The main components of the essential oils are menthol (35–45%), menthone (15–25%), methyl acetate (3–5%) ,neomenthol, isomenthol, menthofuran, terpenes, flavonoids, tannins and phenolic acids (Jahodář 2010). It is used mostly to ease the pain, to suppress the sensitivity of nerve endings, to promote the excretion of bile during digestion and against diarrhea (Wenzel 2014).

MATERIAL AND METHODS

Plant material

To obtain the young barley juice, Francin variety of spring barley has been used, grown at the laboratory conditions at the temperature 22 °C. Juice from the barley grown into the phase DC 29 was extracted by the means of the instrument Healthy Juicer for the green mass processing. Essential oils of selected species of medicinal plants - fennel (*Foeniculum*), cinnamon (*Cinnamonum*), lemon balm (*Melissa*) and mint (*Mentha*) were added into fresh juice in two different concentrations (lower - 0.1 ml/l; higher - 0.2 ml/l).

The volume of barley concentrate created during the juicing was diluted with water to the ratio 1:8. The resulting solution was divided into nine samples where the sample no. 2 was conducted as a control without the addition of any essential oil and to the others essential oils were added (see Table 1). The control sample no. 2 was compared with the sample no. 1, which was prepared from the product available on the market, green barley powder from the Green Ways company (GW). The composition and antimicrobial activity of the essential oils was taken into the account during their selection.

Table 1 List of the samples for sensory evaluation and monitoring of the effects of preservatives

Sample	Form	Amount	Essential oil
1.	Young barley, powder	-	-
2.	Young barley, fresh	-	-
3.	Young barley, fresh	Lower	Cinnamon
4.	Young barley, fresh	Higher	Cinnamon
5.	Young barley, fresh	Lower	Fennel
6.	Young barley, fresh	Higher	Fennel
7.	Young barley, fresh	Lower	Lemon balm
8.	Young barley, fresh	Higher	Lemon balm
9.	Young barley, fresh	Lower	Mint
10.	Young barley, fresh	Higher	Mint

Selected criteria for the sensory testing were the scent, the presence of extraneous scent, the presence of extraneous flavours, flavour intensity and the total impression. 25 respondents were interviewed, of which one third were men and two thirds were women. There were 10 samples to evaluate. Then the samples were stored in the refrigerator and the preservation effects of the essential oils were monitored during a four weeks period.

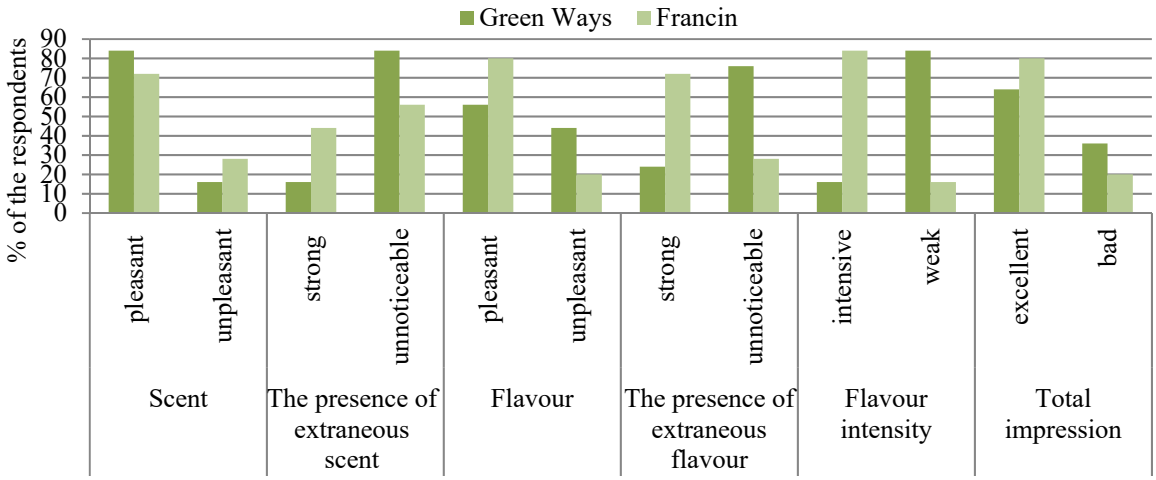
RESULTS AND DISCUSSION

Sensory tests

Sensory tests were evaluated and graphically presented. At first, sensory tests comparison of the beverages prepared from the powder made by Green Ways and the juice freshly pressed from barley plants were performed.

Overview of the results (dilution ratio 1:8)

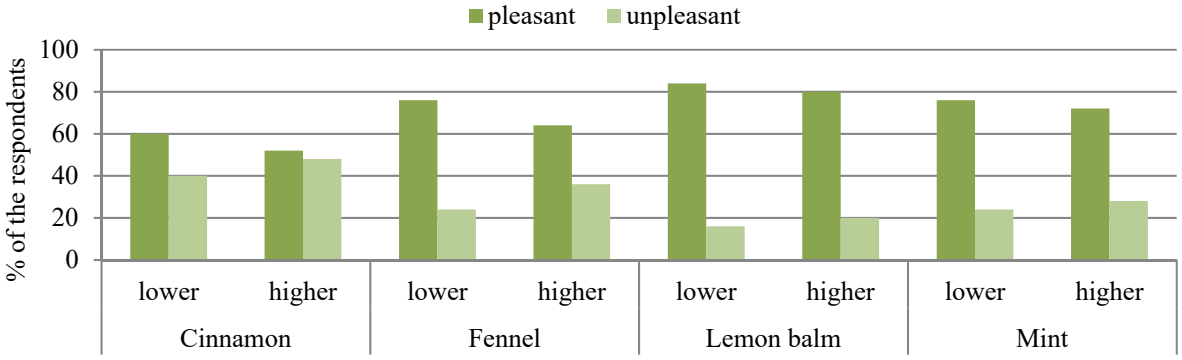
Figure 1 The comparison of fresh juice and the beverage prepared from powder



Results obtained from the monitored samples suggests that barley from GW company had, according to the respondents, better scent than fresh juice from young barley, and some respondents considered the smell of the young barley juice unpleasant. Figure 1 shows that the presence of extraneous scent was imperceptible for respondents in most cases, but 16% of respondents considered the presence of extraneous scent strong in the GW barley and 44% of respondents registered the presence of extraneous scent in the young barley juice. Taste of both investigated samples was mostly pleasant for the respondents (barley GW–56%, juice from young barley–80%).

Another set of sensory tests was performed using young barley juice with the addition of various amounts of essential oil. Cinnamon, fennel, lemon balm and mint essential oils were studied.

Figure 2 The scent of young barley juice with the addition of essential oil



It is evident from the Figure 2 that the respondents found the scent of spring barley juice with lemon balm essential oil the most pleasant, both in lower and higher concentrations (84; 80%). On the other hand, the odour of spring barley juice with higher amount of cinnamon essential oil was found unpleasant by the respondents (48%).

According to the respondents, the strong presence of extraneous scent was found in 84% of the cases in juice with higher amount of mint essential oil. As can be seen in the Figure 3, the most

unnoticeable extraneous scent was reported in the juice with higher amount of fennel essential oil (40% of the respondents).

Figure 3 The presence of extraneous scent in young barley juice with the addition of essential oil

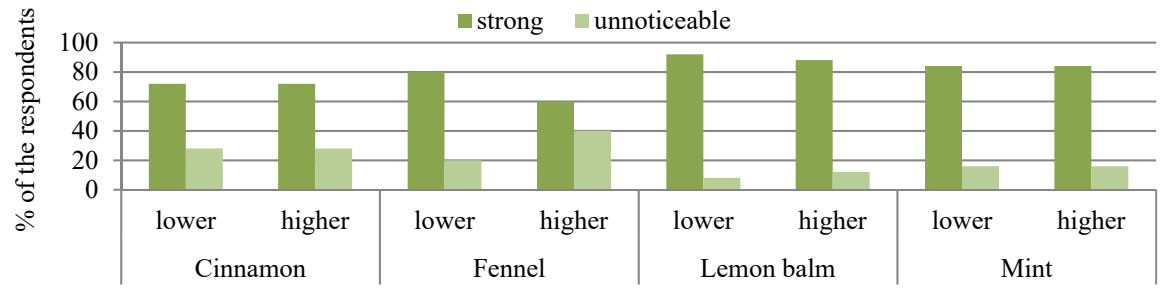
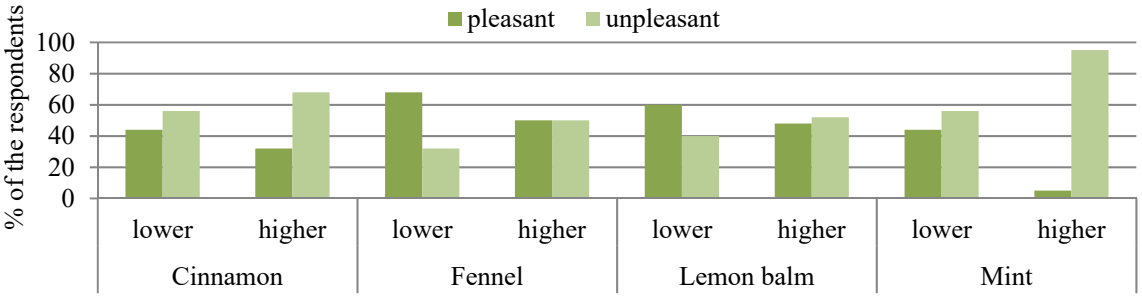
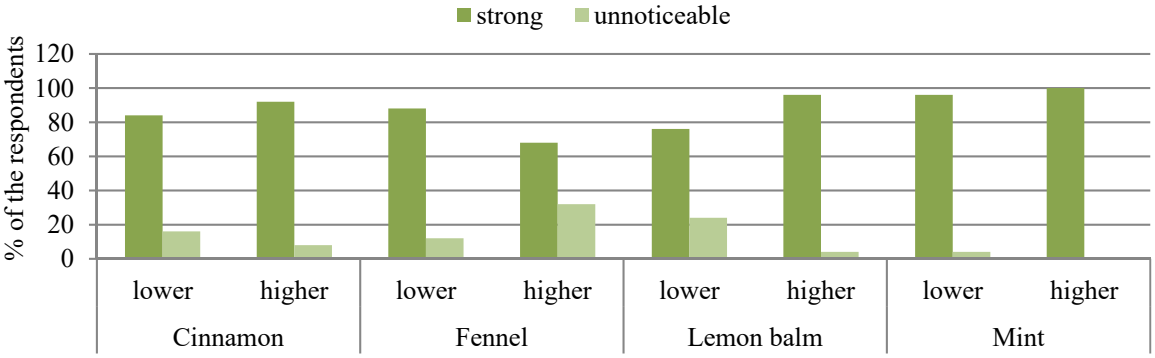


Figure 4 The flavour of young barley juice with the addition of essential oil



During the sensory evaluation, the taste was of one of the most important criteria. As you can see from Figure 4, the juice with fennel essential oil had a pleasant taste in both the lower and the higher concentration (68; 50% of the respondents). The spring barley juice enriched with higher concentrations of mint essential oil was unpleasant for 95% of the respondents.

Figure 5 The presence of extraneous flavour in young barley juice with the addition of essential oil



As can be seen in Figure 5, 100% of the respondents reported the strong presence of extraneous taste in the young barley juice with higher amounts of mint essential oil. In the juices enriched with lower amounts of fennel essential oil, only 12% of the respondents didn't consider the presence of extraneous flavour as unnoticeable.

According to the respondents, the most intense flavour had young barley juice with higher amount of mint essential oil (92% of the respondends). As shown in Figure 6, the weakest taste was found for the sample with a lower concentration of lemon balm oil.

Figure 6 Flavour intensity of the young barley juice with the addition of essential oil

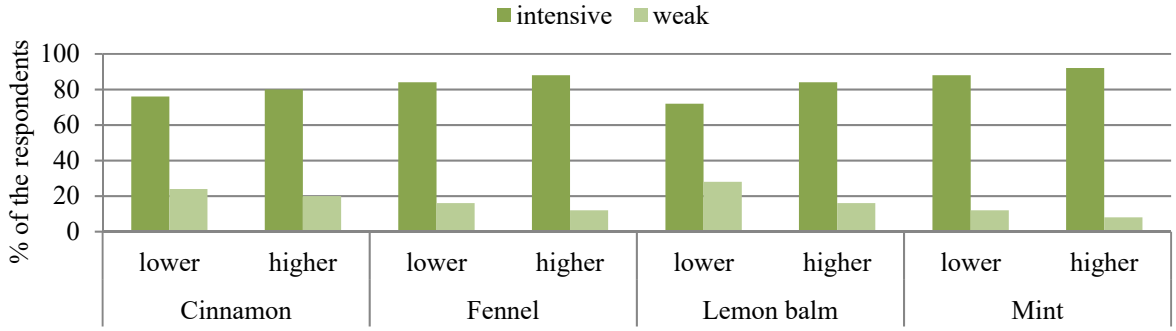
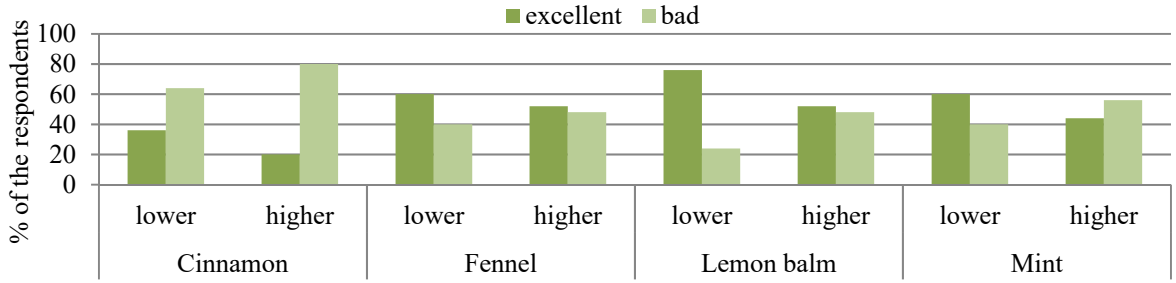


Figure 7 Total impression of the young barley juice with the addition of essential oil



The respondents agreed that the young barley juice enriched with lemon balm essential oil made an excellent impressionin lower amount (76%). On the other hand, 80% of the respondents rated the juice with a higher amount of cinnamon essential oil as bad. However, cinnamon essential oil is important from the technological point of view. Despite the fact that the impression of juice with higher amount of cinnamon essential oil was rated negatively, we can see from the Figure 7 that the juice with a lower amount of cinnamon essential oil was rated as excellent by 36% of the respondents.

The preservative effects of essential oils to the young barley juice

In terms of the preservation effects, the results of this work showed that untreated young barley juice can be stored at temperatures 5–8 °C for about five days. Best preservation effects were found for the cinnamon essential oil.

The samples treated with cinnamon essential oil didn't show any changes during the four weeks period. Fennel and lemon balm essential oils were found to have less effective preservation properties than the cinnamon essential oil, but at temperatures 5–8 °C these essential oils also showed preservation effects for 3 weeks.

The preservation effects of mint essential oil to the young barley juice was found out to be suitable during the four weeks period. The tests of microbiological purity will be also performed as a part of future research.

The preservation of young barley was investigated by Kovářová (2013). They present methods of preservation by freezing and sterilisation in their paper. However, the enzymes and active ingredients present in young barley are very susceptible both to the high temperatures used for sterilisation and low temperatures used for freezing. The exposition to high temperatures leads to sensory changes of the colour of the sample and the taste. Freezing causes the precipitation of samples. The use of the essential oils doesn't require any thermal interference. On the contrary, the essential oils are natural products that enrich young barley juice with favourable substances.

CONCLUSION

It is evident from the obtained results that the best preservative effects were found for the cinnamon essential oil. However, the sensory evaluation of the flavour of young barley juice enriched with cinnamon essential oil was rather negative. The sample with the addition of cinnamon essential oil didn't show any changes during the four weeks of storage. Fennel essential oil proved to have less effective preservation properties than cinnamon, but even this essential oil showed protective effects for 3 weeks. Within the sensory evaluation, the young barley juice enriched with fennel essential oil was assessed rather positively. Lemon balm essential oil, characterized by its typical lemon scent, proved to have preservation effects for 3 weeks similarly to the fennel essential oil. However, young barley juice with the addition of this essential oil was found tasty by the respondents, who rated the flavour as rather pleasant. The preservation effects of the mint essential oil to the young barley juice were also monitored in this work. Mint essential oil is characterized by its refreshing scent, even in very small quantities; its fragrance is very intense. Mint essential oil proved to be very useful preservation agent for up to 4 weeks of storage. However, according to the respondents, young barley juice enriched with mint essential oil was rather unpleasant, especially at higher concentrations of the essential oil in the sample.

The results indicate that cinnamon and mint essential oils proved to have the best preservation effects. The efficiency of essential oils was studied also in low concentrations, because the use of essential oils in larger quantities is not organoleptically acceptable. The treatment of the beverages with trace amounts of essential oil could have a positive effect also on their taste. Although the evaluation was not always positive, in terms of the product conservation the activity of used essential oils is not insignificant.

ACKNOWLEDGEMENTS

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SEED VIGOUR AND ROOT SYSTEM SIZE AS A ATTRIBUTE FOR DROUGHT ESCAPE AND TOLERANCE

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Abstract: Seed vigour is the ability of seeds to germinate and form the basis for future plant growth and development in standard and stressed conditions (drought, low temperatures, lack of nutrients). The root system size of 39 winter wheat genotypes (*Triticum aestivum* L.) planted in field experiment was evaluated in relation to the seed germination and seed vigour and grain yield. Statistically significant inter-varietal differences in average root system size values from the entire growing season were found. Positive relationship between root system size in the grain filling stage and germination of harvested seed was confirmed for early genotypes. Root system size of late genotypes was statistically significantly associated with seed vigour. Grain yield was not affected by the seed vigour and seed germination of sown seeds, but by the root system size. Genotypes with a higher average values of root system size and root system size at stem elongation stage achieved higher grain yield. It is possible to provide selection for cultivar tolerance to stress already at the seed germination stage and on the quality of plant root systems. Seed vigour and quality of the embryonic roots is important for the following growth and plant development.

Key Words: seed germination, abiotic stress, grain yield, wheat, breeding

INTRODUCTION

The most serious abiotic stress causing losses to European crop farmers is currently drought stress. The ability to germinate and produce of vigour plants when the soil moisture conditions are below optimum or stressful may be vital for herbage creation in periods with limited water supply or those when cold episodes occur. Higher seed quality may be particularly important in low-input agriculture because poor early performance is not as readily compensated for later on by mineral fertilisers and pesticides as it is in conventional agriculture. Furthermore, quality and seed vigour are important factors for competitiveness against weeds: the seeds of low vigour resulted in a perceptible increase in weed biomass and decrease in crop yield. Seed vigour is generally described as the sum of the seed properties that determine the potential level of activity and performance of the seed during germination and seedling emergence (Perry 1978). The significance of this trait was documented by Pedersen et al. (1993), who reported that an increase in the mean germination time due to poor seed vigour resulted in a significant loss in grain yield. Biological quality of seeds so often affects the growth of both root and shoot during the whole vegetation period (Bláha and Šerá 2014). It is supposed that genotypes with more vigorous seeds develop, among others, drought tolerance. Sprouted seeds from more vigorous grains are able to escape from possible drought during the initial vegetation stages, develop larger root system and will be more drought resistant in further vegetation stages („drought escape“). At the same time, root system quality along with its size can be an important factor for gaining yield stability by effective water absorption from the soil in critical stages of plant growth. Large root system is also one of the aspects of higher drought resistance of crops.

The aim of this work was (i) to evaluate the differences of root system size (RSS) of selected wheat genotypes, (ii) to quantify the relationship of RSS, germination of seeds and vigour of seeds on the basis of correlation analysis and (iii) evaluate the RSS, seed germination and seed vigour effect on the yield of grain.

MATERIAL AND METHODS

Root system size (RSS), seed vigour and seed germination were evaluated for 39 genotypes of winter wheat (RAGT Czech s.r.o.) in field experiment at Branišovice locality (South Moravia, Czech Republic, GPS location: 48.9534597N, 16.4287542E) in 2015. The experiment was realized in four repetition. Electrical capacity measurement (measured in nanofarads – nF) was used to evaluation of the RSS according to Chloupek

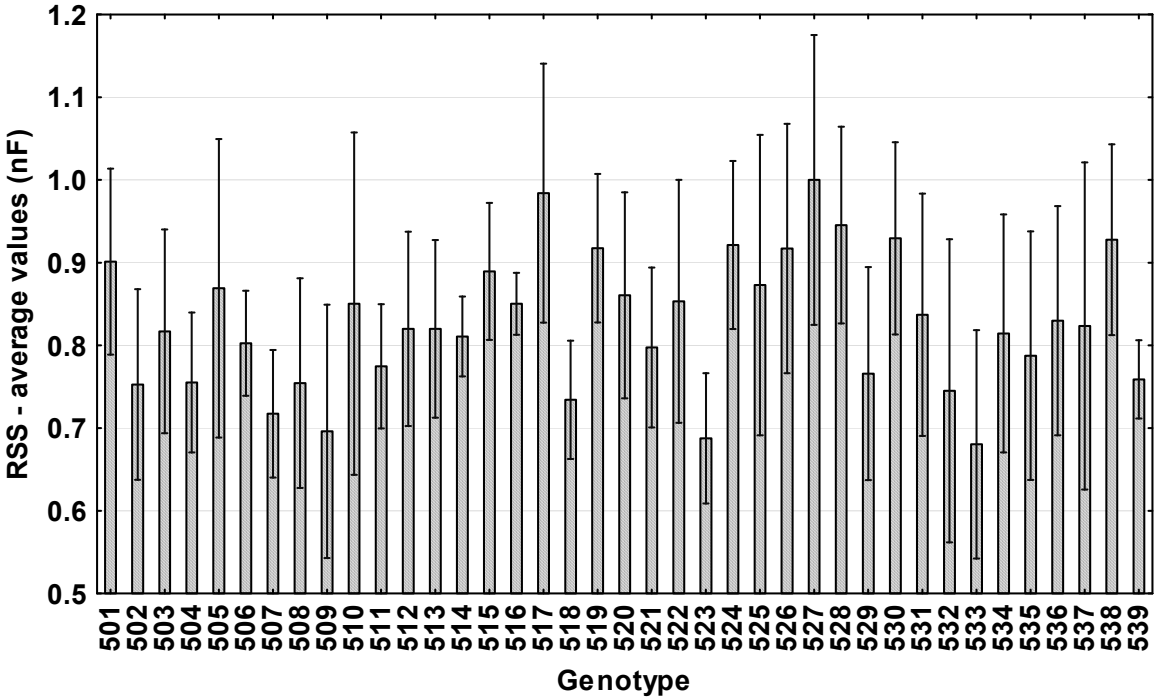
(1972) during the BBCH (Meier 1997) stage 30, BBCH stage 50 and BBCH stage 75, using parallel model of LCR meter at 1 kHz frequency. Grain yield was evaluated at full-ripening stage. After the harvest, seed germination and seed vigour of evaluated genotypes was tested. Seed vigour was defined as the percentage of germinated seeds under stress conditions, e.g. at temperature of 10 °C and physiological drought -0.5 MPa in polyethylene glycol-water solution PEG 6000 (Ullmannová 2013). Simultaneously, other seeds (other variant) were exposed to optimum humidity conditions and temperature of 20 °C. 50 seeds of each variant were set in germinators with filter-paper. This procedure was repeated 3 times. The seeds with germ length at least half the length of the seed and at least 3 embryonic roots were considered properly germinated. Percentage of germinated seeds was evaluated every 2 weeks. The data were then processed in STATISTICA 10 software. Analysis of variance ($p \leq 0.05$) and correlation analysis were carried out.

RESULTS AND DISCUSSION

Root system size of wheat plants was evaluated during main vegetation period (April–July), when precipitation totals reached only 60% of long-term precipitation average and plants were exposed to lack of water. Analysis of variance showed statistically significant differences between RSS of monitored varieties ($p \leq 0.05$), and tendencies to develop either larger or smaller root system. Lower RSS values were observed during the whole vegetation period, while higher RSS values occurrence was dependent on the phenophase. Higher RSS values were observed in genotypes 527, 517, and 528 (measurement in the stem elongation stage), in genotypes 527, 528, 526, and 536 (measurement in the heading stage), and in genotypes 526, 536, 515 (measurement in the grain filling stage). On the other hand, low RSS values during all monitored phenophases was shown by genotypes 523, 509, and 507. Statistically significant difference was found between root system size of genotypes 527, 528, 517, 519 (large RSS) and 523, 533, 507, 509 (small RSS). For average RSS values see Figure 1.

Statistically significant relationship was found between RSS in the first (BBCH 30) and second (BBCH 50) term of RSS measurement (r = 0.445**) and between second (BBCH 50) and third (BBCH 75) term of RSS measurement (r = 0.499**). Root system size (in the stem elongation stage) had essential influence on root development in the following period. Thus it is probable that plants partially adjust their root system size actively during vegetation. This ability is one of the desirable features of varieties resistant to abiotic stressors.

Figure 1 Average RSS values (nF) of monitored common wheat genotypes during vegetation period in 2015 (vertical columns represent confidence intervals at $p \leq 0.05$)



Good seed germination and vigour of the seeds can accelerate the plant growth and drought escape in the initial ontogenetic phases in both spring and autumn. It was found that size and vigour of the seeds are associated to wheat drought tolerance (Rebetzke and Richards 1999). Also large root system is associated to lack of water tolerance and it was discovered that larger root system is related to barley (Svačina et al. 2014)

as well as wheat (Heřmanská et al. 2015) grain yield. Combination of these features may lead to cultivation of a genotypes with higher drought tolerance potential. Larger RSS and higher seed vigour synergy in 39 monitored wheat genotypes was not confirmed. Correlation analysis did not confirm statistically significant relationship of RSS and seed germination or seed vigour (see Table 1; column Total). However, seed vigour and RSS relationship is also influenced by the earliness of the genotype. In the genotype set there were 26 early and 11 late genotypes, while 2 were not tested. Seed germination of the early genotypes was positively correlated with the RSS values (in the grain filling stage; r = 0.411*). Higher seed vigour was shown by genotypes with larger RSS at the end of vegetation. Larger RSS in this phase can thus be a consequence of higher seed vigour and quality in the beginning of growth, which ensured large root system. On the other hand, root system of the late genotypes was not influenced by the seed germination but by their vigour. Interesting is the statistically significant negative relationship of RSS (in the heading stage), average RSS and seed vigour (Table 1). It is possible to assume that RSS of the late genotypes is important from the aspect of production also in latter ontogenetic phases. In case of fast root biomass growth in the beginning of vegetation and the water supply in spring months is not sufficient (April–June 2015), root system growth in the consecutive generative phase can be inhibited in order to save resources and assimilates for the photosynthetic aparatus and grain development. Relationship between seed germination and seed vigour among the late genotypes was not discovered.

Table 1 Relationship (expressed as the correlation coefficients) of wheat RSS in three vegetation stages and seed vigour, seed germination and grain yield of genotypes differentiated by earliness

	Seed vigour			**Seed germination**			**Grain yield**		
	Early genot.	Late genot.	Total	Early genot.	Late genot.	Total	Early genot.	Late genot.	Total
RSS in stem elongation	0.109	-0.497	0.046	0.235	0.123	-0.036	0.494*	0.350	0.414**
RSS in heading	0.229	-0.799**	0.255	0.301	-0.020	0.004	0.028	0.362	0.161
RSS in grain filling	0.388	-0.184	0.150	0.411*	0.211	0.095	0.109	0.029	0.159
Average	0.241	-0.829**	0.138	0.369	0.148	-0.010	0.417*	0.454	0.394*

Legend: correlation coefficient is significant at $p \leq 0.05$; and $p \leq 0.01$***

Seed vigour and seed germination was not related to grain yield, which may have been caused by long vegetation period of winter wheat, which is strongly influenced by weather conditions during winter months (Table 2). Previous results (Ullmannová et al. 2013) indicate the possibility of successful selection for higher seed vigour as an important factor of agronomic and malting quality, even in good years (vigour 93–95%), for the traits given above. However, in the years with generally much lower vigour (61–86%), the success could be more responsive because the effect of the variety prevailed over the effect of the environment for bad years.

Table 2 Correlation relationship of seed vigour/germination and grain yield of monitored wheat genotypes differentiated by earliness

	Seed vigour			**Seed germination**		
	Early genotypes	Late genotypes	Total	Early genotypes	Late genotypes	Total
Grain yield	-0.117	-0.055	-0.258	-0.078	0.142	-0.164

Legend: correlation coefficient is significant at $p \leq 0.05$; $p \leq 0.01$***

Grain yield was considerably positively influenced by RSS during all the vegetation period, in early genotypes mainly (in the stem elongation stage; r = 0.494*). In late genotypes it is possible to see the influence of root system size on the yield also (in the heading stage; Table 1). Higher grain yield in 2015 at an experimental locality in South Moravia was statistically significantly related to larger root system size of the wheat plants.

CONCLUSION

It is possible to provide selection for cultivar resistance to stress already at the seed germination stage and on the quality of plant root systems.

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SPECIES COMPOSITION OF VEGETATION IN VINEYARDS OF THE WINERY VILLAGE POUZDŘANY

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Abstract: The purpose of the work is to compile a list of species growing in vineyards of the winery village Pouzdřany and further to evaluate importance of occurring plant species in view of the ecosystem. According to the winery law, the village Pouzdřany is registered as winery village belonging to the winery region Morava and Mikulov winery sub-region. Within the winery village, three vine lines were delimited: Kolby, Stará hora, Grunty. 102 plant species were found during the monitoring. In vineyards of the vine line Kolby, 88 plant species were found, of this 7 endangered and protected. In vineyards of the vine line Stará hora it was 26 plant species. In vineyards of the vine line Grunty we found 28 plant species.

Key Words: vegetation, vineyards, plant species, Pouzdřany

INTRODUCTION

Plant community is a group of plants, formed by coexistence of individual plant species populations in the given environment. Selection of species and their populations is determined in the phytosociology by conditions of the given environment, thus by the set of factors affecting the phytosociology and mutual competition (Neuhäuslová-Novotná and Guthová-Jarkovská 1980).

Species diversity of the plant community is necessary for creation of stable bonds in the vineyard agroecosystem (Pavloušek 2011). Numerous organisms are present generally in soil, thus also in the vineyard, in microedaphon, mezoedaphon and macroedphon (Jandák 2010). Just the vegetation growing in vineyards above ground as well as under ground contributes to biological diversity here. Important organisms have enough food and therefore stable environment for life here (Hejduk 2009).

The soil would contain approximately 200 earthworms per sq. meter; during our study of biodiversity we encountered a situation in conventionally cultivated vineyard, when 1 m^2 contained in average 5 earthworms only. Suitable vegetation in vineyards solves partly the problem of biologically degraded vineyard (Hluchý 2014a).

Higher number of plant species in vineyards contributes to occurrence of useful insect species helping in fight against pests. These positive species include e.g. predatory heteropters and ichneumon-flies (Ekovín 2016). A three-year study of biodiversity of vineyards in South Moravia proved that just composition and quality of herb vegetation in the inter-rows is the second most important factor influencing occurrence of butterfly species (Hluchý 2014b).

Grass cover brings benefits for earthworms (improved structure of soil), seven-spot ladybird (natural predator for aphis), lacewing (its grubs live on aphis), syrphid fly (its grubs live on thrips, aphids and other parasitic insect), earwig, spiders, ground beetles, frogs, songbirds, etc. These organisms contribute significantly to maintaining of natural balance of vineyards. Moreover, organic substances (above all, humic acids) agglutinate earth aggregates resulting in effective retaining of water (Hejduk 2009).

Live organisms in soil are also necessary in processes of transformation of humus. This releases nitrogen, phosphorus, sulphur and trace elements utilised subsequently by (Kalina 2004). In non-grassed

vineyards the soil dries up and this limits biological activity in the top layer. This earth is also loosened on the surface resulting in destroying corridors and cavities created by the earth edaphon (Hejduk 2009). Loss of species diversity in agricultural country is a big problem now. E.g. populations of free living insect have big importance in vineyards. Parasites of eggs, caterpillars and pupas help to suppress spreading of pests in considerately cultivated vineyards. The pests potato leafhoppers are suppressed by specific parasitoides, etc. (Pavloušek 2014).

The ECOWIN project by a Vienna institute Bioforschung Austria and Association Ekovín was realised in 2009 to 2012. Its purpose was to create conditions for existence of a big number of plant and animal species on more than 1000 ha of vineyards. A group of scientists working on a project VineDivers exists in France in last years. The purpose of this project is to analyse impacts of various farming systems on vineyards on biodiversity and ecosystem services (Vinedivers 2016).

Whereas zoological research activities has already brought a number of interesting results regarding to biodiversity in vineyards, floristical knowledges are still extremely poor and a well-advised research was not done in the Czech Republic up to this time. The purpose of the work is to compile a list of species growing in vineyards of the winery village Pouzdřany and evaluate the importance of occurring species from the point of view of the ecosystem.

MATERIAL AND METHODS

Characteristics of the interest territory Pouzdřany

The cadastral unit of the village Pouzdřany is situated in the South Moravian region, approximately 40 km south of Brno. The altitude varies from 177 to 220 meters. The territory lies in very warm and dry climatic region. Geological subsoil consists of Paleogene claystones and sandstones of flysch zone, covered discontinuously with loess and loess soils. The valley is filled with deluviofluvial sandy clay sediments. The soil types are represented by pararendzina typical and cambisol. In the part Kolby it is brown soil typical and luvisol typical. National natural monument Pouzdřanská steppe – Kolby is a part of the cadastral territory of the village. Decree of the Ministry of the Environment, no. 151/2014 Coll. specifies protected organisms.

Total area of the cadastral territory of the village Pouzdřany is 1349.9 ha, of this farmland 841.7 ha. As for the farmland, arable land makes 742.8 ha, meadows and pastures 38.4 ha and permanent cultures 60.5 ha.

According to the winery law, the village Pouzdřany is registered as winery village, belonging to the winery region Morava and Mikulov winery sub-region. Three winery lines are recognised and studied: Kolby, Stará hora, Grunty.

Methodology of evaluation of vegetation species composition

Evaluation of vegetation was made using a floristic list of the found species. Evaluation was made in the course of July 2016. Scientific names of individual plant species were used according to Kubát et al. (2002), categories of plant rarity and endangerment follow redlist of Grulich (2012). Inspection routes were determined on the selected territories within the winery lines. The found species were registered during the inspections. Occurrence of each recorded species was evaluated using a simple three-point scale after completion of the inspections.

Scale evaluating occurrence of species:

- 3 – very frequently occurring species with dominant occurrence (dominant species)
- 2 – common species with frequent occurrence on some parts on the vineyard only (sub-dominant species)
- 1 – rare species with rare and sporadic occurrence

RESULTS AND DISCUSSION

List of plant species found on the evaluated winery lines

The vine line Kolby was the first evaluated territory. Most of the area belonging to this line consists of vineyards. Most of the vineyard area is arranged in a similar way. Alternating of grassy inter-rows is used here, or inter-rows are cultivated. There were 88 plant species found during the monitoring.

The species with frequent occurrence on vine line Kolby (level 3 of the scale): *Lolium perenne, Amaranthus retroflexus, Achillea millefolium, Falcaria vulgaris, Convolvulus arvensis* and *Erigeron annuus.*

Species with common occurrence on vine line Kolby (level 2 of the scale) were: *Anagallis arvensis, Arrhenatherum elatius, Calamagrostis epigejos, Cirsium arvense, Conyza canadensis, Festuca rubra, Hordeum murinum, Chenopodium album, Chenopodium hybridum, Linaria vulgaris, Lotus corniculatus, Medicago lupulina, Plantago lanceolata, Reseda lutea, Robinia pseudacacia, Securigera varia, Setaria pumila, Silene vulgaris* and *Taraxacum* sect. *Ruderalia*

Species with rare or sporadic occurrence on vine line Kolby (level 1 of the scale) were: *Agrimonia eupatoria, Anagallis foemina, Arenaria serpyllifolia, Artemisia absinthium, Artemisia vulgaris, Astragalus glycyphyllos, Atriplex patula, Berteroa incana, Bromus inermis, Bromus sterilis, Bryonia alba, Capsella bursa-pastoris, Carduus acanthoides, Centaurea stoebe, Cichorium intybus, Clematis vitalba, Cornus sanguinea, Crepis tectorum, Cynoglossum officinale, Daucus carota, Dictamnus albus, Echinops sphaerocephalus, Echium vulgare, Elytrigia intermedia, Erodium cicutarium, Eryngium campestre, Fragaria viridis, Fraxinus excelsior, Galium verum, Geranium pusillum, Hyoscyomus niger, Hypericum perforatum, Chondrilla juncea, Lactuca serriola, Lathyrus tuberosus, Leucosinapis alba, Melica transsilvanica, Melilotus officinalis, Mercurialis annua, Morus alba, Onobrychis viciifolia, Origanum vulgare, Peucedanum alsaticum, Phacelia tanacetifolia, Plantago major, Polygonum aviculare, Potentilla tabernaemontani, Prunus* sp., *Rosa canina, Salvia nemorosa, Sambucus nigra, Scabiosa ochroleuca, Sisymbrium officinale, Sonchus oleraceus, Stachys annua, Tragopogon dubius, Trifolium pratense, Trifolium repens, Tripleurospermum inodorum, Verbascum austriacum, Verbascum blattaria, Vicia cracca* and *Viola arvensis.*

The line Stará hora was the second evaluated winery line. Vineyards are only on a part of this line. These are mostly vineyards that form a part of gardens or lie on small pieces of land and are cultivated by small private wine producers. Cultivated inter-rows with intensive regulation of vegetation is mostly utilised here. During the monitoring we could find 26 plant species on this line.

Species with frequent occurrence on this line include (level 3 of the scale): *Lolium perenne, Amaranthus retroflexus* and *Erigeron annuus.*

Species with common occurrence on vine line Stará hora (level 2 of the scale): *Achillea millefolium, Calamagrostis epigejos, Carduus acanthoides, Cirsium arvense, Convolvulus arvensis, Elytrigia repens* and *Chenopodium album.*

Species with rare or sporadic occurrence on vine line Stará hora (level 1 of the scale): *Anagallis arvensis, Arrhenatherum elatius, Atriplex patula, Bromus sterilis, Consolida regalis, Dactylis glomerata, Dipsacus fullonum, Falcaria vulgaris, Hordeum murinum, Chenopodium hybridum, Lactuca serriola, Papaver rhoeas, Portulaca oleracea, Prunus* sp., *Setaria pumila* and *Urtica urens.*

The line Grunty was the third evaluated winery line. Territory of this line consists mostly of abandoned or very little maintained vineyards, orchards and gardens. Maintenance of vegetation is very extensive here. On this line we could find total 28 plant species.

Species with very frequent occurrence on vine line Grunty (level 3 of the scale): *Calamagrostis epigejos, Carduus acanthoides, Dactylis glomerata, Erigeron annuus* and *Urtica dioica.*

Species with common occurrence on vine line Grunty (level 2 of the scale): *Arrhenatherum elatius, Artemisia absinthium, Bromus sterilis, Cirsium arvense, Clematis vitalba, Elytrigia repens, Falcaria vulgaris, Rosa canina* and *Solidago canadensis.*

Species with rare or sporadic occurrence on vine line Grunty (level 1 of the scale): *Asparagus officinalis, Astragalus glycyphyllos, Cornus sanguinea, Crepis tectorum, Epilobium ciliatum, Galium*

aparine, Galium verum, Lactuca serriola, Melilotus albus, Melilotus officinalis, Onopordum acanthium, Plantago major, Securigera varia and *Setaria pumila.*

Evaluation of occurrence of plant species on the monitored winery lines

The highest number of species was found on the winery line Kolby. Also several endangered and protected plant species were found there. As for the found species, now the following species are considered as endangered: *Anagallis foemina* (C3), *Dictamnus albus* (C3), *Chondrilla juncea* (C3), *Melica transsilvanica* (C4a), *Peucedanum alsaticum* (C3), *Stachys annua* (C2t), *Verbascum austriacum* (C4a), *Verbascum blattaria* (C2b).

Significantly lower number of plant species was found on winery lines Stará hora and Grunty. Species that can be extirpated with difficulties or invasive species dominated there.

Each of the explored winery lines has different arrangement that dominates here. The line Stará hora is typical by smaller vineyards that are separated by gardens, orchards or fields. Regulation of vegetation is very intensive on most of the vineyards. To the contrary, vineyards on the line Grunty are maintained very extensively and many of them are overaged and abandoned. In view of the species spectrum, these two ways of the vineyards arrangement leads to decreasing number of plant species and spreading of invasive plant species.

On the line Kolby the intensity of regulation of vegetation on vineyards is limited on most of the areas to mowing of grassed inter-rows, mechanical cultivation and possibly chemical regulation only in the strip along the stems. The vineyards are arranged in larger continuous areas that are bordering directly with balks, bosks and other dispersed greenery. This greenery functions probably as a resource of new plant fruits and seeds and contributes therefore to higher number of the plant species found on this line. Reasonable regulation of vegetation in the vineyards, applied on continuous areas as well as direct contact with surrounding vegetation as the resource of seeds of various plant species is probable the reason of varied composition of the plant society on this winery line.

CONCLUSION

During the monitoring of vegetation on vineyards in the winery village Pouzdřany we found 102 plant species. Most of the species were found on the winery line Kolby. On this line in the vineyard we found 7 endangered and protected plant species. On other winery lines Stará hora and Grunty we found much less plant species, with dominating species that we consider as weed or invasive species.

This comparison shows that reasonable regulation of vegetation in vineyards, applied on continuous areas is important to preserve high number of plant species in vineyards. Also, important is the border of vineyards with surrounding vegetation as the resource of original plant species.

Plants are the basis of the food chain and resource of food for many animals. Variety of the plant society is important for good functioning of the whole vineyard ecosystems. Maintained vineyards may also be places of occurrence of protected plant species and contribute to their survival in our country.

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SPECIES COMPOSITION OF VEGETATION IN VINEYARDS OF THE WINERY VILLAGE POPICE

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Abstract: The purpose of the work is to compile a list of species growing in vineyards of the winery village Popice and evaluate importance of occurring plant species for growing of vine. According to the winery law, the village Popice is registered as winery village belonging to the winery region Morava and Mikulov winery sub-region. Within the winery village there were delimited 8 vine lines: Mitrberk, Panenský kopec, Písky, Ráfle, Sonberk, Stará hora, Svidrunk and Unédy. During our monitoring we found 104 plant species. As for the found species that can compete directly with vine, we can mention, above all, *Cirsium arvense, Convolvulus arvensis, Elytrigia repens, Elytrigia intermedia, Artemisia vulgaris, Arrhenatherum elatius* and *Medicago sativa.* Further, there were species that can be considered as invasive and may compete negatively with vine, such as *Robinia pseudacacia*, *Lycium barbarum, Calamagrostis epigejos* and *Solidago canadensis.*

Key Words: vegetation, vineyards, plant species, Popice

INTRODUCTION

The Czech Republic is classified as small winery country. Within Europe, this includes winery regions located on the north and spreading over two winery regions, in Bohemia and Moravia. In Bohemia, two sub-regions can be distinguished, Mělník and Litoměřice. In Moravia, sub-regions Znojmo, Mikulov, Velké Pavlovice and Slovácko (Pavloušek 2011). Further the vineyards are divided into winery villages and finally into winery lines (Obůrková 2013). Winery regions of the Czech Republic belong to warm and dry regions with mild winter and slight lack of precipitations (Kraus 1999). These conditions have big influence on quality of grapes. Particularly the course of day and night temperatures is significant (Pavloušek 2011).

Each winery region consists of typical mosaic of ecosystems and each individual winery region consists of a mosaic different from any other (Kraus 1999). Vegetation growing in the vineyard has positive influence on its whole ecosystem. We can see increase of organogenous nitrogen, improvement of permeability and structure of soil, increase of the content of humus in top layers, reduction of water erosion and elution of nitrogen and other nutrients, increase of the content of edaphone, enhancement of thermal regime of soil, improved infiltration of water, increased water capacity of soil, restricted growth of weed, support and stabilisation of fauna and arthropods in the vineyard, etc. (Trioli and Hofmann 2009).

In the years 2009 to 2012 the ECOWIN project was realised by the Vienna institute Bioforschung Austria and Association Ekovín, whose purpose was, among others, to create conditions for existence of a big number of plant and animal species on more than 1000 ha of vineyards. A group of scientists working on a project VineDivers exists in France in last years. The project has to analyse impacts of various farming systems in vineyards on biodiversity and ecosystem services (Vinedivers 2016).

The purpose of the work is to compile a list of species growing in vineyards of the winery village Popice and evaluate the importance of occurring species in view of vine growing.

MATERIAL AND METHODS

Characteristics of the interest territory Popice

The cadastral unit of the village Popice is situated in the South Moravian region, approximately 30 km south of Brno. The altitude varies from 175 to 308 meters. The territory lies in very warm and dry climatic region. Geological subsoil consists of Paleogene claystones and sandstones of flysch zone, covered discontinuously with loess and loess soils. Soil types are represented by pararendzina typical and cambisol, brown soil typical and luvisol typical.

According to the winery law, the village Popice is registered as winery village, belonging to the winery region Morava and Mikulov winery sub-region. Eight winery lines are recognised within the winery village: Mitrberk, Panenský kopec, Písky, Ráfle, Sonberk, Stará hora, Svidrunk and Unédy. On winery lines Písky and Ráfle no vineyards were found in time of the evaluation and their territories consisted mostly of arable land and therefore evaluation of vegetation was not performed there.

Total area of the cadastral unit Popice is 999.2 ha, of this farming land makes 832.6 ha. As for farming land, arable land makes 431.1 ha, meadows and pastureland 36.6 ha, permanent plantation 353.2 ha.

Methodology of evaluation of vegetation species composition

Evaluation of vegetation was made using a floristic list of the found species. Evaluation was made in the course of July 2016. Scientific names of individual plant species were used according to Kubát et al. (2002). Itineraries of the inspection routes were determined on the selected territories within the winery lines. The found species were registered during the inspections. Occurrence of each found species was evaluated using a simple three-point scale after completion of the inspections.

Scale evaluating occurrence of species:

- 3 – very frequently occurring species with dominant occurrence (dominant species)
- 2 – common species with frequent occurrence on some parts in the vineyard only (sub-dominant species)
- 1 – rare species with rare and sporadic occurrence

RESULTS AND DISCUSSION

List of plant species found on the evaluated winery lines

In the course of monitoring, there were found total 104 plant species. Their occurrences on individual winery lines and intensity of their occurrences can be found in Table 1.

Table 1 Occurrence of species on winery lines of the winery village Popice

	Winery line					
Species	Panenský kopec	Unédy	Sonberk	Svidrunk	Stará hora	Mitrberk
Lolium perenne	3	3	3	3	2	3
Setaria pumila	2	2	1	3	3	3
Chenopodium album	3	3	2	1	2	3
Cirsium arvense	2	2	2	2	3	3
Amaranthus retroflexus	2	2	1	1	3	3
Convolvulus arvensis	2	2	3	2	-	3
Elytrigia repens	3	2	1	2	-	3
Achillea millefolium	1	3	2	2	-	2
Tripleurospermum inodorum	1	2	2	-	2	2
Trifolium repens	3	-	-	3	-	3

Taraxacum sect. *Ruderalia*	2	1	2	2	-	2
Falcaria vulgaris	3	2	1	1	1	1
Carduus acanthoides	1	1	1	1	2	2
Echinochloa crus-galli	-	3	3	1	-	1
Plantago lanceolata	2	-	2	-	2	2
Polygonum aviculare	-	2	2	-	2	2
Conyza canadensis	2	1	2	2	1	-
Hordeum murinum	1	-	2	2	-	2
Lactuca serriola	-	1	1	1	1	2
Artemisia vulgaris	2	-	1	1	-	2
Crepis tectorum	-	-	1	3	1	1
Medicago lupulina	-	2	2	1	-	1
Plantago major	-	1	1	1	-	2
Arrhenatherum elatius	1	-	2	2	-	-
Bromus tectorum	-	-	2	2	-	1
Erigeron annuus	1	-	1	1	-	2
Atriplex patula	1	1	-	-	-	2
Arctium tomentosum	1	2	1	-	-	-
Daucus carota	1	-	2	-	-	1
Consolida regalis	1	1	-	1	1	-
Rosa canina	1	1	1	1	-	-
Dactylis glomerata	-	-	2	1	-	1
Medicago sativa	2	-	1	-	-	1
Mercurialis annua	2	1	-	-	-	-
Cichorium intybus	-	1	1	1	-	-
Melilotus officinalis	-	1	1	-	-	1
Astragalus glycyphyllos	1	1	1	-	-	-
Ballota nigra	-	-	1	1	-	1
Malva neglecta	-	-	1	1	-	1
Bromus sterilis	-	1	-	2	-	-
Crepis biennis	1	-	2	-	-	-
Calamagrostis epigejos	1	-	1	1	-	-
Echinops sphaerocephalus	-	1	-	1	-	-
Setaria viridis	-	-	-	2	-	-
Lathyrus tuberosus	1	-	1	-	-	-
Capsella bursa-pastoris	-	1	-	-	-	1
Urtica dioica	-	-	1	-	-	1
Festuca rubra	-	-	2	-	-	-
Tragopogon dubius	-	-	1	-	-	1
Astragalus exscapus	-	1	1	-	-	-
Atriplex prostrata	-	2	-	-	-	-
Linaria vulgaris	1	1	-	-	-	-
Papaver rhoeas	-	1	1	-	-	-
Chenopodium hybridum	1	-	1	-	-	-

Sonchus oleraceus	-	-	-	1	-	1
Fallopia convolvulus	1	-	-	-	1	-
Juglans regia	-	-	1	1	-	-
Arenaria serpyllifolia	-	-	-	1	-	1
Elytrigia intermedia	-	-	2	-	-	-
Phragmites australis	-	-	1	-	-	1
Reseda lutea	-	-	1	-	-	1
Agrimonia eupatoria	1	1	-	-	-	-
Seneciovulgaris	-	-	1	-	-	1
Galium verum	-	-	2	-	-	-
Portulaca oleracea	-	-	1	-	-	1
Lotus corniculatus	2	-	-	-	-	-
Robinia pseudacacia	-	-	-	-	-	2
Dictamnus albus	1	-	-	-	-	1
Onobrychis viciifolia	1	-	-	-	-	1
Epilobium ciliatum	-	-	1	1	-	-
Conium maculatum	-	-	1	-	-	-
Securigera varia	1	-	-	-	-	-
Origanum vulgare	1	-	-	-	-	-
Anagallis arvensis	-	-	-	-	-	1
Scabiosa ochroleuca	1	-	-	-	-	-
Leucosinapis alba	-	-	-	1	-	-
Picris hieracioides	-	-	-	1	-	-
Sisymbrium officinale	-	-	-	-	-	1
Trifolium pratense	-	-	-	-	-	1
Geranium pusillum	-	-	-	1	-	-
Urtica urens	-	-	-	-	-	1
Astragalus cicer	-	-	1	-	-	-
Lycium barbarum	-	-	-	-	-	1
Atriplex sagittata	-	-	-	-	-	1
Poa pratensis	-	-	1	-	-	-
Arctium lappa	1	-	-	-	-	-
Artemisia absinthium	1	-	-	-	-	-
Erodium cicutarium	-	-	-	-	-	1
Cerastium arvenxe	-	-	-	1	-	-
Xanthium strumarium	-	1	-	-	-	-
Dipsacus fullonum	-	1	-	-	-	-
Rumex crispus	-	-	-	-	-	1
Rumex acetosa	-	-	1	-	-	-
Hypericum perforatum	-	-	1	-	-	-
Vicia cracca	-	1	-	-	-	-
Viola arvensis	1	-	-	-	-	-
Cerinthe minor	-	-	-	-	-	1
Solidago canadensis	-	-	-	1	-	-

Evaluation of occurrence of plant species on the monitored winery lines

The winery line Panenský kopec was the first evaluated territory. Most of the area, belonging to this line, is occupied by vineyards. Similar arrangement of vineyards is applied on larger areas, with alternating cultivated and grassed inter-rows; alternatively the cultivated inter-rows were sown by annual plants. During the monitoring there were found 46 plant species on this line. As for the found plant species on this line that can compete directly with vine, we can mention particularly *Elytrigia repens, Convolvulus arvensis, Cirsium arvense, Artemisia vulgaris, Medicago sativa, Arrhenatherum elatius, Rosa canina* and *Calamagrostis epigejos.*

The line Unédy was the second evaluated winery line. Most of the line area consists of vineyards and similar arrangement of vineyards is applied here. Grassy inter-rows are used here with alternating intentionally and spontaneously grassed inter-rows. During the monitoring we could find 37 plant species on this line. As for the plant species found on this line, we can mention particularly *Cirsium arvense, Convolvulus arvensis, Elytrigia repens* and *Rosa canina.*

The line Sonberk was the third evaluated winery line. Most of this line area consists of vineyards and similar arrangement of vineyards is applied here. Grassy inter-rows are used here with alternating intentionally and spontaneously grassed inter-rows. During the monitoring we could find 59 plant species on this line. As for the plant species found on this line that can compete directly with vine, we can mention particularly *Convolvulus arvensis, Cirsium arvense, Arrhenatherum elatius, Elytrigia intermedia, Artemisia vulgaris, Elytrigia repens* and *Calamagrostis epigejos.*

The line Svidrunk was the fourth evaluated winery line. Most of this line area consists of vineyards and similar arrangement of vineyards is applied here. Grassy inter-rows are used here with alternating grassy and cultivated inter-rows. During the monitoring we could find 42 plant species on this line. As for the plant species found on this line that can compete directly with vine, we can mention particularly *Cirsium arvense, Convolvulus arvensis, Elytrigia repens, Arrhenatherum elatius, Artemisia vulgaris, Calamagrostis epigejos* and *Solidago canadensis.*

The line Stará hora was the fifth evaluated line. Most of this line area consists of orchards and lands with other types of utilisation. Vineyards form a minor part here and are newly planted. All inter-rows are cultivated here. During the monitoring we could find 15 plant species on this line. As for the plant species found on this line that can compete directly with vine, we can mention particularly *Cirsium arvense.*

The line Mitrberk was the sixth evaluated line. Vineyards on this line are cultivated in different ways that alternate very frequently. A part of the vineyards is used by small private wine producers. Grassy and cultivated inter-rows are used here including their alternating. During the monitoring we could find 54 plant species on this line. As for the plant species found on this line that can compete directly with vine, we can mention particularly *Cirsium arvense, Convolvulus arvensis, Elytrigia repens, Artemisia vulgaris, Robinia pseudacacia*, *Medicago sativa, Melilotus officinalis, Phragmites australis* and *Lycium barbarum.*

CONCLUSION

During the monitoring of vegetation in vineyards in the winery village Popice we found 104 plant species. As for the found plant species that can compete directly with vine, we can mention particularly *Cirsium arvense, Convolvulus arvensis, Elytrigia repens, Elytrigia intermedia, Artemisia vulgaris, Arrhenatherum elatius* and *Medicago sativa.* Further there were found species that we can consider as invasive and may compete negatively with vine, such as *Robinia pseudacacia*, *Lycium barbarum, Calamagrostis epigejos* and *Solidago canadensis.*

Currently the vineyards are perceived as a plant community, where in addition to vine also other plant species are growing. Occurrence of numerous plant species prevent erosion, provides food for insect and vertebrate species, enriches soil with nitrogen. Some of these plant species may compete with vine, e.g. species with deep root systems and possible species with allelopathic abilities. However, also invasive species occur in vineyards; these represent danger for vegetation of the vineyards as well as for the surrounding ecosystems.

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THE EFFECT OF INOCULATED AND MITIGATED BY PLANTS BIOCHAR ON SOIL MICROBIOTA

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Abstract: Biochar application to the soil and its influence on soil physical, chemical and biological properties remains one of the most arguing topics last decades. Nowadays it is used as soil amendment in the terms of the strong negative impact mitigation of anthropogenic activities. Especially it may be found a lot of controversial statements regarding biochar's impact on soil microorganisms. Main representatives of soil microbiota such as key groups of microorganisms along with arbuscular mycorrhizal fungi considered to be sensitive indicators of soil state changes. Thus, in this study there is a try to enlighten the biochar's effect on soil microbiota and colonization of roots by arbuscular mycorrhizal fungi (AMF). Investigation involved five types of soil treatments with the lettuce as a model indicator plant (*Lactuca sativa)*. Two types of bacterial inoculums applied to model plants in combination "with" and "without" the addition of the mineral fertilizer were exposed in controlled pot experiment. In order to avoid the estimation of the primary effect on plants immediately after the fresh biochar application, which could be partly deleterious, there were estimated only the results from the second generation, after the first plant generation has been harvested. The second generation of plants have been seeded into the same soil with the redosing the inoculum and fertilizers addition only. It has been analysed the colonization of roots by AMF and the total number of microorganisms including nitrogen-fixing bacteria, actinomycetes, spore-forming bacteria and micromycetes. Research results have shown of root colonization by AMF in the second plant generation with no significant differences between the treatments. The enumeration of different microorganism groups demonstrated sharp increase in all the applied treatments that corresponded to the root biomass increase as well.

Key Words: arbuscular mycorrhiza, biochar, soil fungi, soil microorganisms

INTRODUCTION

Strong anthropogenic impact on the environment and especially on its soil component force to find new sustainable ways to improve their state. Thus a charred carbon-enriched material or biochar is used last decades as a soil amendment to sequester carbon in soil and enhance its quality. Quite big amount of studies exists describing positive influence of biochar on soil physical and chemical properties and remains a lot of questions concerning its effect on soil microbial habitat (Jien and Wang 2013). It is known, that biochar changes the physical and chemical environment of the soil and consequently affects the characteristics of soil biota acting. All the possible biochar impacts on soil organisms can potentially interpret changes in nutrient availability and crop productivity (Domene et al. 2014). Biochar's structure with its pores can provide a safe habitat for many microorganisms by protecting them from predators and by providing many of them with carbon, energy and mineral nutrients (Warnock et al. 2007). Soil microorganisms directly interact with plants in the rhizosphere, and this may occur as a result of mutualistic associations between plant roots and microorganisms, as for example with the arbuscular mycorrhizal (AM) fungi or the nitrogen N_2-fixing rhizobia bacteria (Lehmann and Joseph 2009). It depends upon the composition of biochar's residual pyrolysis compounds: they may serve as substrates for microbial growth and metabolism, (Steiner et al. 2008). From the other side, these compounds may also be toxic to plants and probably to some types of microorganisms (McClellan et al. 2007). Some researchers have shown that soils have a higher microbial biomass and abundance of culturable bacteria and fungi, adding biochar could indicate that the biochar is inhibiting the activity of biochar-colonizing

microorganisms, changing bacterial to fungal ratios Moreover, microorganisms colonizing fresh biochar that has post-pyrolysis condensates on its surfaces will differ from ones colonizing the biochar surfaces after the substances have been metabolized (Lehmann and Joseph 2009). A lot of biochars are very low in inorganic N content and this gives diazotrophs a competitive advantage for surface colonization (Lehmann and Joseph 2015).

Mycorrhizae are sensitive to management interventions, for example adding biochar, and it can speculate on the possible synergistic effects of mycorrhizal inoculation and biochar application in improving soil quality and plant growth (Schwartz et al. 2006).

The aim of our research is to investigate the lagging of the biochar influence after the first generation of plants harvesting along with inoculums adding on arbuscular mycorrhiza fungi (AMF) and microbiota of soil in general.

MATERIAL AND METHODS

Characterization of sampling locality, experimental design

Soil samples have been collected from the plots situated in the protection zone of underground drinking water source "Brezova nad Svitavou". Experimental soil samples have been taken according to CSN ISO 10 381-6 (CSN is "Czech Technical Standard"). After they have been homogenized and sieved through a sieve with a grid size of 10 mm. Four different types of treatments have been prepared that included four replications of each treatment resulted into twenty plastic containers (10x10x11) filled with 800 g of topsoil. An overview of the applied inoculums and fertilizers including their active ingredients are displayed in Table 1.

Table 1 Overview of applied treatments

Treatment	Amendment	Application rate	BBCH	Active ingredients
T1	Control without any additives	-	-	-
T2	Biochar + "Bactofil" inoculum	50 t/ha 1 l/ha	13 15–18	*Azospirillum brasilense, Azotobacter vinelandii, Bacillus megaterium, Bacillus polymyxa, Pseudomonas fluorescens, Streptomyces albus*
T3	Biochar + "Bactofil" inoculums + DAM 390	50 t/ha 1 l/ha 140 kg/ha (N)	13 15–18	*Azospirillum brasilense, Azotobacter vinelandii, Bacillus megaterium, Bacillus polymyxa, Pseudomonas fluorescens, Streptomyces albus, mineral nitrogen*
T4	Biochar + "NovaFerm" inoculum	50 t/ha 10 l/ha	13 15–18	*Azospirillum* spp., *Azotobacter* spp., *Bacillus megaterium, Bacillus subtilis*
T5	Biochar + "NovaFerm" inoculums + DAM 390	50 t/ha 10 l/ha 140 kg/ha (N)	13 15–18	*Azospirillum* spp., *Azotobacter* spp., *Bacillus megaterium, Bacillus subtilis, mineral nitrogen*

Taking into account our previous research results we have decided to apply the same soil with already mitigated biochar and to plant the second generation of plants to see its effect (Mikajlo et al. 2015). It is important to notice, that after first generation of plants harvesting again and before the second generation of plants seeding all the root biomass has been removed and experimental soil has been homogenized again. It may be observed from the table with the treatments overview that soil has been amended with biochar in all the treatments except the first control one. Beech wood biochar made at slow pyrolysis with the use of low temperature 470 °C has been applied to experimental containers. The

inoculation by “Bactofil” and “NovaFerm” additives took place at the beginning of vegetation of the second generation of plants. After one week of exposition mineral N have been applied in a DAM 390 liquid fertilizer form that contains 30% of nitrogen; the ratio of ammonium, nitrate, and amidic nitrogen is 1:1:2. Lettuce (*Lactuca sativa)* has been chosen as an experimental plant – the same as in our previous studies with the first generation of plants.

Experimental set up has been conducted in the growth box phytotron with the following laboratory ambient conditions: 22 °C daily temperature, 19 °C night temperature, 65% humidity with a day length of 12 h and light intensity of 380 μmol/m/s in the period from June 2015 until October 2015.

Estimation of roots colonization by arbuscular mycorrhizal fungi

Roots colonization by arbuscular mycorrhizal fungi has been determined by taking samples from the root system of each experimental plant after harvesting according to (Koske and Gemma 1989). Lettuce roots in 0.5 g of each sample and about 3 cm in length have been washed-out with water and stored in FAA solution (formaldehyde – acetic acid – ethanol 50%). Then samples have been rinsed and incubated with 10% (w/v) KOH solution for 1 h at 90 °C aiming to increase stain penetration and mycorrhizal roots clearing. The acidification process with 10 ml of 1% HCl was following. With the next step roots have been transferred directly into a beaker containing 10 ml of 1% trypan blue in lacto glycerol and incubated for 1 h at 90 °C. After the incubation stained roots have been cut into 15 mm long segments that have been randomly taken from each plant and adjusted to slides (20 segments from each root). Experimental segments have been studied with the routine microscope Olympus CX41. We have analyzed the presence or absence of root colonization by arbuscules, vesicles, extra and intraradical hyphae (see Figure 1). Total dry root biomass has also been measured.

Figure 1 Root segment colonization by AMF

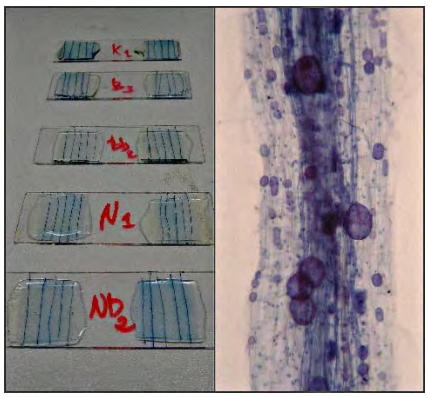
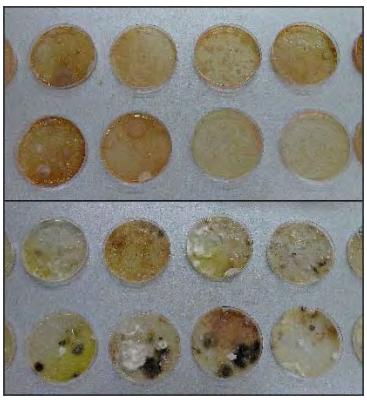


Figure 2 CFU: actinomycetes and micromycetes



Microbiological analysis

Colony forming units (CFU) dilution plate method has been used for microbial diversity determination in soil samples according to CSN EN ISO 6887-1 (Czech/International Technical Standard – “Part 1 – Preparation of test samples, initial suspension and decimal dilutions for microbiological examination). Total number of soil microorganisms including spore-forming bacteria, microscopic fungi, actinomycetes and nitrogen-fixing bacteria have been estimated. MPA nonselective medium has been prepared to estimate total number of microorganisms and spore-forming bacteria that have been heated at 85 °C for 15 minutes before their seeding. The number of microscopic fungi has been seeded on Czapek Dox agar, actinomycetes on starch and ammonia agar, nitrogen-fixing bacteria on Ashby agar (see Figure 2). Values have been expressed in CFU per g.

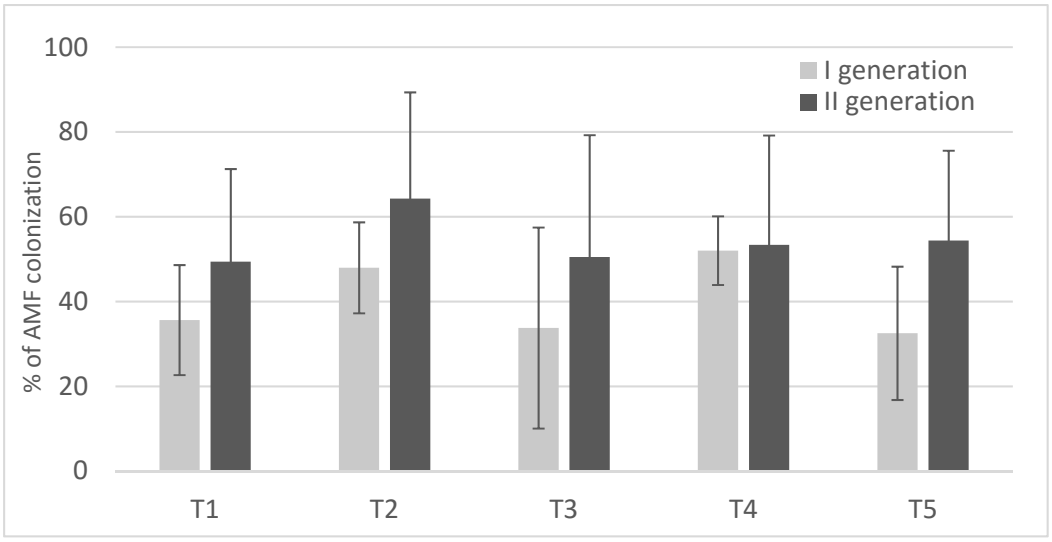
RESULTS AND DISCUSSION

Root AMF colonization values

Previous research has showed that in the first plant generation the roots were colonized by AMF only by 30–50% (Mikajlo et al. 2015). Only “Bactofil” inoculum has showed colonization stimulation of 25%. Comparing two generations of plants, that have been grown in the same amended with biochar and bacterial inoculums soil, we may notice the following differences (see Figure 3). Second generation

of plants has shown the increase of AMF colonization in all the treatments, except the "Novaferm" treated one where the values stayed on the same level of 52–53%. We may state, that the importance of AMF for the plants nutrition in the second generation was higher rather in the first generation.

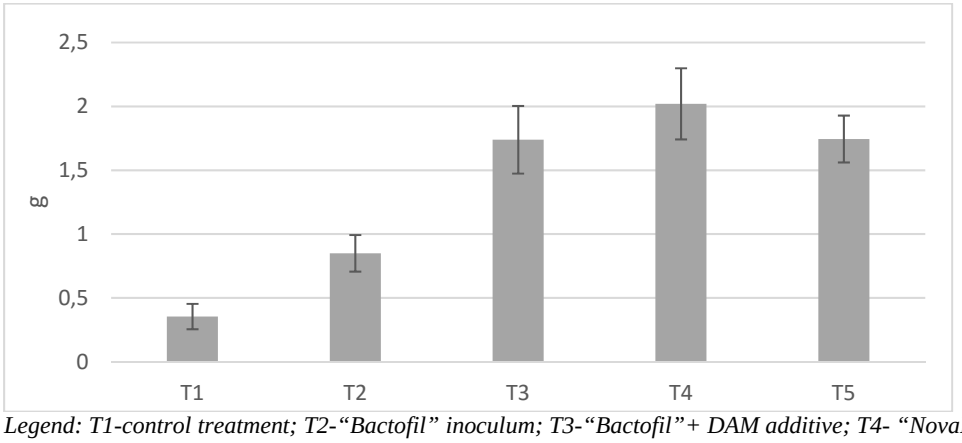
Figure 3 Root colonization percentage by AMF in I and II generation of plants (in %), 2015



Legend: T1-control treatment; T2-"Bactofil" inoculum; T3-"Bactofil"+ DAM additive; T4- "NovaFerm" inoculum; T5- "NovaFerm"+ DAM (mean values ± SD, n = 3)

Basically there were estimated rather big differences between the treatments from AMF colonization values from the first and the second generation of plants. Based on the literature (Covacevich et al. 2012) there were probably no optimal offers of the key nutrients and/or sufficient collaboration with other groups of soil microorganisms, and therefore it was efficient for the control plants to invest carbohydrates into the mycorrhizal partners (especially in the case of "Bactofil" inoculum treatment where the AMF colonization reached almost 65%). Rondon et al (2007) examined whether arbuscular mycorrhizal fungi colonization was increased by adding biochar, but did not observe any significant effects which confirms our results. From the other hand AMF percentage with a "Bactofil" treatment has been higher which can be related to a low underground biomass production (see Figure 4). Thus, active ingredients of this additive invested to arbuscular mycorrhizal fungi growth.

Figure 4 Plant root biomass production (in g), 2015



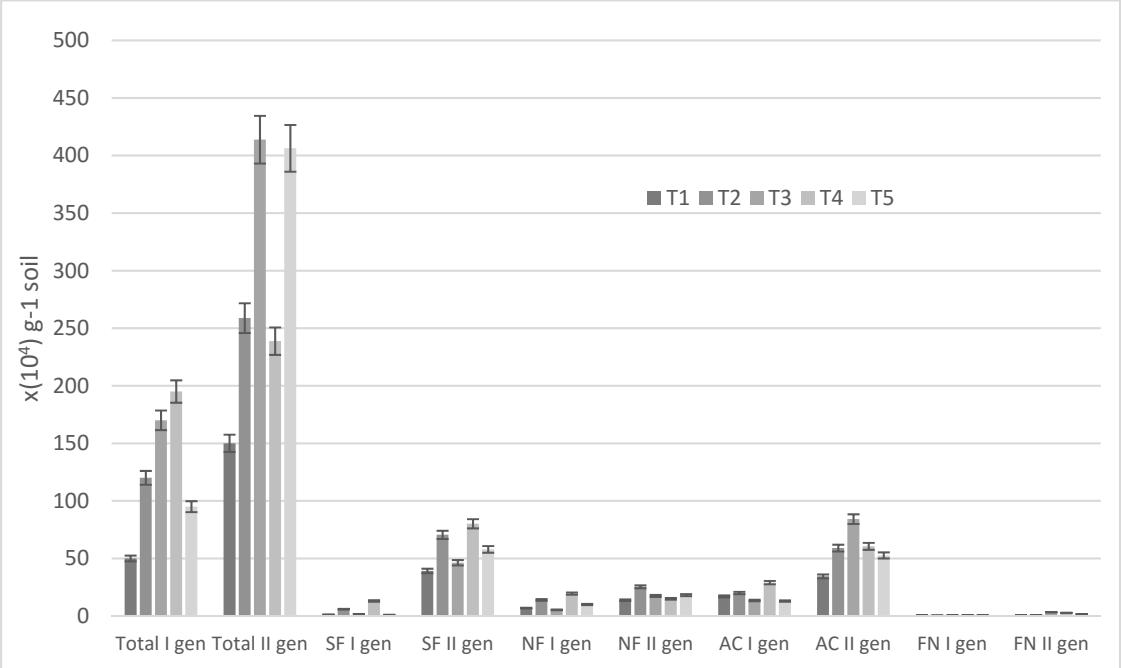
Legend: T1-control treatment; T2-"Bactofil" inoculum; T3-"Bactofil"+ DAM additive; T4- "NovaFerm" inoculum; T5- "NovaFerm"+ DAM (mean values ± SD, n = 3)

Microorganisms diversity assessment

Taking into account results after two plant generation vegetation we may notice that all the groups of microoorganisms have risen in the second generation (see Figure 5, Table 2). We may argue that the second additive application definitely influenced this increase trend in a positive way. Soil amendment with DAM fertilizer mobilized sources in soil. Spore-forming bacteria and the total bacteria amount have grown in almost 3 times higher comparing to the first generation results. These specific kinds of

bacteria are helping all the plants to improve nutrition in general that resulted in the aboveground biomass growth as well. Overall, soil microbiota activity intensively affects soil function and as a consequence crop growth and yield. Our investigation results have common conclusions with the other research (Domene et al. 2014).

Figure 5 Differences between I and II plant generation microbiological diversity (in CFU x (10^4) g soil), 2015



Legend: T1-control treatment; T2-"Bactofil" inoculum; T3-"Bactofil"+ DAM additive; T4- "NovaFerm" inoculum; T5- "NovaFerm"+ DAM (mean values ± SD, n = 3); I gen-first generation, II gen-second generation; SF- spore-forming bacteria, NF- nitrogen-fixing bacteria, AC- actinomycetes, FN- microfungi

Table 2 Comparing I and II plant generations data of CFU analysis

Treat	Total I gen	Total II gen	SF I gen	SF II gen	NF I gen	NF II gen	AC I gen	AC II gen	FN I gen	FN II gen
T1	50±6.4	150±7.6	1.2±0.4	39.12±15.2	6.85±0.3	13.75±8.2	17±5.5	34.37±13	0.55±0.03	0.64±0.2
T2	120±7.7	258.75±9.1	5.9±0.7	70.5±22	13.97±0.9	16.25±6.8	20±4.7	59±18	0.4±0.02	0.66±0,2
T3	170±10	413.75±19.8	1.76±0.1	46.25±21	5.34±0.1	17.5±8.5	13,5±2.4	84.12±34	0.46±0.03	3.33±0.7
T4	195±3.4	238.75±9	13.125±3	80.12±12.8	19.45±6.2	14.93±7	29±8.8	60.5±24	0.62±0.04	2.76±0.8
T5	95±7.3	406.25±17.8	1.04±0.5	57.87±22	10,06±2.8	18.12±7.9	13±1.6	52.6±14.5	0.55±0.02	1.53±0.9

CONCLUSION

Amending soil with biochar starting to be one of the most promising soil management strategies. Definitely it should be concentrated on a support of soil biota to carry out the key ecosystem functions. Microorganisms ensure long-term soil fertility and sustained crop production. Research results have stated on undoubtedly better biochar and inoculums influence on soil microbiota including AMF after first generation of plants harvesting. We may observe bright rise of all the representatives of microorganisms in terms of CFU analysis. Though there was no significant difference between the

treatments while analysing AMF colonization. Still the results showed higher values comparing to the previous studies with the first plant generation. Taking into consideration dry biomass results it may be also admitted positive influence of treatments especially with "Novaferm" inoculum amendment.

Our next investigations will take into consideration different biochar concentrations and lagging of its effect in the soil by adding/mixing it with organic matter and consequently completing the lacking nutrients.

ACKNOWLEDGEMENTS

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NITROGEN FATE IN TERMS OF MITIGATED INFLUENCE OF BIOCHAR IN SOIL

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Abstract: Biochar is formed in the thermochemical transformation of plant biomass through pyrolysis. It is considered that biochar improves soil properties, and that its application in the soil increases the carbon sequestration by pumping CO_2 out from the atmosphere. It was confirmed in our previous studies, that the freshly applied biochar to soils can worsen the growth of plants. Because of it, the real influence of biochar after dissipation of its initial adverse effects was examined in the next generation of plants cultivated in pots. After the addition of identical inoculums to model plants as was made in the first generation, the soil was tested concerning the fate of nitrogen - in terms of the amount of its mineral forms, their availability in the soil and the rate of leaching. For the second generation of plants five kinds of soil treatments have been prepared with the same indicator plant salad (*Lactuca sativa*) as in the first generation experiment. Estimation of nitrogen availability in soil and assessment of mineral nitrogen leaching have been investigated using ion exchange resin method. Experimental results have showed the lowest values of nitrogen leaching in terms of inoculum additives along with biochar application that lead to microbial development and consequently to nitrogen immobilization. Nitrogen availability investigation has indicated the increase of its amount released from the microbial biomass after second generation of plants harvesting. Hence, it may be stated that the interactions between soil mixture with biochar, native soil microorganisms and/or bacterial inoculums and experimental plants have been improved during the second plant cultivation.

Key Words: biochar, mineral nitrogen, inoculum, soil

INTRODUCTION

Industrialised and developing countries almost in equal way suffer from soil productivity loss and degradation that form an acute problem nowadays (Obalum et al. 2012, Lehmann and Joseph 2009). This occurs mainly due to the strong anthropogenic impact with the negative consequences that include irrigation, and input of fertilizers, pesticides and intensive use of agrochemicals (Vaičys and Mažvila 2009, Sobral et al. 2015). Thus, finding new sustainable ways to overcome these destroying soil actions becomes one of the main targets today. Biochar believes to play a key role in expanding options for sustainable soil management by improving soil productivity, decreasing environmental impact with positive consequences for soil properties in general. (Verhejien et al. 2009, Lal 2015) Biochar is produced from sustainably provided waste biomass such as crop residues, manures, timber, forestry residues and green waste with the use of modern pyrolysis technologies (Woolf et al. 2010). Actually this material provides a unique opportunity for soil fertility and nutrient-use efficiency increase with the use of locally available and renewable materials in a sustainable way. Biochar has also an impact on soil nitrogen cycling that is highly recognized as plants require this element for growing (Prommer et al. 2014). In the agriculture nitrogen makes the main annual input in the form of inorganic or organic fertilizers for crop nutrition. Except for biochar does not supply nitrogen, as it is one of the elements that is volatile at pyrolysis temperatures. Moreover, nitrogen is eliminated as gas, or integrated with carbon into the stable molecular structures that is uppermost in biochar. Nevertheless, biochar interacts with soil mineral nitrogen and mineral nitrogen that has availability through organic matter mineralisation. Mineral nitrogen of soil consists only small per cent of total nitrogen in soil exactly in the form accesible for the plant roots (Harmsen 2003). Nitrate is a product of ammonium transit by

microbes. Occasionally evident biochar effect on nitrous oxide emissions and leaching, in a case of their decrease, has been contributed to the indirect effect on ammonium sorption. A decrease in nitrate concentrations of soil can be fractionally explained by the ammonium capture and it depends on the biochar rate application in general. At the same time, ammonium sorption impact can have negative influence in terms of plant growth, taking into account the short term (Lehmann and Joseph 2009). Since ammonium can in general be accessible for plants, sorbed to internal pores of biochar ammonium could be physically inaccessible to roots.

The main aim of the investigation is to study the effect of biochar with the mitigated properties mixed with the inoculums on nitrogen availability for soil microorganisms and to investigate mineral nitrogen leaching influenced by the same conditions.

MATERIAL AND METHODS

Characterization of sampling locality, experimental design

Plots where we have collected our samples are situated in the protection zone of underground drinking water source "Brezova nad Svitavou". These soil samples have been taken according to CSN ISO 10 381-6 (CSN is "Czech Technical Standard"). Next step is based on samples homogenization and sieving through a sieve with a grid size of 10 mm. Four different types of treatments have been mixed with four replications per each treatment. As a result, we obtained twenty plastic containers (10x10x11 cm) filled with 800 g of topsoil. An overview of the applied treatments is displayed in Table 1.

Table 1 Overview of applied treatments

Treatment	Amendment	Application rate	BBCH	Active ingredients
T1	Control without any additives	-	-	-
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T3	Biochar + "Bactofil" inoculums + DAM 390	50 t/ha 1 l/ha 140 kg/ha (N)	13 15–18	*Azospirillum brasilense, Azotobacter vinelandii, Bacillus megaterium, Bacillus polymyxa, Pseudomonas fluorescens, Streptomyces albus, mineral nitrogen*
T4	Biochar + "NovaFerm" inoculum	50 t/ha 10 l/ha	13 15–18	*Azospirillum* spp., *Azotobacter* spp., *Bacillus megaterium, Bacillus subtilis*
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It may be seen from the table with the applied treatments that soil has been amended with biochar in all the treatments except the first control one. Biochar made from beech wood at slow pyrolysis with the use of low temperature 470 °C has been applied to experimental containers. The repeated inoculation by "Bactofil" and "NovaFerm" additives after the first plant generation harvesting inoculation" took place at the beginning of vegetation of the second generation of plants. After one week of additives persistence soil mineral N have been applied in a DAM 390 liquid fertilizer form. It contains 30% of nitrogen; the ratio of ammonium, nitrate, and amidic nitrogen is 1:1:2. Lettuce (*Lactuca sativa)* has been chosen as an experimental plant – the same as in our previous studies with the first generation of plants (Mikajlo et al. 2015). It is significant to point out, that after first generation of plants harvesting and

before the second generation of plants seeding all the root biomass has been removed and experimental soil has been homogenized again.

Research has been conducted in the growth box phytotron with the following laboratory ambient conditions: 22 °C daily temperature, 19 °C night temperature, 65% humidity with a day length of 12 h and light intensity of 380 μmol/m/s in the period from June 2015 until October 2015.

Nitrogen availability measuring

The main approach of nitrogen availability in soil determination is based on the method that has been established and described by the authors Bundy and Meisinger (1994). The specific procedure includes two stages of the experiment. One helps to estimate mineral nitrogen content before soil incubation. While another one serves to measure ammoniacal nitrogen content in the term of soil incubation within 7 days with the 4 M potassium chloride application. Basically available soil nitrogen NH_4^+ is liberated foremost from the cytoplasm of microbial biomass where it has been formed.

Mineral nitrogen leaching estimation

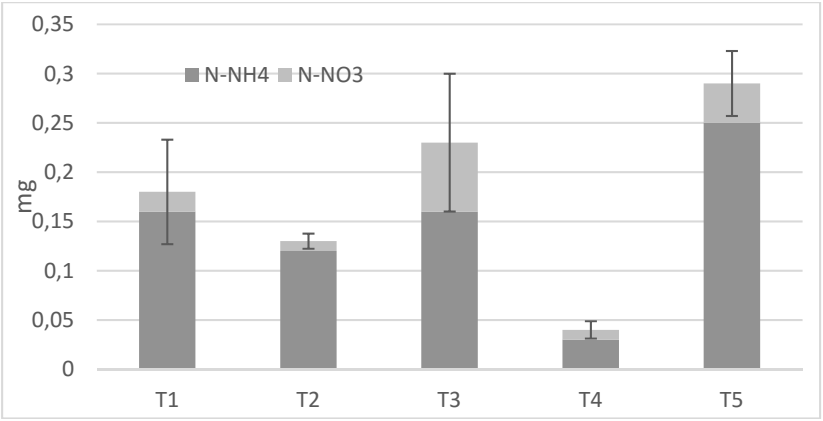
In accordance with the authors Novosadova et al. (2011) we have measured mineral nitrogen (N_{min}) leaching. Ion Exchange Resins (IER) have been used to evaluate these particular values. In total mineral nitrogen content, have been measured in the soil with two basic values ammonium nitrogen (NH_4^+-N) and nitrate nitrogen (NO_3^--N). Special discs with IER (75 mm diameter and is 5 mm thick) are made from plastic (PVC) and nylon mesh (grid size of 0.1 mm) cutted tubes and filled with CER – Cation Exchange Resin and AER – Anion Exchange Resin in the ratio 1:1. After the experiment finish these discs have been dried at laboratory temperature at 20 °C for seven days. N_{min} has been extracted using 100 ml of 1.7 M sodium chloride. Method of distillation and titration has been used for the estimation of released N_{min} according to Peoples et al. (1989). Investigation results have been expressed in mg of N_{min}. After the first generation of plants harvesting we have applied to the same soil (with the redosing of amendments) IER discs to measure nitrogen leaching in terms of second generation of plants growing.

RESULTS AND DISCUSSION

Mineral nitrogen leaching from soil

Previous studies on the estimation of mineral nitrogen in soil that have been conducted with the first generation of plants with the same additives (biochar, inoculums and mineral nitrogen) have shown relatively low amounts of mineral nitrogen in leachetes which are displayed in Figure 1 (Mikajlo et al. 2015). Lower amounts of percolating nitrogen, that significantly differ from the other applied treatments, have been caused mainly by the immobilization of soil nitrogen via the active bacteria which are added in the treatments B and N.

Figure 1 NH_4^+-N and NO_3^--N leaching from arable soil in I generation of plants (in mg), 2015



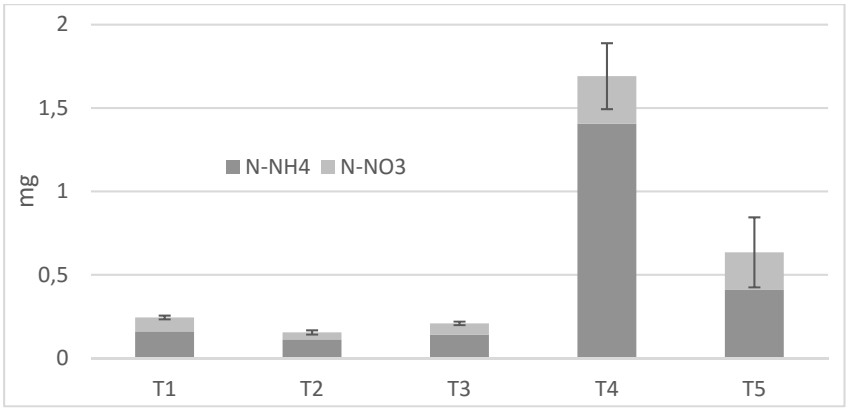
Legend: T1-control treatment; T2-"Bactofil" inoculum; T3-"Bactofil"+ DAM additive; T4- "NovaFerm" inoculum; T5- "NovaFerm"+ DAM (mean values ± SD, n = 3)

Microbial control of the fate of mineral nitrogen during the second plant cultivation has not been so precise as during the first generation of plants cultivation in the case of Novaferm inoculum

amendment. Investigation data shows that the highest N leaching up to 1.69 ± 0.19 mg occurred with the "Novaferm" application comparing to the control unamended soil (0.24 ± 0.01 mg) (see Figure 2). It is important to notice that basically N leaching have not changed in first and second generation taking into account the control soil without any treatments with just a slight difference (0.18 ± 0.05 mg and 0.24 ± 0.01 mg). Soil treated with "Bactofil" inoculum showed no significant changes between the first and second generation. Thus no remarkable nitrogen leaching values have been occurred compare to the control. Moreover, application of "Bactofil" amendment with biochar indicated on the lowest level of N leaching. "Bactofil" with DAM treatment stated on a decrease of N leaching comparing to the first generation of plants (0.23 ± 0.05 to 0.2 ± 0.01 mg). Slight N leaching in 0.63 ± 0.2 has been occurred in soil treated with "Novaferm" inoculum and DAM amendment.

Thus, it may be seen from the results that "Bactofil" inoculum with or without DAM adding along with biochar application leads to microbial activity development and nitrogen immobilization. Based on these results we may suppose that the real soil nitrogen availability would be better during the repeated cultivation in the soil mixture with biochar.

Figure 2 NH_4^+-N and NO_3^--N leaching from arable soil in II generation of plants (in mg), 2015

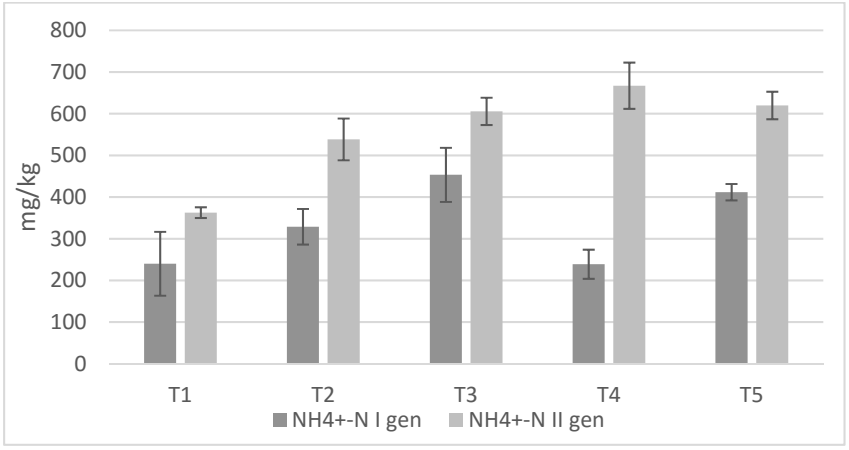


Legend: T1-control treatment; T2-"Bactofil" inoculum; T3-"Bactofil"+ DAM additive; T4- "NovaFerm" inoculum; T5-"NovaFerm"+ DAM (mean values ± SD, n = 3)

Availability of nitrogen for soil microbes

Previous studies have indicated on N availability increase in soil treated with bacterial inoculums along with biochar and mineral fertilizer amendment (Mikajlo et al. 2015). Investigation results on N availability in soil after the first and second generation of plants growing can indicate the following trends (see Figure 3).

Figure 3 Differences between I and II plant generation NH_4+-N availability in microbial biomass (in mg/kg), 2015



Legend: T1-control treatment; T2-"Bactofil" inoculum; T3-"Bactofil"+ DAM additive; T4- "NovaFerm" inoculum; T5-"NovaFerm"+ DAM (mean values ± SD, n = 3); I gen-first generation, II gen-second generation; SF- spore-forming bacteria, NF- nitrogen-fixing bacteria, AC- actinomycetes, FN- microfungi

It may be seen that all the values after the second generation of plants harvesting have been higher comparing to the first generation values. Thus N index has gradually increased with each treatment while the control unamended soil values remained the lowest (362.53 ± 12 mg/kg). The highest N increase has been fixed in the soil treated with "Novaferm" inoculum (667.16 ± 55 mg/kg), almost twice higher comparing to the control soil. Treatment with "Novaferm" additive and with DAM fertilizer also resulted in high values of N availability (619.72 ± 33 mg/kg). "Bactofil" inoculum with and without mineral fertilizer treatments have indicated on an increase of N availability as well (605.59 ± 64 mg/kg and 538.34 ± 42 mg/kg respectively). Comparing the first and second generation of plants it can be noticed that N availability has risen in the second generation practically in each treatment application. Hence, it may be argued that definitely inoculated biochar with mitigated properties after the first generation of plant harvesting has positive influence on microbial activity development and thus on soil state improve and plant growth.

CONCLUSION

Taking into account the results of the previous studies it has been decided to continue research on biochar, inoculums and mineral fertilizer with the same soil and the same plants cultivated in consequent second generation (Mikajlo et al. 2015). Investigation data claim on a slight increase of soil nitrogen amount using the treatment with biochar and "Bactofil" inoculum with and without mineral fertilizer amendment. No great difference in mineral nitrogen leaching has been found between the treatments applied in the first and second generation. Nitrogen availability results have stated on the rise of nitrogen indexes after the second generation of plants harvesting and thus its positive influence on microbial activity development. Especially it may be admitted that biochar with "Novaferm" inoculum have prospered to the rise of nitrogen availability particularly while applying this kind of treatment.

Next planned research will be aimed to study various concentrations of biochar and its mitigation influence by organic matter adding to complete the lacking nutrients.

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THE INFLUENCE OF ANTHROPOGENIC LEAD ON CONTAMINATION OF SOIL

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Abstract: In this study was investigated the state forest soils around Lenora (district Prachatice) of risk of elements lead. The aim of your experiment was to assess the influence of glassworks in Lenora (1834–1995) on the pollution of forest soils. From the neighborhood of Lenora were collected 42 samples from 5 locations, further sorted out into 8 sampling points up to 3 km in area of Velké Nivy, Radvanovické saddle, the hill Chlustov, the hill Ptáčník, Zátoňská mountain. EDTA-extractable (bioavailable) lead in the collected soil is the most accumulated in humus (H) organic horizons due to its high sorption capacity. The found average content of EDTA-extractable lead in the evaluated forest soils are in the range 8.5 to 28.6 mg/kg DM with a mean value of 17.6 mg/kg DM. Only three of the eight sampling points (Velká niva, Hill Chlustov east and Radvanovické saddle-spruce forest) exceeds the determined content of 30 mg/kg DM.

Key Words: EDTA, lead, soil, glassworks

INTRODUCTION

In nature, the lead is pervasive as most of the trace elements, the average concentration is about 36th place of elements in the Earth's crust. In the last 50 years it was extracted and concentrated a large amount of lead from ore and it was re-released into the environment in the form of eg. tetraethyl in leaded gasoline. Thus, animals are exposed to the health risks and their body tissues and fluids can contain more lead than would correspond to the natural background. Infants and children may be at risk if they inhale the dust-bound pollutants. The lead content in the blood is generally accepted as the indicator organism load (Komárek et al. 2008).

The main source of error in the determination of lead in environmental and biological samples could be the secondary contamination that may occur during sampling and during their own analysis. Lead enters the environment during production, use and recycling of the lead compounds, the combustion of fossil fuels (coal, gas), the use of mineral fertilizer, sewage etc. The estimation of emissions from individual lead sources shows that anthropogenic sources in the atmosphere are more important in contrary with the natural sources (Chen et al. 2009).

The lead contamination of the atmosphere is estimated to be 5,000 years old (younger Stone Age-Neolithic). It began when the first imperfect smelting was done in Southwest Asia (Mesopotamia). The former world lead production was approximately 200 tons per year. Isotopic studies of lead can provide information on the pollution of the various parts of the environment. Lead is a toxic element, which has no known function in biological systems (Hansmann and Köppel 2000).

The aim is to obtain information about the properties of lead and its geochemical position especially in forest soils, which are characterized formed soil horizon.

MATERIAL AND METHODS

Description of locations

The examined locations are located in the area former glassworks of Lenora. In the nearby of Lenora village on Prachatice region, is located eight sampling sites (Figure 1) at a height of 786 m

AMSL. The samples were collected mainly on forest soils according to the developed horizons. From the area of the top of the hill Chlustov located on the west side of Lenora at a height of 1094 m AMSL, the samples were collected from the beech stand on the western side of the hill and on the east side of beech-fir forest. From the area of the hill Ptáčník which lies eastward from the village at a height of 868 m AMSL were collected samples of spruce forest as well as from the experimental area around ZF JCU directly above the Lenora and from the area of forest Velká Niva. Additional sampling site is located at Zátoňské mountains (1028 m AMSL), where is the vegetation change on spruce-beech. Next samples were collected from the site of Radvanovické saddle lies from the south side of the village. Examined samples were collected from the two places characterized by different vegetation of spruce and beech stands. Mentioned sampling areas were located about 100 m apart. Considering the fact, the location belongs to Šumava National Park, there is necessary to have an entry permit from the competent forester from Zátoň.

Figure 1 Map of locations

Table 1 GPS locations around Lenora

Location	GPS coordinates
The hill Chlustov – west (VCH-Z)	N 48° 55.612'; EO 13° 45.167'
The hill Chlustov – east (VCH – V)	N 48° 55.734'; EO 13° 45.649'
Zátoňská mountain (ZH)	N 48° 56.664'; EO 13° 50.108'
The hill Ptáčník (VP)	N 48° 55.572; EO 13° 48.481'
Experiment area ZF JCU	N 48° 55.535'; EO 13° 48.418'
Velká niva (VN)	N 48° 55.505'; EO 13° 48.709'
Radvanovické saddle (RS)	N 48° 53.706'; EO 13° 47.460'

MATERIAL AND METHODS

Soil samples of forest sites were sort out by soil horizons, which are created in the soil. Samples were divided to the the litter (L), fermentation horizon (F), humification horizon (H), the first mineral horizon (A1) and the second mineral horizon (A2). The strength of horizons were different, and it was depended on the type of vegetation, the rate of decomposition of substances in these horizons and stand age. Other forest soils samples were divided by the depth of sampling to 15 cm, 30 cm and 40 cm. Horizons L, F, H of forest soils were collected using a garden shovels, mineral horizons and other soil samples were collected using a soil sampling probe. All the samples were stored in plastic bags with a

precise description of the location and the date of collection. Subsequently, they were allowed to air dry, to be prepared for further processing Kudravá and Rúriková (2005).

Preparation of the sample for analysis

Firstly, well-dried samples were processed using a mortar and pestle or a laboratory mixer (for horizons L and F). They were pulverized to a fine part and then sieved through a sieve of mesh size 2 Mesh screen (Bollhöfer and Rosmann 2001). In this process, the samples were rid of coarse impurities. The treated samples were re-sieved in a mortar and then they were passed through sieve 0.5 Mesh screen.

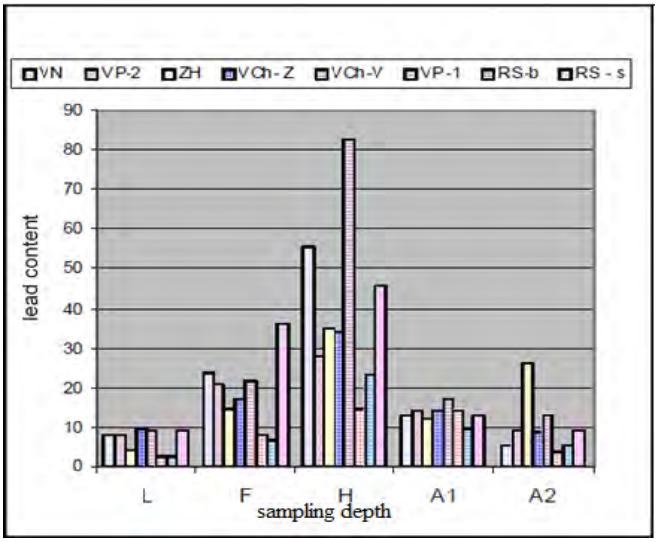
Analytical methods

The 0.05 M EDTA solution was prepared. 18.6 g of Chelatone-3 was dissolved and transferred into a 1 l volumetric flask. pH of the solution was determined on pH meter (Mettler DL25, Switzerland) and adjusted by adding approximately 6 ml of ammonia solution (p. a. Penta manufacturer) until stabilization of pH = 7. The 0.5 g of soil was transferred to vials for extraction (Falcon tubes) then the 25 ml of extraction solution EDTA was added. Samples were mixed on a shaker for 60 minutes (Bermond et al. 1998). After that, the samples were centrifuged on ultracentrifuge (type 2–5, Sigma, Germany) for 10 minutes on 3900 rpm. The supernatant was filtered through a filter paper with a blue stripe type (Watmann, GB). Overall lead content in the extract were determined by ICP - OES (ICAP 6000, Thermo Scientific Cambridge UK). Prior the analysis, the samples were diluted according to the lead concentration approximately of 20 µg/l (Chrastný et al. 2008).

RESULTS AND DISCUSSION

EDTA-extractable content (Organic accessible) of lead in soils is shown in the following graph (Figure 2). The graph shows the most of the lead is accumulated in humus (H) organic horizons. This trend could be explained by the high sorption capacity of this kind of soil.

Figure 2 The content of EDTA-extractable lead in forest soils near Lenora

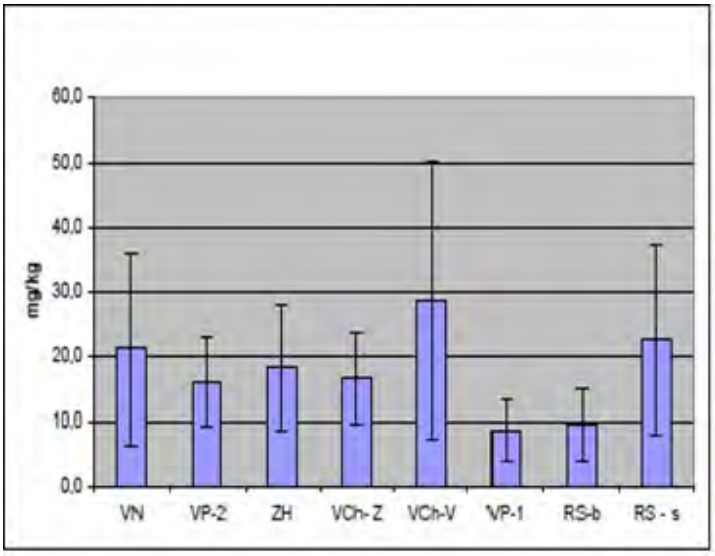


Legend: VN - Velká niva, VCh -V hill Chlustov east, VP - 2 hill Ptáčník, VP-1 hill Ptáčník over the experimental area, ZH - Zátoňská mountain, RS-b Radvanovické saddle – beech forests, VCh - Z The hill Chlustov west, RS - S Radvanovické saddle - spruce forest

The total content of lead in the most soils of the Czech Republic regardless of type (forestry, agriculture), its diameter does not exceed 20 mg/kg DM (Sanka and Materna 2004). The found average content of EDTA-extractable lead in the evaluated forest soils are in the range 8.5 to 28.6 mg/kg DM with a mean value of 17.6 mg/kg DM. Only three of the eight sampling sites (Velká niva, the hill Chlustov east and Radvanovické saddle - pine grove) exceed the determined content of 30 mg/kg DM (Figure 2). Velká niva, Radvanovické saddle are comparable stands (spruce) and also the order of the contents of lead large floodplain corresponds to 21.1 ± 14.7 mg/kg DM, Radvanovické saddle - spruce corresponds to 22.6 ± 14.6 mg/kg DM. Velká niva is closer to the source of the order of about 2 km from the glassworks in the direction of the prevailing winds than Radvanovické saddle pine

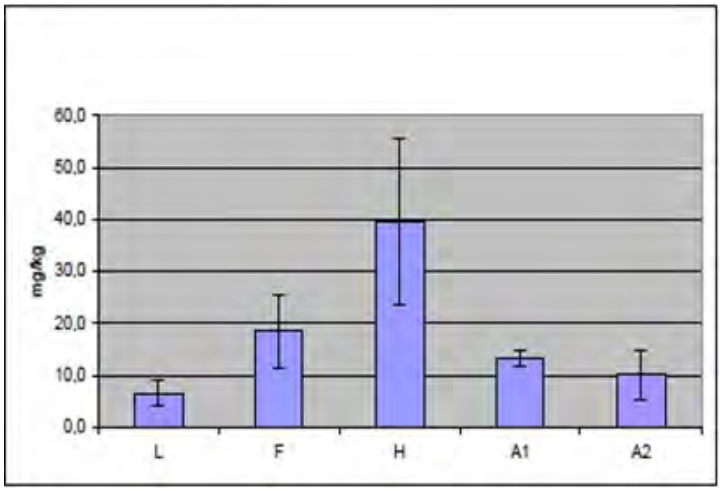
grove. Radvanovické saddle spruce forest is about 4 km north of the source from which it follows that it should not be equally affected by glassworks. In terms of soil horizon (Figure 3) is the most contaminated horizon H which reaches 39.7 ± 15 mg/kg DM, which is twice higher the lead content in soils according to Weiss et al. (1999). However, H-term horizon forest soil is very thin (a few centimeters). The minimum value of lead concentration at the site of the Velký Ptáčník experimental area was estimated to 14.4 mg/kg DM and the maximum value of the lead concentration on the area of hill Chlustov east was estimated to 82.4 mg/kg DM. Other horizons, except F horizon, is varied in the range 6.5 ± 2.6 mg/kg DM, 13.4 ± 1.4 mg/kg DM, which is lower than results by Maříková (2008). The average value of the content of Pb^{2+} in all horizons at all locations is approximately a half lower in the comparison with H horizon 18.5 ± 7.1 mg/kg DM. This result is in good agreement with the results by Papanikolaou et al. (2005).

Figure 3 Average content of EDTA-extractable lead in the soils at all locations



Legend: VN - Velká niva, VCh-V hill Chlustov east, VP - 2 hill Ptáčník, VP-1 hill Ptáčník over the experimental area, ZH - Zátoňská mountain, RS-b Radvanovické saddle - beech forests, VCh- Z The hill Chlustov west, RS-S Radvanovické saddle - spruce forest

Figure 4 Average content of EDTA-extractable lead in soil horizons



Legend: litter (L), fermentation horizon (F), humification horizon (H), the first mineral horizon (A1) and the second mineral horizon (A2)

Determined levels of lead in selective locations Radvanovické saddle in the most contaminated H-horizons (spruce 45 mg/kg DM, beech 23 mg/kg DM) could be explained by the higher adsorptive capacity of greater leaf area spruce stand.

CONCLUSION

All samples were collected from soil horizons L, F, H, A1 and A2. It was found that EDTA-extractable (bioavailable) lead in the collected soil is the most accumulated in humus (H) organic horizons due to its hight sorption capacity. The obtained average content of EDTA-extractable lead in the evaluated forest soils was in the range from8.5 to 28.6 mg/kg with a mean value of 17.6 mg/kg DM. The EDTA-extractable Pb^{2+} usually represents only about one tenth of the total content. Only three of the total eight sampling sites (Velká niva, the hill Chlustov east and Radvanovické saddle - pine grove) exceed the limit 30 mg/kg DM of lead.

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THE EFFECT OF DROUGH ON TGW, PROTEIN AND STARCH CONTENT IN BARLEY EXPERIMENTAL LINES

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Abstract: Drought is considered as one of the most important abiotic stress factors. The severity of drought is unpredictable as it depends on many factors such as occurrence and distribution of rainfall, temperature, evaporation and moisture storing capacity of soil. Understanding of drought stress and water use in relation to plant growth is important for sustainable agriculture. Currently, the breeding of new varieties of barley is increasingly focused on improving the level of resistance to abiotic stresses, especially drought while maintaining good health and corresponding yield and grain quality. New genetic resources with higher resistance to drought are searched for among genotypes well adapted to dry conditions. In this work there were evaluated lines derived from reciprocal crosses between cv. Tadmor (originating from Syria, with potentially high tolerance to drought) and cv. Jersey (advanced European spring malting barley). The obtained lines (F5 and F6 generation) with parent genotypes were cultivated at two locations (Brno, Žabčice) differing in the water retention capacity of soil. The assessment was aimed at traits associated with yield and quality – thousand grain weight (TGW), protein and starch content.

Key Words: barley, drought, proteins, starch

INTRODUCTION

Water is necessary for all life processes of plants. Drought reduces photosynthesis and also reduces the potential harvest index, especially the dry weight of the grain. Lack of moisture in cereals suffering from stress leads to a reduction of yield and damage to the grain (Keyvan 2010, Pour-Siahbidi and Pour-Aboughadareh 2013).

Drought is unpredictable because it depends on many factors, such as the occurrence and distribution of precipitation, temperature, soil type and structure etc. The seriousness of damage depends on the particular stage of plant growth, which is affected by drought (Dolferus et al. 2011). Kilic and Yagbasanlar (2010) claim that although drought stress usually reduces grain yield, it can significantly increase the level of the other constituents of economic yield, as the protein content.

Barley (*Hordeum vulgare* L.*)* is widely used as food or feed for animal. For malting and brewing purposes specific quality parameters are very important, including protein and starch content (Gous et al. 2015). Grain protein content varies greatly across different environments. Barley suitable for malting should have moderately low grain protein content (10–11.5%). High protein content will not only reduce malt extract, but also deteriorate final beer quality (Molina-Cano et al. 1997, Wu et al. 2015). Abiotic stress can negatively effects starch biosynthesis, resulting in starch structural changes, which may make the grain less suitable for malting and brewing (Hollmann et al. 2014).

MATERIAL AND METHODS

For the experiment F5 and F6 generation lines were chosen, which were derived from reciprocal crosses between Tadmor and Jersey variety.

Variety Tadmor was developed from Syrian landrace Arabi Aswad under the breeding program by ICARDA (International Center for Agricultural Research in the Dry Areas). It can be distinguished by its black colour of hull and is characterized by very good adaptation to drought, increased resistance to oxidative stress from excessive radiation due to lower content of chlorophyll in leaves, higher osmotic

potential and high efficiency of water use (Tardy et al. 1998, Teulat et al. 1997, Teulat et al. 1998, Teulat et al. 2001, Teulat et al. 2002). Variety Jersey (Limagrain Advanta Nederland BV, NL) belonged to the most grown malting varieties in the Czech Republic till the 2008 year. It is characterized by high malting quality, resistance to powdery mildew (mlo) and relatively higher sensitivity to drought.
Evaluation of F5 and F6 generation lines was carried out in field conditions at two locations Brno (GPS position of the locality: 49°12'42.2"N 16°36'57.1"E) and Žabčice (GPS position of the locality: 49°01'18.6"N 16°37'01.9"E), which differ in terms of weather and soil conditions. In both experiments the same set of 85 lines (TxJ designated as lines 1 and combination JxT designated as lines 2) was planted in two rows of 21 seeds each.

All plants were harvested by hand, then threshed and cleaned using laboratory thresher Haldrup LT-20. The grain samples were used for determination of thousand grain weight (TGW). Starch and protein contents were evaluated by the FT-NIR spectrometer (Nicolet). Reference method for starch was used according to Ewers and for protein by Kjeldahl.

RESULTS AND DISCUSSION

Climate conditions

Temperatures in both years were higher by 1–2 °C in comparison with the normal temperature for the years 1960–1990. Precipitation was lower than normal rainfall in both years and in 2015, the precipitation was only 50% of normal precipitation (Table 1).

Table 1 Average temperature and precipitation on location Žabčice

	Normal temperatures (1961–1990)	Normal precipitation (1961–1990)	2014		2015	
			Average temperature (°C)	Precipitation (mm)	Average temperature (°C)	Precipitation (mm)
March	4.3	23.9	8.5	5.6	5.4	28.0
April	9.6	33.2	11.8	11.2	10.1	9.4
May	14.6	62.8	14.4	62.8	14.7	33.8
June	17.7	68.6	18.8	43.4	19.1	22.4
Total	-	188.5	-	123.0	-	93.6

Thousand grain weight (TGW)

The thousand grain weight is an important character determining the yield and product quality in barley. The lack of water together with high temperatures can shorten the length of the grain filling and thus reduce the weight and size of the grains (Doganlar et al. 2000).

Table 2 Evaluated parameters in parental varieties of barley

Character	Variety	2014		2015	
		Brno	Žabčice	Brno	Žabčice
Thousand grain weight (g)	Tadmor	49.4	43.9	51.4	49.4
	Jersey	48.2	41.9	49.3	44.2
Protein content (%)	Tadmor	16.5	17.8	13.8	13.4
	Jersey	13.9	18.7	11.7	11.2
Starch content (%)	Tadmor	55.2	53.5	61.4	61.2
	Jersey	61.2	56.6	65.3	66.7

Between parental varieties no distinct differences were observed, however, there was a significant difference between locations (Table 2). Variety Jersey showed a lower TGW at both locations, but variety Tadmor showed only slight difference at dryer location Žabčice (2 g). Figure 1 and 2 show variation in TGW among evaluated lines on both locations in 2014 and 2015 year. The significant effect of location on TGW was observed only in 2014. Lines 1 (TxJ) showed higher TGW as contrast lines 2 (JxT), especially at location Žabčice. In 2015 only slight reduction was in Žabčice and difference between evaluated lines was not significant. It could be explained by higher precipitation in May

and June (Table 1). Thousand seed weight is one of the characters, which is mainly influenced by the genes. Under certain conditions some genotypes are able to compensate the yield loss caused by drought by increasing the TGW (Pasam et al. 2012).

Figure 1 Thousand grain weight in evaluated lines of barley in 2014

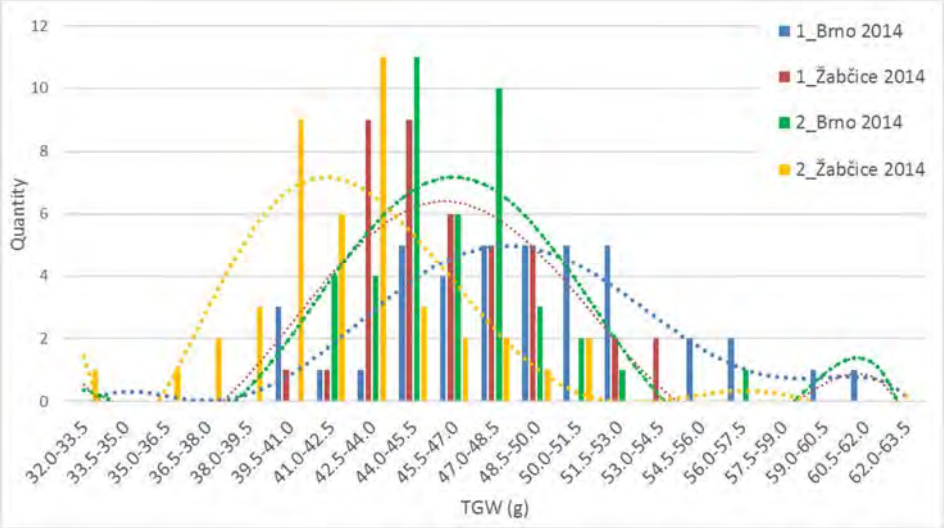
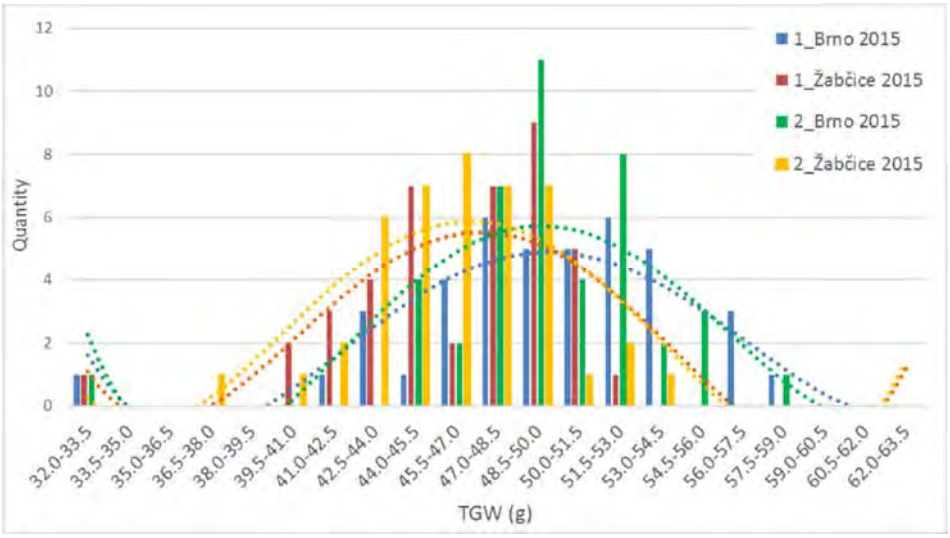


Figure 2 Thousand grain weight in evaluated lines of barley in 2015



Legend: 1_Brno = Tadmor x Jersey, 2_Brno = Jersey x Tadmor, 1_Žabčice = Tadmor x Jersey, 2_Žabčice = Jersey x Tadmor

Protein and starch content

Lower protein content and high starch content is important for malting purposes (Goodall et al. 2013). The concentration of both substances is partly influenced by genotype, but environment has a great impact has – nutrition, the effect of abiotic and biotic stresses (Psota and Kosař 2002).

In 2014, grain harvested at dry location (Žabčice) showed generally higher protein content than grain harvested at wetter location in Brno (Figure 3 and 4). It is thus apparent that greater drought caused an increase of protein content and also reduced starch content of the grain. It is consistent with the results of e.g. Robredo et al. (2011) and Ahmed et al. (2012). In 2015, when precipitation was higher, the protein content was consequently lower than in 2014. In 2014, lines 2 (JxT) showed higher protein content at location Brno. At location Žabčice lines 2 (JxT) showed higher nitrogen content in 2015.

Figure 3 Protein content in evaluated lines of barley in 2014

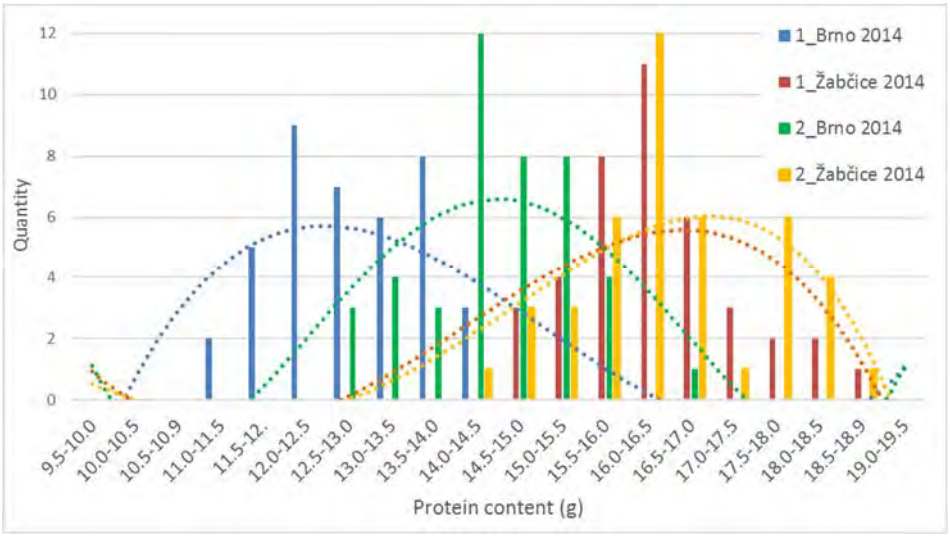
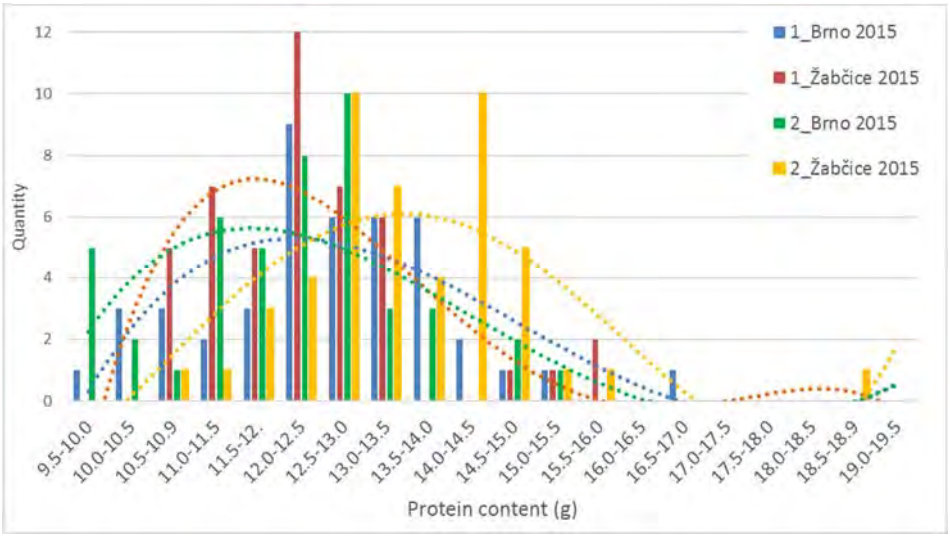


Figure 4 Protein content in evaluated lines of barley in 2015



Legend: 1_Brno = Tadmor x Jersey, 2_Brno = Jersey x Tadmor, 1_Žabčice = Tadmor x Jersey, 2_Žabčice = Jersey x Tadmor

Figure 5 Starch content in evaluated lines of barley in 2014

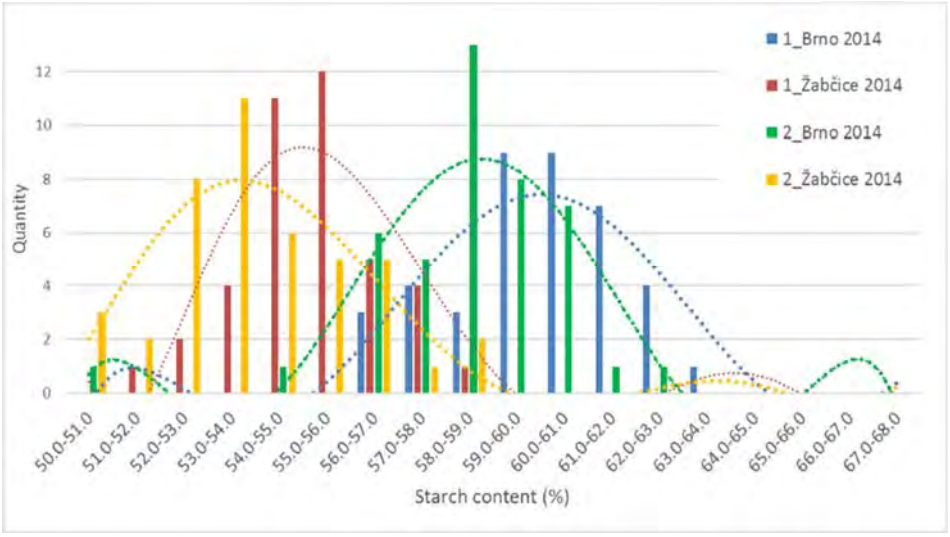
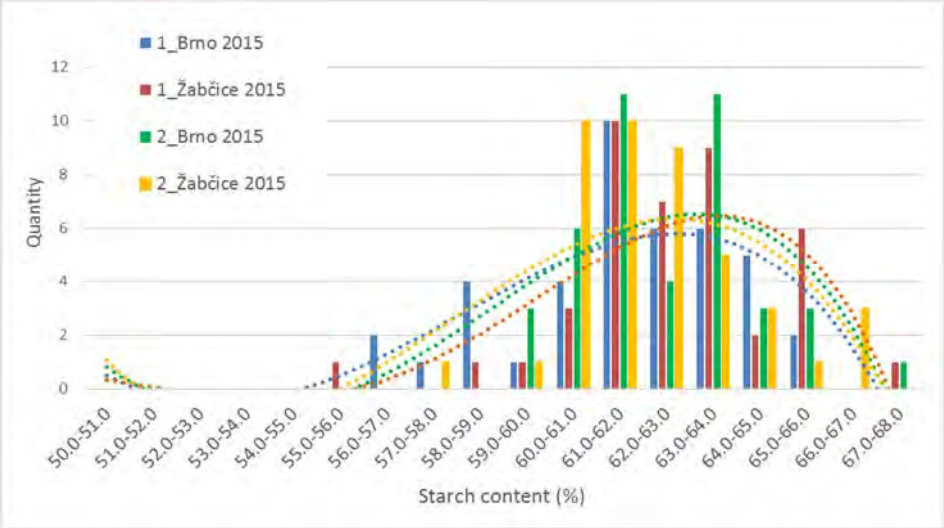


Figure 6 Starch content in evaluated lines of barley in 2015



Legend: 1_Brno = Tadmor x Jersey, 2_Brno = Jersey x Tadmor, 1_Žabčice = Tadmor x Jersey, 2_Žabčice = Jersey x Tadmor

Occurrence of water deficit in cereals can reduce the total starch content up to 40% (Thitisaksakul et al. 2012). This is in accordance with obtained results. On drier site the reduction in starch content was observed in almost all lines (Figure 5 and 6). Malting variety Jersey retained at both locations high potential starch accumulation compared to Tadmor variety (Table 2). In 2015, starch content was similar at all locations and in all lines. However, lines 2 (JxT) had apparently lower starch content at both locations in 2014. Negative correlation between the starch and protein content was described in many works, e.g. Hubík and Mareček (2002), which was consistent with our findings.

CONCLUSION

Two generations of experimental lines were grown at location Brno and at drier location Žabčice in 2014 and 2015 year. TGW, protein and starch content were evaluated.

Parental varieties and evaluated lines showed decrease of TGW at location Žabčice in both years. In 2014 lines 2 showed higher TGW at both locations, but in 2015 TGW was similar between lines 1 and lines 2. Experimental lines showed higher variability than parental varieties. TGW in evaluated lines was 30–60 g, but in parental varieties only 40–50 g.

As the qualitative parameters protein and starch concentrations in harvested grain were assessed. Tadmor showed higher protein content and lower starch content compared with Jersey at both locations. Grain of experimental lines harvested at drier location Žabčice showed a higher protein content than grain harvested at location Brno. In 2014 decrease in starch content was apparent at dry location Žabčice and lines 2 (JxT) showed lower starch content at both locations. Significant differences between lines 1 and 2 were not detected in 2015.

Some of these experimental lines of barley could be serve as a source of genetic variability for breeding to improve drought tolerance.

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THE INFLUENCE OF DEFICIENT NUTRITION ON GROWTH AND ROOT ACTIVITY OF MAIZE (*ZEA MAYS* L.) UNDER HYDROPONIC CONDITIONS

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Abstract: Root system plays important role in uptake of nutrients which influences the crop quality and yield. On the other hand, soil bioavailable nutrient supply is one of main limiting factor affecting development of the roots. The aim of hydroponic cultivation experiment was the determination of the nutrition deficient impact on the root system of maize (*Zea Mays* L.). In this experiment the root system was described by electric capacity due to we are able to find the active parts of root which are responsible for main uptake of nutrition. Three deficiency variants (nutrition solution without nitrogen, phosphorus and potassium) and one variant with all nutrition (control variant) were observing. LCR meter was using for measuring the root electric capacity and also the weight of dry matter of whole plant and root was determined. The weight of root dry matter of variant with P deficiency was increased in time (from 20 to 77 mg/plant), on the contrary the weight of root dry matter of variant with K deficiency was decreased in time. The electric capacity has similar trend, it was increased in variant with P deficiency and it was decreased in variant with K deficiency in the 3rd term (0.078 nF).

Key Words: maize, hydroponic cultivation, deficient nutrition, root dry matter, root electrical capacity

INTRODUCTION

Maize (*Zea Mays* L.) is plant with huge root system. The root system has essential importance for water and nutrition uptake. The nutrition is one of the limiting factor involves the growth and development of the roots. The bioavailability of nutrients in the soil solution may determine growth, size and activity of root system. Important developmental processes, such as root-hair formation, primary root growth and lateral root formation, are particularly sensitive to changes in the internal and external concentration of nutrients (Lopez-Bucio et al. 2003). Contents and bioavailability of soil nutrients are critical factors for plant growth and productivity (Baligar et al. 1998). Nitrogen (N), phosphorus (P) and potassium (K) are the nutrients that are responsible for alter post-embryonic root developmental processes (Zhu et al. 2005, Wissuwa et al. 2005, Liu et al. 2008, Hawkesford et al. 2012, Kellermeier et al. 2013).

For evaluation of the size of root system could be use measurement of root capacitance which is a nondestructive method to estimate the size of plant root systems (Chloupek 1977, Dalton 1995). This method enables to find only the active (life) part of root because the polarization of life membranes or cells is realising there, so the live parts are electric active (Středa and Klimešová 2016). The positive relative between root electric capacity and the weight of root system was found during many experiments, for example at sunflower (Rajkai et al. 2005) or at durum wheat (Nakhforoosh et al. 2012).

The aim of this work was to characterize the effect of primary nutrients (N, P and K) deficiency on the maize root system weight and electrical capacity in the earliest stages of development.

MATERIAL AND METHODS

Vegetation pots experiment in the form of an aqueous culture with maize (*Zea Mays,* L.) was commenced in growth chambers of the Department of Agrochemistry, Soil Science, Microbiology and Plant Nutrition, Faculty of AgriSciences, Mendel University in Brno in 2014.

Maize was sown into a nutrient-free substrate and when the plants´ roots reached approximate length 3 cm (5 days after beginning of germination – DAG), they were put into vegetation pots with nutrient solutions of different composition (Table 1). The solution had been prepared by using the method of Hoagland (Hoagland and Arnon 1938). 11 litre glass pots, which were wrapped around in non-transparent film, were used as vegetation pots. In each pot the nutrient solutions were aerated at regular time terms (5 minutes in each 3 hours). The vegetation pots were in growth chambers (PlantMaster, CLF Plant Climatics GmbH, Germany) in controlled temperature, humidity and light mode (12 h day length, temperature of 23/18 °C (day/night) and relative humidity of 55/70%, photosynthetic photo flux density of 350 μmol/m^2/s). When the experiment was set up, 0.5% solution of iron (ferric chloride) was added to all solutions (Laštůvka and Minář 1967). The pH value of all solutions was monitored and it was constant during the entire experiment.

Table 1 Treatments of the experiment and weights of chemicals (g per 1 litre of nutrition solution) according to Hoagland and Arnon (1938)

Nutrient solutions	Chemicals						
	$Ca(NO_3)_2$	KNO_3	K_2SO_4	KH_2PO_4	$Ca(H_2PO_4)_2$	$CaSO_4.2H_20$	$MgSO_4$
complete	0.821	0.506	–	0.136	–	–	0.120
without N	–	–	0.871	–	0.117	0.344	0.060
without P	1.231	–	0.861	–	–	–	0.241
without K	1.231	–	–	–	0.117	–	0.241

As model crop was used maize variety SY ONDINA. Syngenta (2004) presents this variety like mid-early hybrid with FAO 290, for grain and silage use, flinty dent grain type, hybrid is stable, adapted to growing in various climatic zones, moderately high plants, high harvesting potential, high starch content, highly tolerant to rust and helminthosporiosis. During the experiment the sampling of plants was in regular terms of 7 days (13 DAG, 20 DAG, 27 DAG).

Immediately after sampling, the electrical capacity of the root system was determined (Chloupek 1977, Dalton 1995). It was measured by LCR meter ELC-131D at a frequency of 1 kHz in nanofarads (nF) in distilled water of constant composition (in a bottle according to Woulf). One electrode was attached to the plant hypocotyl and the other electrode was inserted in a constant position at the bottom of the bottle. The electric capacity was measured in the electrical circuit where the alternating current passes between the root system and water. The plants were divided into root and aboveground parts after determination of electrical capacity and afterwards the dry matter weight of these parts were established.

The Statistica 12 CZ programme was used for statistical evaluation of the electrical capacity of the root system. The effect of the deficient nutrient on the formation of the root system was evaluated by ANOVA analysis of variance. The differences among the treatments were evaluated by follow-up tests according to Fisher (LSD test) at 95% ($P<0.05$) level of significance.

RESULTS AND DISCUSSION

Table 2 shows that the root dry matter weight of plants from complete solution and plants with P deficiency were increasing during the experiment. The root architecture of plants can undergo several changes in response to P deficiency. The long-term P deficiency can cause the excessive root elongation (Anuradha and Narayana 1991) which might be responsible for increasing the dry matter of plants with P deficiency. The increase of lateral root growth and secondary root branching at the expense of primary root elongation were observed in maize (Mollier and Pellerin 1999, Zhu et al. 2005), beans (Lynch and Brown, 2001) and rice (Wissuwa 2005). The ratio between the root weight and the whole plant weight in variant with P deficiency did not change a lot during the terms (25.78–28.95) in contrast to the variant with complete solution (the ratio was descending during the terms).

First, the weight of plants with nitrogen deficiency was decreased (in 1st and 2nd term) but the raising of weight is obvious in the last 3rd term. When the plant is long-term in the condition of N deficiency, it starts to change the structure of root system. The plant enhances the creating of lateral root in order to extend the nitrogen uptake (Chun et al. 2005, Hawkesford et al. 2012). Maize reduces the number of primary roots but increase the total root length under low-N conditions (Liu et al. 2008).

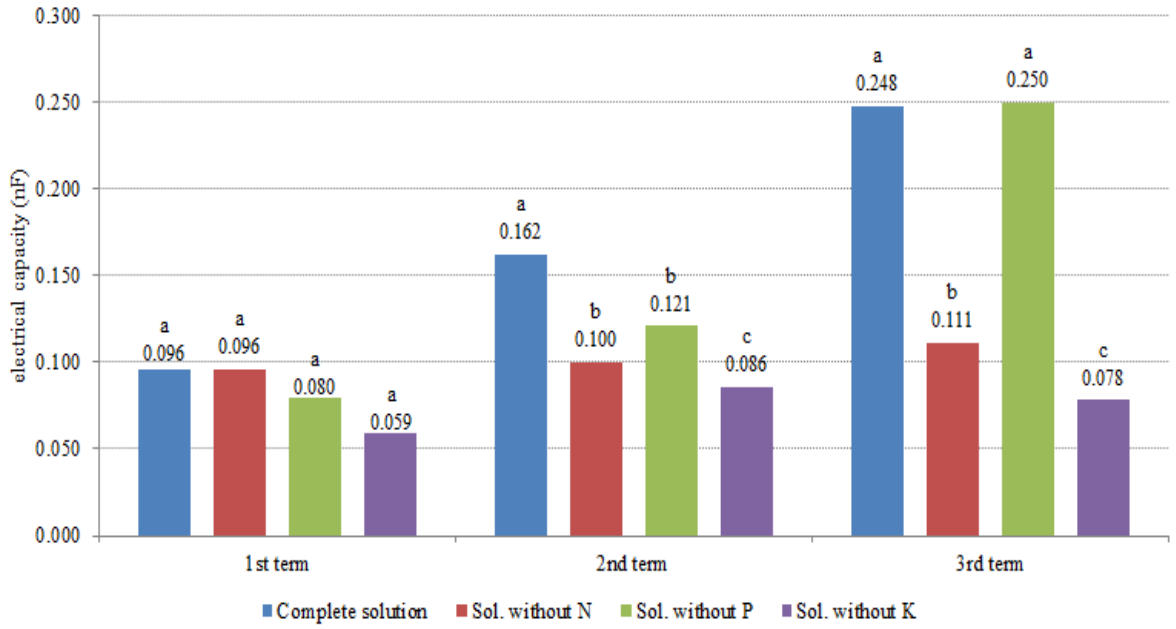
Maizlish et al. (1980) present when numbers of primary root per plant of maize increased with increasing nitrogen, but the elongation rate of an individual primary root did not respond strongly to increased N.

The bigger reduction of root weight was found in the K deficiency variant. The ration between dry matter weight of whole plant and root was 20.63% in the last term. Baligar et al. (1998) present that plants with insufficient K nutrition reduce the root growing. The seedlings of *Arabidopsis* showed a strong reduction of lateral root elongation in potassium deficiency (Kellermeier et al. 2013). Similarly Armengaud et al. (2004) present plants of *Arabidopsis* grown on K-free medium developed visible symptoms potassium deficiency on 10 days after germination, which included chlorosis of older leaves and a typical growth arrest of lateral roots.

Table 2 Dry matter weight of whole plant and root (mg/plant) and proportion of root dry weight to total plant weight (%) , 1st term 13 days after sowing, 2nd term 20 days after sowing and 3rd term 27 days after sowing

Treatment	Part of plant	Dry matter weight (mg/plant)		
		1st term	2nd term	3rd term
Complete solution	Whole plant	55	180	502
	Root	18 (32.73%)	54 (30.00%)	118 (23.51%)
Nutrient solutions without N	Whole plant	56	75	194
	Root	24 (42.86%)	19 (25.33%)	70 (36.08%)
Nutrient solutions without P	Whole plant	76	128	266
	Root	20 (26.32%)	33 (25.78%)	77 (28.95%)
Nutrient solutions without K	Whole plant	47	116	160
	Root	16 (34.04%)	37 (31.90%)	33 (20.63%)

Figure 1 Electrical capacity of maize root system (nF). Means followed by the different letters are significantly different (P<0.05)



From Figure 1 is obvious the difference in electric capacity among each term. The value of electric capacity was rising during the experiment, excepting K deficiency variant. The plants from deficiency solution have almost ever lower electric capacity then control plants, only in the last 3rd term) P deficiency plants had little bit higher electric capacity than control plants (not significance). Baligar

et al. (1998) present that P deficient nutrition induces enhancing length of primary root and the secondary root branching which increases the density of root-hair. Similarly Johnson et al. (1996), Dubrovsky (1997) and Zhu and Lynch (2004) reported increasing the root system forms of short lateral roots with large numbers of root hairs, when plants were exposed to low P conditions. The mentioned changes in root architecture increased absorptive surface which means higher electric capacity.

The plants from N and K deficient variants have significantly lower electric capacity in contrast to control plants. The most expressive different is in the last term, N deficient plants have of 55.24% lower electric capacity than control variant and K deficient plants have of 68.55% lower electric capacity in contrast to control plants. Similar small electric capacity was noticed in K deficient sunflowers (Škarpa 2011). Potassium significantly influences the cell turgor that drives cell expansion and elongation. The low K concentration in cytosol decreases the cell turgor, which is needed for the elongation of root hairs (Lew 1991).

Figure 2 Relation between the electrical capacity (nF) of root maize and their root dry matter weight; a – complete solution, b – nutrient solutions without P, c – nutrient solutions without N, d – nutrient solutions without K

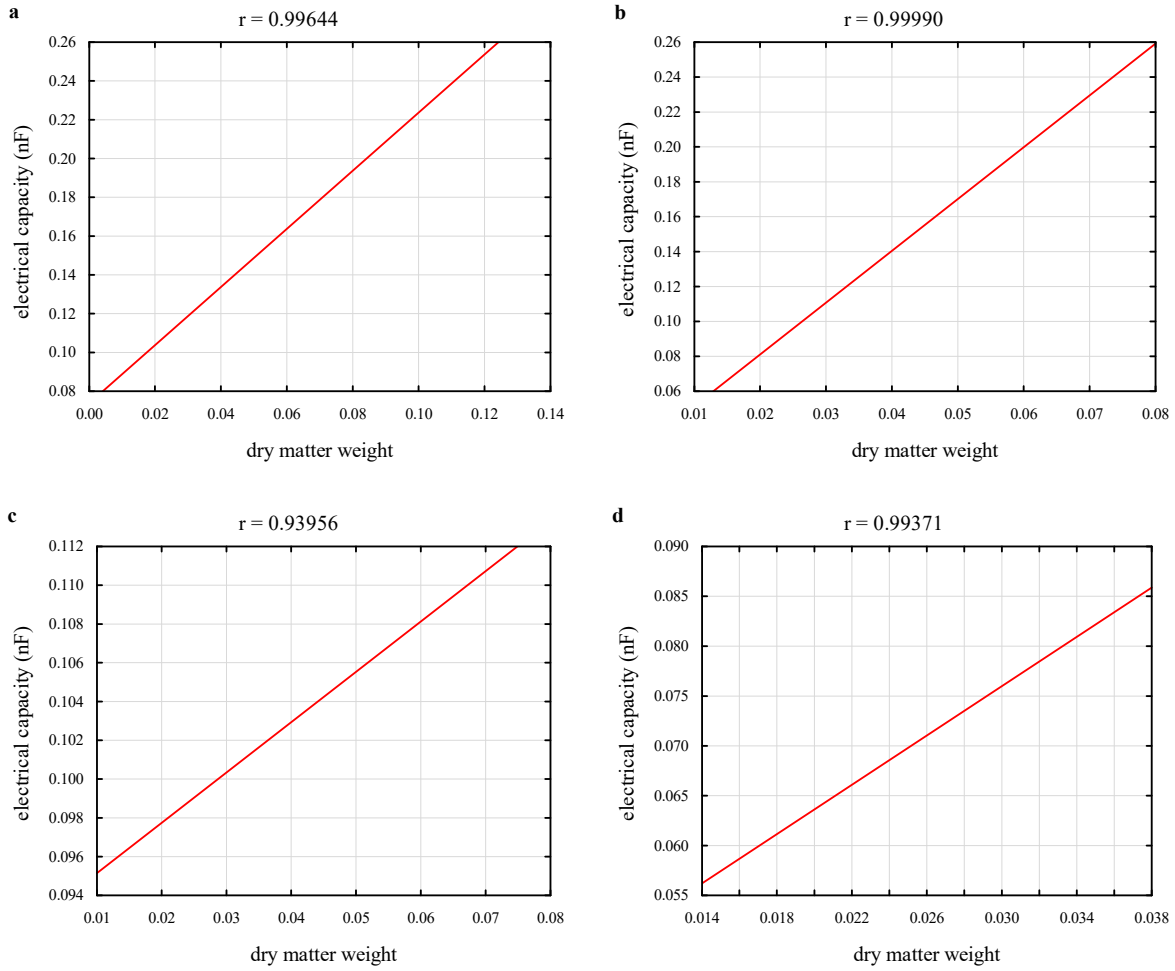


Figure 2 shows high correlation between the electrical capacity and root dry matter weight of maize. The significant relation was measured in variant with nutrient solution without phosphorus ($r = 0.999$; $P<0.01$), the relation of the others variants were not significant ($P<0.05$). McBride et al. 2008 studied four maize genotypes in the pots experiment and they were determinated significant relation between electric capacity and root weight. Really close correlation was also found at sunflowers (Rajkai et al. 2005). The relation between electric capacity and root dry matter weight was also noticed by Ozier-Lafontaine and Bajazet (2005) at spinach.

CONCLUSION

Nutrients deficiency in maize nutrition had significant effect on dry matter weight of whole plant and root and root electrical capacity. The root dry matter weight of plants from complete solution and plants with P deficiency were increasing in time. First, the roots weight in variant with N deficiency was decreased, but it was enhanced in 3rd term. The biggest roots weight reduction and their share in the whole plants weight was detected in variant with K deficiency. High correlations between the root electrical capacity and root dry matter weight were found in variants with all nutrients deficiency. This relation was significant in variant with phosphorus deficiency. Knowledge of roots development in deficient environment can be exploited by breeding of varieties for different deficiency conditions.

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INVADED PLANTS COMMUNITIES IN THE BEREK FLOODPLAIN FOREST (NOVÉ ZÁMKY DISTR., SLOVAKIA)

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Abstract: The study presents result of the research that was realised in year 2015 (May–October) in the Berek floodplain forest (SE edge of Nové Zámky). Research was aimed at invasive plant communities on 3 differently managed areas of forest cover. We were able to document 114 species on 9 permanent research plots. There were identified 35 non-native species and 20 taxa were in the category of invasive species. Using expert systems, we identified some potential plant communities: *Sambuco nigrae-Aceretum negundo*, *Rhamno catharticae-Cornetum sanguineae*, *Hyoscyamo nigri-Conietum maculati*, *Festuco arundinaceae-Althaeetum officinalis*. Factors of non-native species dispersion are mainly caused by uneven flooding of the floodplain forest during the spillage of river Nitra, exploitation of wood and maintain felled sites under power lines. Spreading of non-native especially invasive species in the lowland floodplain forest reduces species diversity of plant and animal communities and economic use cover. Therefore, we consider it necessary to manage non-native especially invasive species.

Key Words: plants invasions, floodplain forest, clear-cuts, Slovakia

INTRODUCTION

Biotical invasive plants are a phenomenon in the world, threatening biodiversity and only few ecosystems are occurrence free of invasive species (Catford et al. 2012). In context of the spread of invasive plant species the floodplain forests belong to the most threatened ecosystems. Permanent disruption of these native communities creates ideal conditions for introduction, occurrence and spread non-native especially invasive plant species which are threats for native biotopes. Machar (2002) states, that the greatest risk of spread of non-native species are all the new forest roads, buildings and bridge structures which involve the disruption of the surface of the soil. Chytrý and Pyšek (2009) state, that clear-cut areas are the worst way of forest management, because the excess of free resources is used by species that can easily and quickly spread here. Dominance of invasive plants on attacked areas is a phenomenon at the community level that talks about the suppression of natural species. The richness of species and its decline in specific areas depends on the identity of non-native species, which expanded in the area (Hejda et al. 2009). Problematics of invasive species with significant consequences for nature protection, forestry and agriculture can be fully understood only when we realize how intensely interrelated biotic invasive and landscape changes are. Invasions are also the driving force behind the landscape changes and their partial consequence (Pyšek and Sádlo 2004).

The aim of the study was to identify the species richness of invasive plant species and determine invaded plant communities in the Berek floodplain forest (district Nové Zámky, SW Slovakia).

MATERIAL AND METHODS

Floodplain forest Berek of approximately 100 ha is situated on the SE edge of Nové Zámky town (centre of the site has coordinates 47°58'20.544" N, 18°7'39.970" E, cover length is 2.6 km, the largest width is 0.6 km). Forest is located in the Nové Zámky in the Nitra region, on Danubian Flat in the southern part of western Slovakia. The Berek forest is remnant of original native floodplain forest complex and part of Special Protection Areas (bird areas) Dolné Považie (NT 2016). Climatic conditions of Nové Zámky town and surroundings are characterized as area of warm, very dry with mild winter (Lapin et al. 2002). Mean annual values of climatic moisture indicator for the area of Nové Zámky point

to the lack of rain (Tomlian 2002). Average precipitation in January is only about 30 mm and in July is about 60 mm (Faško and Šťastný 2002). Mean annual temperature of active soil surface for Nové Zámky town and surroundings is 12 °C (Tomlain and Hrvoľ 2002).

Areas were selected in 3 different habitats and those are: (i) clear-cut (C1–C3), (ii) forest cover (F1–F3) and (iii) poplar monoculture (M1–M3). Forest cover areas (F1–F3) represent remnant of original native floodplain forest complex. Phytocenological relevés we have made repeatedly in the period May–October 2015 in monthly intervals within the diploma thesis (Sofková 2016). Size of relevés we selected is 20 x 20 m in each plot. The method of the Zurich-Montpellier school with seven membered Braun-Blanquet scale coverage and frequency was used for phytocenological relevés (Moravec et al. 1994). Categorization of non-native species was made by Gojdičová et al. (2002) and compare properties by Medvecká et al. (2012).

The relevés were processed and imported into the program JUICE (Tichý and Holt 2006). By using TWINSPAN procedure's crispness value we specified the optimal number of clusters, grouped by the Sörensen similarity index. Limits for division into clusters is coverage rate (%) of values 0, 5, 15 and 25 (in JUICE the so-called values of cut level). Using expert system we have assigned communities (associations) with indices (FPFI - combined index frequency and fidelity, FPD - positive index fidelity, FQI - frequency index) under formal definitions to the clusters of relevés. Slovak expert system does not have forest vegetation processed, therefore we used the comprehensive expert system for the Czech Republic. To analyse the relationship (correlation) of species/relevés, we used indirect linear analysis (PCA) in the program Canoco (Ter Braak and Šmilauer 2002), the discovered length of the gradient of species data (SD = 3.835). Graphical representation was created using the tool CanoDraw.

RESULTS AND DISCUSSION

On 9 research plot, we created 64 phytocenological relevés from which we identified 114 taxa. Thirty-five species were identified in the list of non-native species out of them 20 were invasive (Gojdičová et al. 2002). The most occurring species were: *Solidago gigantea*, *Impatiens parviflora*, and *Aster lanceolatus.* The lowest frequency of invasive species has been in remnant of original native areas in the forest cover, specifically F2 with only 2 species. The highest number of frequency was on clear-cuts, specifically on the area C1 with 13 species and on area C2 was 9 species. The Table 1 shows the categories of species and information about their properties, such as: invasion status, time of introduction, life forms and origin of the taxon.

Table 1 Overview of the characteristics of identified non-native species (Medvecká et al. 2012) and their categorization by Gojdičová et al. (2002)

Latin title	Medvecká et al. (2012)					Gojdičová et al. (2002)
	IS	RT	TI	LF	Origin	Categories
Abutilon theophrasti	nat	neo	1865	T	As	2
Amaranthus retroflexus	inv	neo	1830	T	C Am SAm	2
Ambrosia artemisiifolia	inv	neo	1949	T	NAm	1a)
Arctium lappa	nat	arch	-	He	E As	-
Arrhenatherum elatius	-	-	-	-	-	8
Artemisia vulgaris	-	-	-	-	-	8
Aster lanceolatus	-	-	-	-	-	1a)
Aster novi-belgii	inv	neo	1865	He	NAm	1a)
Atriplex sagittata	nat	arch	B	T	E As	1b)
Bidens frondosa	inv	neo	1947	T	NAm	1b)
Bromus sterilis	nat	arch	N	T He	E As	1b)
Calamagrostis epigejos	-	-	-	-	-	8
Celtis occidentalis	nat	neo	1840	Ph	NAm	3
Cichorium intybus	nat	arch	-	He	E As Af	1b)
Cirsium arvense	-	-	-	-	-	8
Cirsium vulgare	-	-	-	-	-	1b)
Conium maculatum	nat	arch	M	T He	E As Af	1b)

Latin title	Medvecká et al. (2012)					Gojdičová et al. (2002)
	IS	RT	TI	LF	Origin	Categories
Convolvulus arvensis	nat	arch	I	He G	E As Af	-
Conyza canadensis	inv	neo	1791	T	NAm	1a)
Capsella bursa-pastoris	nat	arch	-	T	E	-
Chelidonium majus	nat	arch	R	He	E As	-
Datura stramonium	cas	neo	16c	T	?	2
Echinocystis lobata	inv	neo	1933	T	NAm	1a)
Epilobium ciliatum	inv	neo	1946	He	NAm CAm	2
Galega officinalis	nat	neo	1791	He	E	6
Galinsoga parviflora	inv	neo	1853	T	SAm	1a)
Helianthus tuberosus	inv	neo	1830	He	NAm	1a)
Impatiens parviflora	inv	neo	1897	T	As	1a)
Iva xanthiifolia	nat	neo	1934	T	NAm	1a)
Juglans nigra	nat	neo	1770	Ph	NAm	3
Lamium purpureum	nat	arch	R	T	E As Af	-
Malva sylvestris	nat	arch	-	T	E As Af	-
Negundo aceroides	cas	neo	1794	Ph	NAm	1a)
Parthenocissus quinquefolia	nat	neo	1897	Ph	NAm	2
Populus x canadensis	nat	neo	1800	Ph	H C	-
Phleum pratense	-	-	-	-	-	8
Robinia pseudoacacia	inv	neo	1720	Ph	NAm	1a)
Rumex confertus	-	-	-	-	-	8
Sambucus nigra	-	-	-	-	-	8
Solidago gigantea	inv	neo	1909	He	NAm	1a)
Stenactis annua	-	-	-	-	-	1a)
Tanacetum vulgare	-	-	-	-	-	1b)

Legend: IS - invasive status: cas - casual, nat - naturalized, inv - invasive. RT - residence time: neo - neophyte, arch - archaeophyte. TI - time of introduction: B - Bronze Age, I - Iron Age, N - Neolithic and Aeneolithic era, M - Medieval period, R - Roman and Migration period. LF - life forms: G - geophyte, He - hemicryptophyte, Ph - phanerophyte, T - therophyte. Origin of the taxon: Af - Africa, As - Asia, C - from cultivation, E - Europe, Cam - Central America, H - hybrid, Nam - North America, Sam - South America. Categories: 1a) invasive taxa neophyte, 1b) - invasive taxa archaeophyte, 2 - potential invasive taxa, 3 - often accomplishing taxa, 6 - domesticated taxa, 8 - expansive taxa.

Using TWINSPAN analysis we sorted phytocenological relevés into 6 clusters, their numbers were found using crispness value analysis. Table 2 indicates the number of relevés allocated to each cluster site on which the vegetation has been studied and their corresponding coordinates.

Table 2 Overview of phytocenological relevés and their localization

Cluster	Relevés	Research plot	N. of relevés	Coordinates
1	43, 44, 45, 46, 47, 48, 49	F1	22	47°58'27.5"N 18°07'55.4"E
	50, 51, 52, 53, 54, 55, 56, 57	F2		47°58'19.9"N 18°08'12.4"E
	58, 59, 61, 60, 62, 63, 64	F3		47°58'20.2"N 18°08'21.6"E
2	1, 2, 3, 4, 5, 6, 7	C1	7	47°58'22.0"N 18°08'26.9"E
3	8, 9, 10, 11, 12, 13, 14	C2	7	47°58'18.9"N 18°07'39.8"E
4	15, 16, 17, 18, 19, 20, 21	C3	7	47°58'20.9"N 18°07'40.5"E
5	36, 37, 38, 39, 40, 41, 42	M3	7	47°58'24.9"N 18°07'50.6"E
6	22, 23, 24, 25, 26, 27, 28	M1 M2	14	47°58'17.2"N 18°08'25.5"E
	29, 30, 31, 32, 33, 34, 35			47°58'18.4"N 18°08'26.9"E

Based on analysis of synoptic tables in program JUICE we found diagnostic, constant and dominant species for each associated entry with the cluster. Expert systems generated potential communities (see Table 3). Higher precision device relevés in the communities were shown using the Czech expert system that assesses the woody vegetation as well. Quotation marks next to community

codes indicate the likelihood of inclusion in the syntaxon, which can be explained by the transitional nature of the analysed covers. Their species composition and structural properties are modified by the introduction of invasive plants and they are also communities greatly affected by people (Janišová et al. 2007).

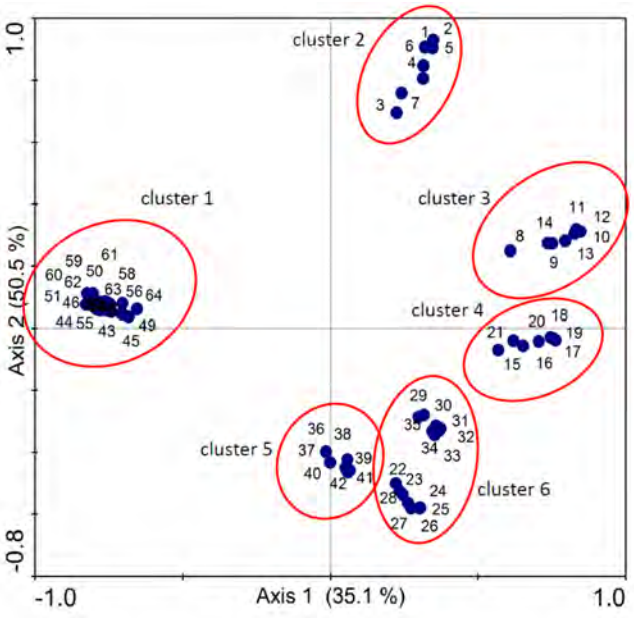
Table 3 Competence phytocoenosis types of research plots, clusters and number of relevés the communities

Communities	Cluster	N. of relevés	Research plot
?KBD03 *Sambuco nigrae-Aceretum negundo* - C	6, 5	21	M1 M2 M3
?KBB05 *Rhamno catharticae-Cornetum sanguineae* - C	6, 4	21	M1 M2 C3
?XCE03 *Hyoscyamo nigri-Conietum maculati* - C	3, 2	14	C2 C1
?XCB08 *Artemisio vulgaris-Echinopsietum sphaerocephali* - C ?MCA04 *Phragmitetum australis* – C	2	7	C1
?XDD03 *Anthriscetum trichospermae* - C ?KBE01 *Chelidonio majoris-Robinietum pseudoacaciae* - C ?XDC05 *Urtico dioicae-Parietarietum officinalis* - C	1	22	N1 N2 N3
?MAH02 *Festuco arundinaceae-Althaeetum officinalis* - S	6, 4, 3	28	M1 M2 C3 C2
?MAE13 *Filipendulo ulmariae-Menthetum longifoliae* - S	5	7	M3
?MAH04 *Agropyro repentis-Rorippetum austriacae* - S ?MAH08 *Potentilletum reptantis* – S	2	7	C1
Non - identified woody vegetation by the system - S	1	22	F1 F2 F3

Legend: C - Czech expert system, S - Slovak expert system

For graphical representation of the correlation relevés we used nonlinear principal component analysis. Clumps of relevés were assigned to the clusters (see Figure 2) by TWINSPAN clustering methods.

Figure 2 PCA scatter plot phytocenological relevés and their assignment clusters



The *Sambuco-Aceretum negundo* community in synecologically characterized by Valachovič (2012) in regards to occurrence on different habitat. Invasive neophyte tree species *Negundo aceroides* aggressively invades onto ruderal habitats in urban and industrial areas. It massively invades the banks of watercourses, it prefers bank cover and floodplain forests, where it is the dominant part of shrubs and lower tree floor. *Sambucus nigra* is the most prevalent in lower shrub floor.

The *Hyoscyamo nigri-Conietum maculati* community occurs in sufficiently sunlit and dry habitats with soil rich on nitrogenous compounds (VEG 2016). The work Šibík and Kliment (2012) confirms in the record correlating the area of cover near the edge of the forest the occurrence of ruderal communities consisting of species *Conium maculatum*, *Iva*

xanthiifolia, *Ambrosia artemisiifolia*, *Urtica dioica*, *Galium aparine*, which are the same as in our studied areas.

The *Chelidonio majoris-Robinietum pseudoacaciae* settles community the edges of Berek's cover. At present, it massively invades the inner parts of the forest cover and it substitutes some of the autochthonic woody vegetation, especially those that were panted in the past. It often occurs as the substitute for oak cover in the valleys of some rivers and streams. Vítková (2014) states, that *Robinia pseudoacacia* has the ability to change the composition of species of herbal vegetation. The numbers of weaker rivalling species are shrinking and nitrophilous species are becoming more prevalent, e.g. *Chelidonium majus*, *Sambucus nigra*, *Galium aparine*, *Urtica dioica*, *Impatiens parviflora*.

The *Artemisio vulgaris-Echinopsietum sphaerocephali* community is the typical species of *Echinops sphaerocephalus* which is categorized in the invasive species neophyte in Czech Republic (Pergl et al. 2013), in Slovakia it still has a nondescript status (Medvecká et al. 2012). Only a singular subject was found in Berek's clear-cut, but it can be assumed that it will be able to build a viable population in the coming vegetation periods. The vegetation settles anthropogenic sunlit habitats.

The *Festuco arundinaceae-Althaeetum officinalis* community was observed both in anthropogenic and natural habitats along the rivers and their alluviums and also next to backwaters. The main factors were groundwater and sufficient mechanical treading (Hegedüšová et al. 2014).

CONCLUSION

Out of the 20 found invasive species the most common: *Solidago gigantea*, *Impatiens parviflora* and *Aster lanceolatus*. The lowest presence of invasive species has been in remnant of original native areas in the forest cover. It can be concluded that the most invaded communities were found in the clear-cuts. There were 12 communities found in all. The most commonly occurring invaded communities were *Chelidonio majoris-Robinietum pseudoacaciae* and *Hyoscyamo nigri-Conietum maculati* with invasive species: *Robinia pseudoacacia* and *Conium maculatum*. The study provides findings about the disrupted communities and may be an asset in further observation and comparison of occurrences on differently used areas.

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AN EVALUATION OF THE EFFECT OF SELECTED ROOTSTOCKS ON THE GROWTH AND HARVESTING ON HIBERNAL VARIETY

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Abstract: Hibernal is an interspecific cultivar of grapevine that has been cultivated in Germany. It was created by crossing the cultivars Rheinriesling and Seibel 7053 and has very promising potential in terms of fungus-resistant (PIWI) cultivars, so that it can be used for organic wine-making. The study is focused on the impact of seven selected rootstocks (125AA, Amos, Börner, CR2, 5BB, K1SO4, and T5C) on the quantitative characteristics of variety Hibernal. The experiment was carried out in 2015. The aim was to evaluate the influence of rootstocks in the selected locality, based on the following traits: plant vitality, yielding capacity of individual plants, number and weight of bunches per vine, and the weight of 50 berries. The results show that rootstock cultivar 125AA and K1SO4 performed best in the evaluation.

Key Words: Hibernal, quantitative characteristics, PIWI varieties, rootstock

INTRODUCTION

Currently, there are an increasing number of people, who are at the forefront of breeding new varieties and rootstocks with higher resistance. These varieties, called PIWI (from German world "pilzwiderstandsfähige Rebsorten"), are more resistant against pest *Viteus vitifolie* and pathogens *Erysiphe necator* and *Plasmopara viticola*. The bases in turn affect the grafted varieties, not only in terms of the size of fruit and earliness of harvest, but more importantly they solve problems concerning resistance to drought and frost, pests and diseases, and the different chemistry of the soil (Brighenti et al. 2011).

The effect of rootstocks is dependent on the ratio between the leaf area of the plant and its yield. Examinations of the uptake of nutrients and water show them to be closely related to changes in the phenology of plants and it is well known that these processes are significantly influenced by rootstocks (Pulko et al. 2012).

In quantitative terms, it is therefore important to monitor the growth vitality of the varieties of rootstocks used, since the quantity and quality of the harvest depends on it (Brighenti et al. 2012).

When establishing a new vineyard, a well-chosen rootstock and scion combination represents one of the first important steps on the way to success (Keller et al. 2012).

The aim of this work was to evaluate the influence of rootstock used for quantitative parameters on monitored grafted variety of Hibernal at a given location. Hibernal is medium-late to late, interspecific wine grape vine variety, used for producing white wine, first bred in 1944 in Germany (Hillebrand et al. 2003).

MATERIAL AND METHODS

Characteristics of the vineyard

The grapes used in individual analyses were harvested in the vineyard with the gene-pool collection facilities of the research station Mendeleum in Lednice na Moravě (Czech Republic). The altitude of this locality is 176 m a.s.l. and – from the bioclimatological point of view – it is characterised

as a dry region (sub-region dry, district warm and dry with mild winters). The locality is open and well insulated. The plot is predominately flat and slightly sloping to the north-east. The soil is classified as a sandy clay loam with 20–24% of clay particles.

Climatic characteristics

The average annual number of rainy days is 90. A long-term average of the annual sum of precipitation is 524 mm (of this, 61% occurs within the growing season. The period with air temperatures higher than 10 °C lasts from the April 10 to October 10 - i.e., 175 days.

Experimental design

The cultivar under study (i.e., "Hibernal") was grafted onto 7 different rootstocks (125 AA, Amos, Börner, CR2, 5BB, K1SO4, and T5C). Vines were planted in the year 2005. The vine training was of the medium height with one cane (8–10 eyes per cane). The spacing of vines was 1m x 2.2m.

Cultivar Hibernal

This variety was bred by H. Becker et al. in Geisenheim (Germany) as the second filial generation seedling crossed with varieties of Chancellor (Seibel 7053) x Rheinriesling 239. In the Czech Republic, the variety has been allowed since 2004 (Sotolář 2006).

Rootstocks

Altogether, 7 rootstock varieties were tested. All of them are routinely used in viticultural practice. Two were very vigorous (5BB, Börner), two showed medium vigour (125AA, CR2), and the growth of the remaining three (Amos, K1SO4, and T5C) was weak (Pospíšilová et al. 2005).

125AA - *Vitis berlandieri x Vitis riparia*

Amos - Severnyj (Malingre x *V.amurensis*) x Schwarzmann (*V. riparia x V. rupestris*).

Börner - *Vitis riparia* 183G x *Vitis cinerea* „Arnold"

CR2 (Craciunel 2) - *Vitis berlandieri x Vitis riparia*

5BB (Kober 5BB) - *Vitis berlandieri x Vitis riparia*

K1SO4 - LE/K1 x SO4

T5C (Teleki 5C) - *Vitis berlandieri x Vitis riparia*

Sampling

Samples were collected at regular weekly intervals (0–5), namely on September 2, September 9, September 16, September 23, and September 29, 2015. Average samples of berries were collected on 50 plants from different parts of grapes. Each sample consisted of 200 berries. From these berries were randomly selected 3 x 50 berries and these berries were processed in the laboratory on the day of sampling. The evaluation of the harvest (i.e., the number and weight of grapes and the number and weight of annual shoots) was performed on September 29, 2015. In each combination of rootstocks and Hibernal grafts, parameters were evaluated from samples collected from 7 plants.

Evaluation of uvological parameters

Individual vines were monitored from the agrotechnical and ampelographical points of view. The following basic parameters were evaluated: number of annual shoots per vine, weight per plant, average weight of one annual shoot, number and weight of grapes per vine, average weight of one bunch of berries, and weight of 50 berries (this was a modified classificator CPVO-TP/50/1).

Statistical analysis

A statistical analysis was performed using the Excel 2007 package (Microsoft Office, USA) and table sheets and graphs were produced using the statistical software Statistica 10 (Copyright © StatSoft). Results in figures are expressed by mean. It was counted from 7 plants of each rootstock. Error bars represent the 95% confidence interval of a mean. Statistical significance was determined by examining the basic differences between among individual rootstocks using ANOVA and Scheffé's test.

RESULTS AND DISCUSSION

Evaluation of grapes and annual shoots

In 2015, an extensive study of the effects of 7 rootstocks on a selected grapevine cultivar (Hibernal) was performed in the Mendeleum vineyards in Lednice na Moravě (Czech Republic). The ripening of grapes of this cultivar was monitored at weekly intervals. Simultaneously, the yields and some other quantitative parameters were recorded. The results obtained were processed statistically and are presented below.

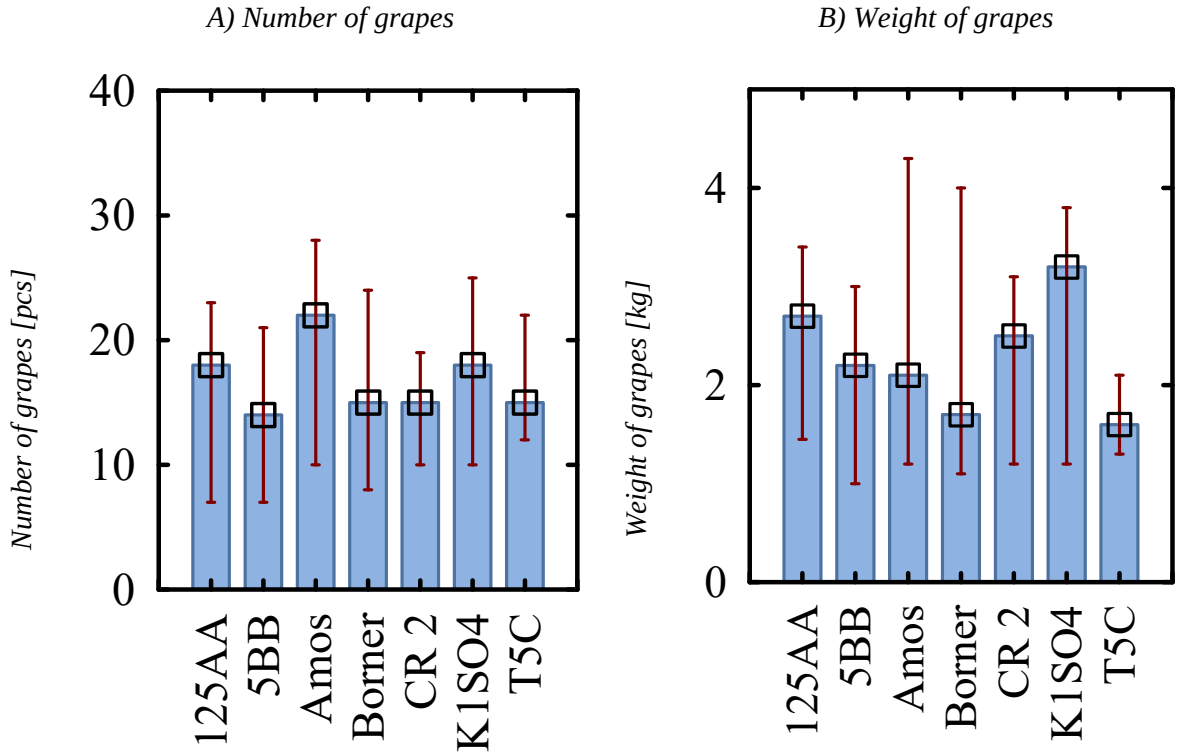
The number and weights of the grapes were evaluated because only these characteristics were influenced by tested rootstocks. The growth of the plants was also evaluated; specifically, the number of annual shoots per vine, and their weight.

The number and weight of grapes per plant

As shown in Figure 1A, the highest number of grapes in 2015 was obtained using the rootstock Amos, with 22 pcs per plant. Other high values were also obtained by rootstocks 125AA and K1SO4 (19 pieces).

Figure 1B shows that the highest weight of the grapes on the plant was achieved with rootstock K1SO4 (3.2 kg). Low yields were obtained with rootstocks Borner and T5C (weighing less than 2 kg).

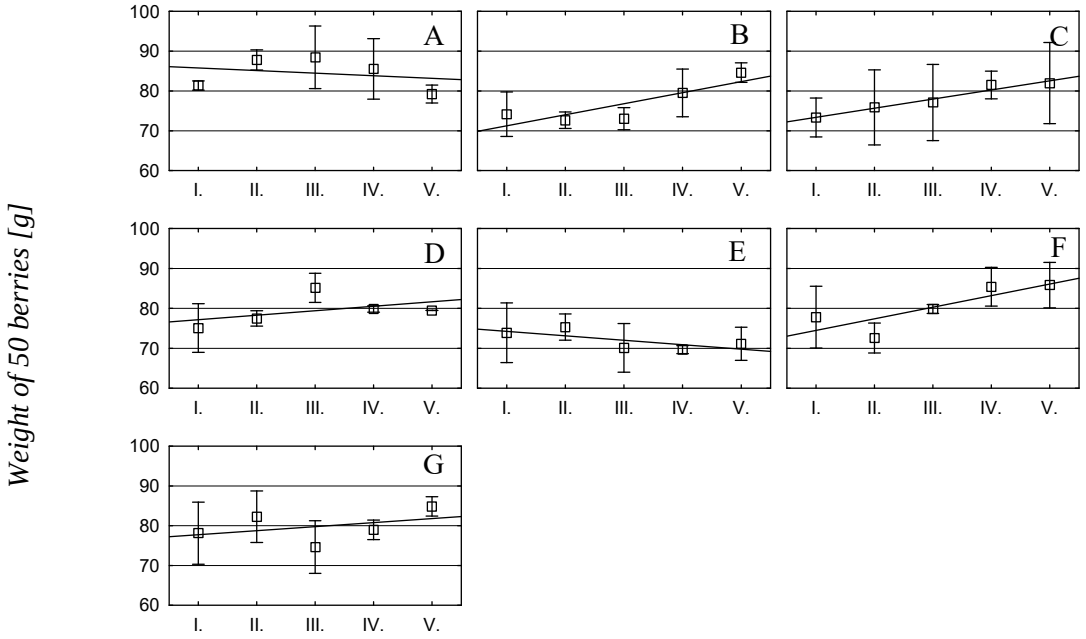
Figure 1 Number and weight of grapes per plant



The weight of 50 berries

The lowest and highest values of the weight of 50 berries were recorded in the beginning in variants with rootstocks Amos and 125AA respectively (72 and 81 g.). In the course of the ripening period, the highest value of the growth rate of berries was observed with the rootstock K1SO4. On the other hand, the lowest growth rate (negative growth rate) was recorded in the variant crossed with the rootstock CR2. The graphs presented in Figure 2 illustrate the growth rates of individual combinations of rootstocks.

Figure 2 Weight of 50 berries (in grams), as determined on different sampling dates (I.–V.) in experimental combinations with individual rootstocks – (A) 125AA; (B) 5BB; (C) Amos; (D) Börner; (E) CR2; (F) K1SO4 and (G) T5C.

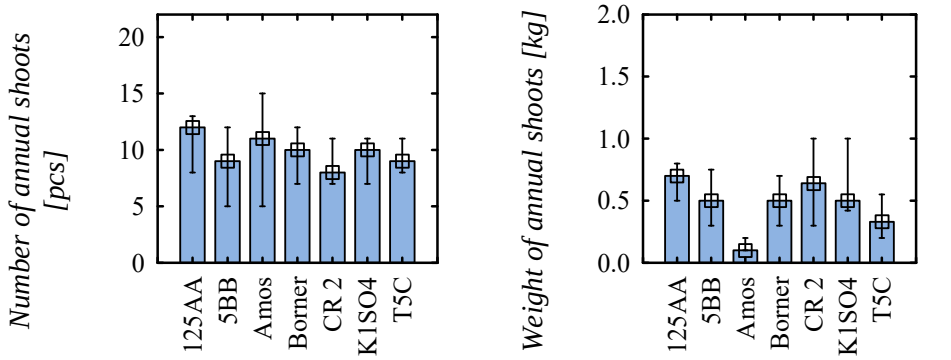


The number and weight of annual shoots per plant

The average number of shoots between rootstocks was very balanced, at around 10 pcs. A slightly higher yield of 12 pcs was obtained with rootstocks Amos and 125AA. The highest weight based on trimmed vines was achieved using rootstocks 125AA and CR2, at 0.7 kg. The lowest weight was with the rootstock Amos. Other rootstocks have balanced weights.

Figure 3 Number and weight of annual shootsper plant

A) *Number of annual shoots* B) *Weight of annual shoots*

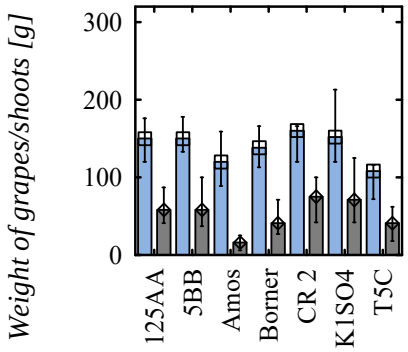


Average weight of one grape and annual shoot

The weight per vine corresponds in this case with the weight of all cropped cuttings. The highest values were from rootstock CR2, and the lowest were again from rootstock Amos, with only 16 g per shoot.

The weight of one grape ranged from 100 to 150 g. The highest weights were obtained by rootstocks K1SO4, 5BB, and CR2.

Figure 4 Weight of one grape (first blue column) and one annual shoot (second grey column)



The number of shoots on the plant should be roughly constant (this is also dependent on the cutting). If there are more annual shoots on the plant, it has shorter internodes and there will be more grapes on the plant, but they are smaller and lighter. If there is a lower number of annual shoots, it has longer internodes, meaning the shoots are larger, there are large leaves, and the grapes are fewer but larger and heavier. By contrast, stronger shoots need more nutrients. The number and weight of the shoots should be regulated using appropriate measures (Cohen and Naor 2002). It was also found that if row spacing (distance) was reduced to 1 meter for a number of shoots on the vine, yields per unit area (i.e., per hectare) were higher, while qualitative parameters remained unchanged (Kliewer et al. 2000). Tethal et al. studied effects of rootstock varieties on qualitative parameters of Cerason juice in 2011 in vineyards of Mendel University in Lednice. In the juice of the berries, the following parameters were monitored: concentrations of sugar, total acids, tartaric acid, malic acid, ratio of tartaric to malic acid (β-ratio), concentration of yeast assimilable nitrogen, and pH value. The best results were recorded in 125AA and 5BB rootstock varieties (Tethal et al. 2015).

The varieties grafted on the rootstock Amos consist of shorter internodes, resulting in the expected higher number of shoots. At the same time, however, they lead to weaker growth shoots, and their weight reached lower values. The fertility has good characteristics, in that it is high, and creates medium-sized grapes of a favourable weight. The T5C base is suitable for loamy soils with ground water; it grows weakly and also forms shorter internodes. Their numbers were comparable to other rootstocks, but their weight was the second lowest. The average weight of one annual shoot was also the lowest in these two rootstocks. In a given year, the T5C rootstock has average numbers of grapes, but their weight is among the lowest. The size of the grape was also the smallest. The 125AA rootstock is an average in lush of growth. The number of shoots left on the plant was the highest, but their weight was too high. The number of bunches and the weight was the second highest. The grapes are medium-sized, creating the assumption that there will be ideal ripening, i.e., there is a good balance between quantity and quality. The luxuriant rootstock 5BB showed a lower number of shoots, but the mass of shoots on the plant was middling. Regarding the grapes, their number was on this rootstock high, but their weight shifted her to diameter.

Börner is an exuberant, moderate to strong-growing rootstock, which reached the average number of annual shoots with less weight on the annual shoots per plant and the weight of one annual shoot. The number of grapes was average and their weight was very low, suggesting small grapes. This is not an ideal level of fertility for the given conditions. The CR2 base is an average lush, with the low number of annual shoots compensated by a higher weight. It is therefore logical that they will achieve the maximum weight, reflecting their very strong growth. The number and weight of grapes from this rootstock was average; the average weight of one grape, however, was the highest. The K1SO4 base had an average number of annual shoots and weight. This rootstock was the third best. It was the highest rated in terms of the weight of bunches on the plant, and was among the highest concerning the average weight of one grape.

CONCLUSION

This study was intended to evaluate the suitability of a combination of Hibernal variety with seven grapevine rootstocks. The best combinations were chosen on the base of an evaluation of a number of

selected parameters. The results of the yields also played a key role. Rootstocks 125AA and K1SO4 performed the best, while 5BB could also be a suitable rootstock for Hibernal. The experimental values obtained could have been influenced by the weather conditions in the year 2015 or by the pedological conditions of the experimental vineyard.

ACKNOWLEDGEMENTS
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THE ANTIOXIDANT ACTIVITY OF ANCIENT WHEAT VARIETIES AND MODERN WHEAT VARIETIES

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Abstract: Wheat is a crucial dietary stable and economic commodity around the globe. It plays an important role in health benefits to combat oxidative stress in the human body by maintaining a balance between antioxidants and oxidants. The objective of this study was to determine the contents of antioxidant activity (tocopherols) in varieties of einkorn, emmer, spelt and *Triticum aestivum* L. and identify the richest sources for improving the nutritional value of bread, pasta and other wheat products. The field experiment were arranged in Ceske Budejovice from 2010 to 2012 with 26 wheat varieties. 2,2-diphenyl-1-pycrylhydrazyl assay was used to evaluate the level of antioxidant activity. The results revealed that antioxidant activity (AOA) ranged from 225.45 mg/kg Trolox DM to 400.83 mg/kg Trolox DM and its values were significantly different among varieties, ploidy level and wheat accessions. Also, modern wheat varieties showed higher AOA than ancient wheat varieties apart from emmer varieties.

Key Words: Antioxidant activity, wheat, food grain sources, phytochemical, reactive oxygen species

INTRODUCTION

Wheat is plant grown on more land area than any other commercial crop. It is also one of the most important food grain sources for people all over the world because of the universal use of wheat for a wide variety of products such as bread, noodles, cakes, biscuits, etc. Wheat kernel is composed of endosperm (81–84%), bran (14–16%), and germ (2–3%) (Pomeranz 1988). Endosperm is the inner part playing a role as storage of energy and functioning protein. Bran is outer layer protecting the grain and germ is the kernel's reproduction system. Whereas wheat endosperm contains mostly starch and protein, bran and germ are rich in dietary fiber, vitamins, minerals and phytochemicals playing an important role in nutrition and health benefits for humans (Pomeranz 1988). The customers are, therefore, strongly recommended to consume whole-grain foods with at least three servings per day. The recent studies have showed that regular consumption whole wheat grain has been found to be associated with reduced total mortality, as well as reduced risk of coronary heart disease, ischemic stroke, type 2 diabetes (Archie et al. 2006), hypertension in women and colorectal cancer (Schatzkin 2007).

The aim of this study was to determine the level of antioxidant activity (tocopherols) in varieties of einkorn (*T. monococcum* L.), emmer (*T. dicoccum* Schuebl [Schrank]), spelt (*T. spelta* L.) and *T. aestivum* L. and identify the richest sources for improving the nutritional value of bread, pasta and other wheat products.

MATERIAL AND METHODS

Used varieties

The varieties came from the Gene bank of the Crop Research Institute in Prague-Ruzyne. In the precise three-year field experiments in 2010, 2011 and 2012 four varieties of wheat einkorn, eight varieties of emmer, seven varities of spelt, four varieties of landraces of bread wheat and three varieties of spring wheat as control (SW Kadrilj, Vanek, Jara) were used.

Field Trials

The field experiments were arranged in a randomized complete design with two replications. Plot size of this treatment was 10 m^2. Varieties were sown on the organic certified research area of the University of South Bohemia in Ceske Budejovice, the Czech Republic. The seeding rate was adjusted for a density of 350 germinable grains per m^2. The crop stands were treated in compliance with the European legislation (the European Council Regulation (EC) No. 834/2007, the European Commission Regulation (EC) No. 889/2008. Characteristics of the conditions of the University of South Bohemia in Ceske Budejovice research area: Mild warm climate, soil type – pseudo gley cambisols, kind of soil – loamy sandsoil, altitude of 388 m.

Laboratory analysis

Finely ground wheat samples (ca 5.0 g) were weighed into 100 mL volumetric flasks and dissolved in methanol. The flasks were filled up with methanol to volume of 100 mL. For AOA determination, 100 µL aliquots of sample solutions were pipetted. Determination of AOA with DPPH assay. Indirect method described by Roginsky and Lissi (2005) was used. Sample containing antioxidants reacts with a solution of stable synthetic radical being converted to a colourless product (DPPH assay). Methanolic DPPH solution [absorbance (t_0) 0.600 ± 0.01] was prepared and 100 µL of the sample were added. Reaction time was 20 min. Absorbency was measured at wavelength λ = 515 nm. AOA was calculated as the decrease of absorbency according to the equation (1): AOA (%) = 100 – [(A_{t20}/A_{t0}) × 100] (1)Where: At_{20}– absorbency in time 20 min; A_{t0}– absorbency in time 0 min. Calculated AOA was expressed in mg Trolox/kg DM. At_0 and A_{t20} were determined from the standard calibration curve ($r^2 \geq 0.9945$). Calibration curves were prepared using working solutions of Troloxin methanol between 5-25 µg Trolox/mL (LOD = 0.601 µg Trolox/mL, LOQ = 2.000 µg Trolox/mL, RSD = 1.83%). All samples were analysed in duplicates.

Statistical analysis

The data were subjected to analysis by using software Minitab 17.0. Specifically, ANOVA multiple factorial analysis, Turkey's HSD test and t-test were used for analyzing the parametric data and non-parametric data.

RESULTS AND DISCUSSION

Whole grain phytochemicals have antioxidant activity, the ability to scavenge free radicals that may oxidise biologically relevant molecules (Liu 2007). Thank to this, whole wheat foods could to contribute to the health benefits of people such as reducing the risk of heart disease, diabetes type 2, cancer and etc. In the present study, there were highly significant differences ($p < 0.05$) among 26 varieties for antioxidant activity (Table 1).

Table 1 Content of antioxidant activity in different wheat grains.

Variety	D11*	D12*	D13*	D14*	D17*	D18*
AOA (mg Trolox/kg DM)	400.83^{a}	364.15^{ab}	341.60^{bc}	288.36^{e-g}	304.56^{c-f}	351.62^{b}
Variety	D19*	RUDICO*	J1**	J2**	J4**	J6**
AOA (mg Trolox/kg DM)	339.92^{bc}	332.90^{b-d}	247.42^{gh}	306.16^{c-f}	293.23^{df}	327.73^{b-e}
Variety	P1***	P2***	P3***	P4***	SP1****	SP2****
AOA (mg Trolox/kg DM)	345.88^{bc}	362.25^{ab}	365.26^{ab}	360.95^{ab}	225.45^{h}	226.55^{h}
Variety	SP3****	SP6****	SP7****	SP8****	SP9****	JARA
AOA (mg Trolox/kg DM)	232.63^{h}	265.56^{f-h}	280.63^{fg}	281.10^{fg}	248.82^{gh}	357.36^{ab}
Variety	SW	VANEK				
AOA (mg Trolox/kg DM)	336.98^{b-d}	353.70^{b}				

Legend: Values marked with different small letters are significantly different at P ≤ 0.05

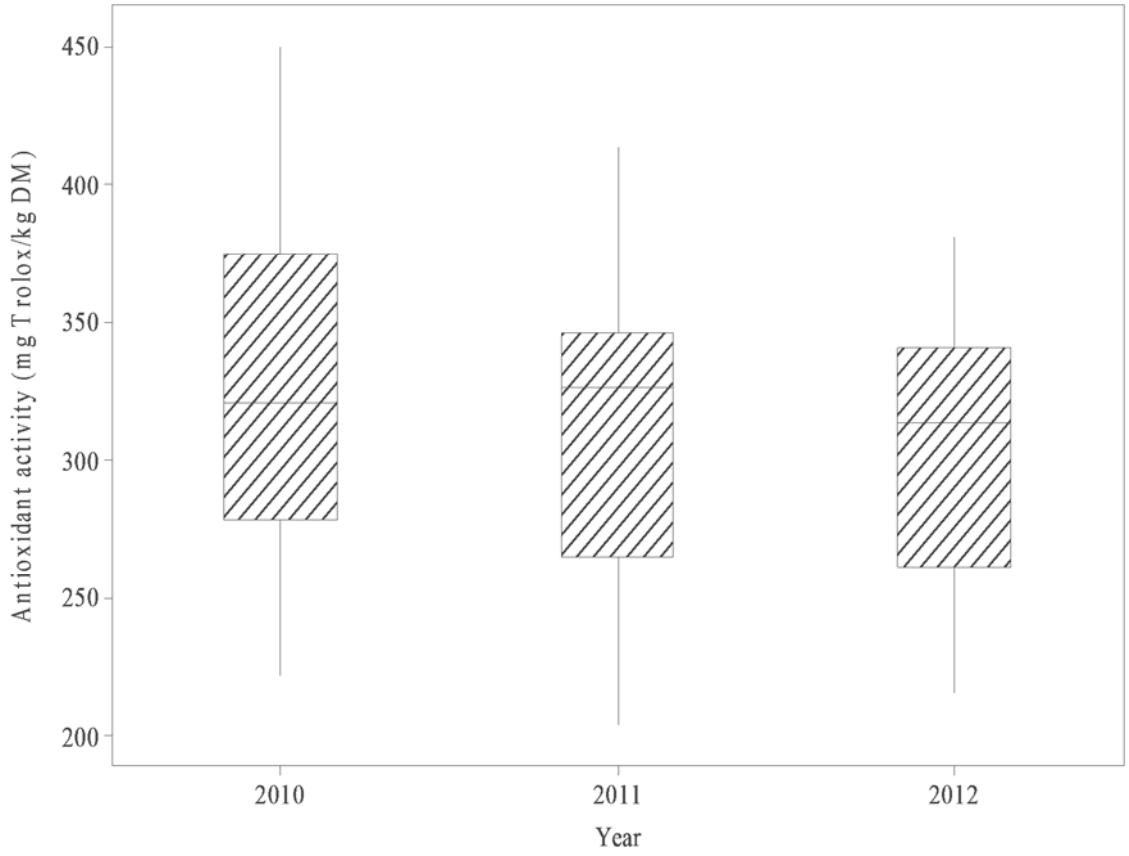
** Emmer varieties; ** Einkorn varieties; *** Landrace of T. aestivum; **** Spelt varieties*

Mean antioxidant activity among varieties ranged from 225.45 mg Trolox/kg DM to 400.83 mg Trolox/kg DM. This demonstrates a broad range of antioxidant content in wheat species. There were eight groups in which the means were not significantly different from one another. Having 400.83 mg Trolox/kg DM, D11 variety belonged to lead group and was significantly different from all other varieties except P3, D12, P2, P4 and JARA. In contrast, the varieties containing the lowest content of antioxidant were SP6, SP9, J1, SP3, SP2 and SP1 with 266.57 mg Trolox/kg DM, 248.82 mg Trolox/kg DM, 247.42 mg Trolox/kg DM, 232.63 mg Trolox/kg DM, 226.55 mg Trolox/kg DM and 225.45 mg Trolox/kg DM, respectively.

According to the findings of Lachman et al. (2012) the antioxidant activity content of 7 varieties ranged between 134.0 and 197.5 mg Trolox/kg DM. Obviously, our results are approximately two-time higher than these ones. This means that the varieties in our experiment are potential to breeding new wheat varieties, as well as its essential as a source of functional food ingredients.

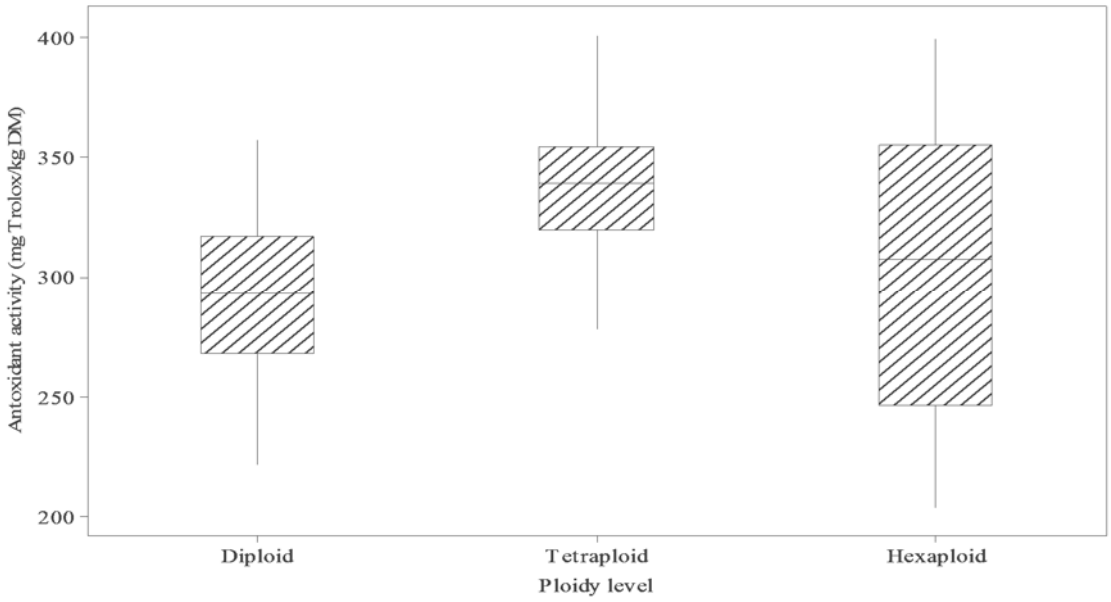
It is known that antioxidant activity content can be influenced by stress factors of the weather conditions during the vegetation period and genotype effects. Comparing the data collected from 2010 to 2012 of four species (Figure 1) show that there is a decrease gradually the mean of antioxidant during the three-year period with 23.26 mg Trolox/kg DM. These differences are, however, not statistically significant.

Figure 1 Antioxidant activity in 26 varieties harvested in 2010, 2011 and 2012



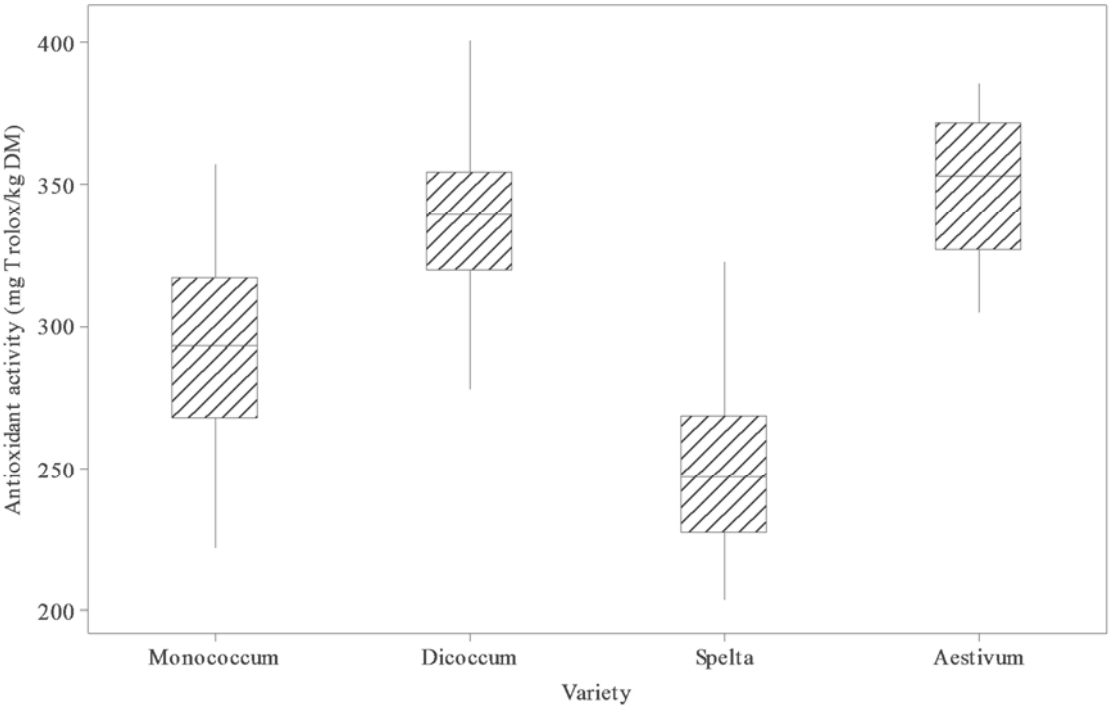
The cultivated diploid (einkorn), tetraploid (durum wheat), hexaploid (bread wheat) and varieties possess antioxidant activity due to their content of hydrophilic (phenolics, selenium) and lipophilic (carotenoids, tocopherols) antioxidants (Hidalgo et al. 2008).

Figure 2 Content of antioxidants in wheat grains from the harvests 2010, 2011 and 2012



Analysing ANOVA Tukey's HSD revealed statistically significant differences between Tetraploid and Diploid as well as between Tetraploid and Hexaploid (Figure 2). The mean antioxidant activity of tetraploid from 2010 to 2012 (340.49 ± 39.11 mg Trolox/kg DM) was higher than the value of diploid and hexaploid (293.64 ± 34.82 mg Trolox/kg DM) and (303.08 mg Trolox/kg DM), respectively. Our results are different to those of Lachman (2012). While antioxidant values in our findings increase from diploid (einkorn) to tetraploid, the reverse is true for Lachman's results. This is because our experiment used 26 varieties in three years compared to 7 varieties in two years of Lachman's experiment.

Figure 3 Antioxidant activity values of four species



The figure 3 illustrates the differences of four varieties. *T. aestivum* and emmer wheat shared the highest value with 354.44 ± 24.97 mg Trolox/kg DM) and 340.49 ± 39.11 mg Trolox/kg DM, respectively. The second high value belonged to *T. monococcum* (293.64±34.82 mg Trolox/kg DM). With 251.54 ± 29.60 mg Trolox/kg DM), *T. spelta* had the lowest value in total four species ($P < 0.05$)

CONCLUSION

Wheat contains a huge essential antioxidants such as dietary fiber, tocopherols, tocotrienols, and etc. The consumption of wheat is associated with reducing risk of chronic diseases including type 2 diabetes, obesity, and cardiovascular disease. In this study, the content antioxidant activity of 26 varieties of whole wheat are reported. Antioxidant activity ranged from 225.45 mg Trolox/kg DM to 400.83 mg Trolox/kg DM. The antioxidant activity values were significantly different among varieties, ploidy level and wheat accessions. Also, this study showed a genotypical variation in the antioxidant activity of einkorn, emmer, spelta and *T. aestivum*.

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SPECIES SPECTRUM OF PLANTS ON SELECTED LAND OF PHOTOVOLTAIC POWER PLANT

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Abstract: This paper focuses on the evaluation of weed species diversity on selected land with photovoltaic power plant in 2013, 2014 and 2015. Furthermore, it is aimed on the evaluation of representation of individual species depending on different conditions within the area. The area is located in South Moravia, Brno-venkov district in the village Unin. Evaluation of vegetation was carried out by multivariate analysis of ecological data, concretely by a redundancy analysis (RDA). Overall, 65 plant species were identified on monitored land. Plant species with highest cover values in phytocoenology relevés were: *Taraxacum* sect. *Ruderalia*, *Dactylis glomerata, Cirsium arvense* and *Digitaria sanguinalis*. The regular management of land with photovoltaic power plant is crucial to prevent the occurrence of tall species causing overshadow of photovoltaic cells which would result in reduction of the production of electricity.

Key Words: vegetation, photovoltaic power plant, species diversity

INTRODUCTION

Photovoltaics is the science using a sunlight for conversion into electricity. Basic components of photovoltaic power plant are photovoltaic modules and inverters. In terms of environmental impact, it is one of the cleanest sources of energy, which is known so far (Quaschning 2010).

The reason for the massive support of photovoltaics is a fact that renewable energy sources help us to protect the environment. One kWh produced from solar sources saves 0.6 kg of CO_2. It is very likely that the expansion of photovoltaics will continue (Hernandez 2014). Turney and Fthenakis (2011) reported, that the savings of CO_2 for production of 1 kWh from photovoltaic module is up to 1100 g, compared with production from black coal.

According to forecasts, photovoltaics have constantly untapped potential and its development will increase in the future. Development of connected stations has reached such an extent in 2010, that the distribution companies have declared a stop state on February. This situation lasted a year and a half, afterwards roof power plants up to 30 kW were allowed to join (Solární novinky 2011).

One of the most important factor for green plant is light, important for photosynthesis, which does not take place in the dark. The amount of radiation depends on the angle of sun rays, clarity of atmosphere and day length. These patterns are influenced by an altitude, a latitude and a condition of atmosphere. The light rays fall on the ground from half as a direct radiation and half as diffuse radiation (Rokia et al. 2014).

Although light is not one of the factors causing germination, it can encourage it or slow down (Attridge 1990). Rokia et al. (2014) stated, that an amount and type of vegetation depends on the specific habitat condition. According to (Baskin and Baskin 2014) these conditions are specified as climatic factors. These factors are determined by climatic processes and modified by climatic factors of territory. The basic factor is the solar radiation that is applied directly as an environmental factor in the form of heat radiation and light.

The aim of this paper is to determine the differences in species composition of vegetation in habitats with different conditions within the property of photovoltaic power plant, caused by prolonged overshadow of photovoltaic panels.

MATERIAL AND METHODS

Characteristics of the area

The monitored area is located in the South Moravia, in the village Unin, which is located northwest from Brno. Climate region is moderately warm and humid. The average annual temperature reaches 6–7 °C and an annual precipitation is 650–750 mm. The area of interest falls geographically into Boskovicka furrow with an altitude of 461 m asl (mapy.nature.cz 2016).

The entire plant is located on a total land area of 18 147 m^2. 2 593 m^2 are arable lands and 2 373 m^2 are permanent grasslands. The rest is consisting of other areas (Nahlížení do katastru nemovitostí 2016).

Evaluation of vegetation and statistical processing

Evaluation of vegetation was carried out by method of phytocoenology relevé. The size of each relevé was 20 m^2 and coverage was estimated in percentage. Monitoring took place in 2013, 2014 and 2015 and three observations were done each year. The first in spring, the second in summer and the third one took place in autumn. 45 observations were conducted in total. Within the area of photovoltaic power plant, three different stands were used for monitoring, area between panels (Mezi_panely), area under panels (Pod_panely) and area around panels and alleyway (Volne). Scientific names of weed species were used according to Kubát et al. (2002).

The obtained data were processed by multivariate analysis of ecological data. Selection of the optimal analysis followed the lenght of the gradient, which was detected by segment analysis DCA. Furthermore, redundancy analysis (RDA) was used, which is based on linear response. A total number of 999 permutations were calculated in a Monte Carlo test. Tested environmental factors were individual stands: under photovoltaic panels (Pod panely), alleyways or between photovoltaic panels (Mezi panely), on open area (Volne). Monitored years were used as covariates (*covariables*) in analysis. The collected data was processed by a computer program called Canoco 4.0 (Ter Braak 1998).

RESULTS AND DISCUSSION

Sixty-five plant species were found in total. The average coverage of identified species on monitored stands in particular terms of evaluation is indicated in Table 1.

Table 1 The average coverage of weeds on different stands within 3 years of monitoring (% - average coverage)

Species	Abbrev.	Stands		
		Volne	Pod_panely	Mezi_panely
Acer campestre	*Ace camp*	0.03		0.01
Achillea millefolium	*Ach mill*	14.06	8.42	11.61
Alopecurus pratensis	*Alo prat*	1.78	4.21	3.15
Anthemis arvensis	*Ant arve*	2.89	0.03	1.39
Anthoxanthum odoratum	*Ant odor*	0.11	1.32	0.60
Anthriscus sylvestris	*Ant sylv*	2.50	0.05	1.03
Apera spica-venti	*Ape spic*		7.58	8.33
Arctium tomentosum	*Arc tom*	1.56	0.05	0.65
Armoracia rusticana	*Arm rust*	0.72	0.53	0.75
Calamagrostis epigejos	*Cal epig*	4.44	0.53	2.02
Capsella bursa-pastoris	*Cap burs*	0.06	3.47	1.50
Cirsium arvense	*Cir arve*	11.67	0.39	4.88
Crepis biennis	*Cre bien*		0.13	0.06
Dactylis glomerata,	*Dac glom*	39.17	0.79	16.49
Digitaria sanguinalis	*Dig sang*	11.67	10.53	14.94
Epilobium ciliatum	*Epi cili*	0.28		0.54
Equisetum arvense	*Equ arve*	0.17	0.26	0.18
Erigeron annuus	*Eri annu*	0.06	0.42	0.20

Fallopia convolvulus	*Fal conv*		0.26	0.11
Festuca rubra	*Fes rubr*	38.61	3.16	17.82
Fragaria vesca	*Fra vesc*	0.56	6.05	3.44
Galium aparine	*Gal apar*	12.89	2.37	6.22
Galium mollugo	*Gal moll*	0.00	1.32	0.64
Geranium pusillum	*Ger pusi*	1.78	1.84	1.55
Chenopodium album	*Che albu*	0.72	0.53	0.73
Chelidonium majus	*Che maju*	0.00	0.11	0.04
Impatiens parviflora	*Imp parvi*	0.61	0.11	0.29
Lamium album	*Lam albu*	5.56	0.00	2.25
Lamium purpureum	*Lam purp*	2.22	0.26	1.01
Lathyrus pratensis	*Lat prat*		0.34	0.37
Leucanthemum vulgare	*Leu vulg*			0.19
Lolium perenne	*Lol pere*	3.33		1.35
Malva neglecta	*Mal negl*	0.11		0.22
Medicago lupulina	*Med lupu*	10.28	0.79	4.49
Convolvulus arvensis	*Con arv*	0.83	0.89	0.72
Phleum pratense	*Phl prat*	3.17	0.00	1.49
Plantago major	*Pla majo*		3.42	1.46
Plantago media	*Pla medi*	2.44	5.79	3.45
Potentilla anserina	*Pot Anse*	3.22		1.56
Prunus domestica	*Pru dome*		0.03	0.14
Ranunculus acris	*Ran acri*		0.53	0.31
Rosa canina	*Ros cani*	0.89	0.05	0.38
Rubus idaeus	*Rub idae*	2.06	0.00	0.83
Rumex crispus	*Rum cris*	2.22	0.11	0.94
Salix alba	*Sal alba*	0.06	0.13	0.72
Salix cinerea	*Sal cine*		0.11	0.39
Salix triandra	*Sal tria*	0.11		0.04
Sambucus nigra	*Sam nigr*	2.58		1.04
Senecio vulgaris	*Sen vulg*	0.83	6.53	3.11
Silene latifolia	*Sil Lat*			0.06
Solanum nigrum	*Sol nigr*	0.06		0.02
Sonchus oleraceus	*Son oler*	2.06	2.89	2.49
Tanacetum vulgare	*Tan vulga*	4.50	4.37	4.74
Taraxacum sect. *Ruderalia*	*Tar offi*	16.17	14.47	21.84
Trifolium hybridum	*Tri hybr*	5.83	2.63	8.16
Trifolium pratense	*Tri prat*	0.83	1.26	1.19
Trifolium repens	*Tri repe*	9.72	8.42	15.39
Tussilago farfara	*Tus farf*	0.33		0.13
Tripleurospermum inodorum	*Trip ino*		0.89	0.55
Urtica dioica	*Urt dioi*	6.44	2.84	3.81
Urtica urens	*Urt uren*	13.72	6.05	8.12
Veronica chamaedrys	*Ver cham*	0.22	0.32	0.31
Vicia cracca	*Vic crac*	1.44	0.32	0.72
Vicia sepium	*Vic sepi*	0.11	2.63	3.61
Viola arvensis	*Vio arve*	0.56		0.22

Results of evaluation of vegetation were firstly processed by Detrended Correspondence Analysis (DCA). Based on this calculation was determined the length of gradient, which was 2.609 and it is used for selection of further processing. Redundancy analysis was chosen for subsequent processing.

Based on the RDA analysis and frequency of occurrence of found species was created the spatial arrangement of found species that have been graphically displayed using the ordination diagrams. Plant

species are shown as vectors (arrows), which have a different direction and colour, and selected stands are displayed as points. In case the vector of relevant species tends to a direction of specific point, representing the stands, the occurrence of this species was increased just on this area.

The results of RDA analysis, which evaluated the relation between stand and plant species, are significant at the level $\alpha = 0.001$ for all canonical axes. Other factors of environment (year of observation and maintenance mode) were analysed as covariates. The results are highly statistically significant (Figure 1).

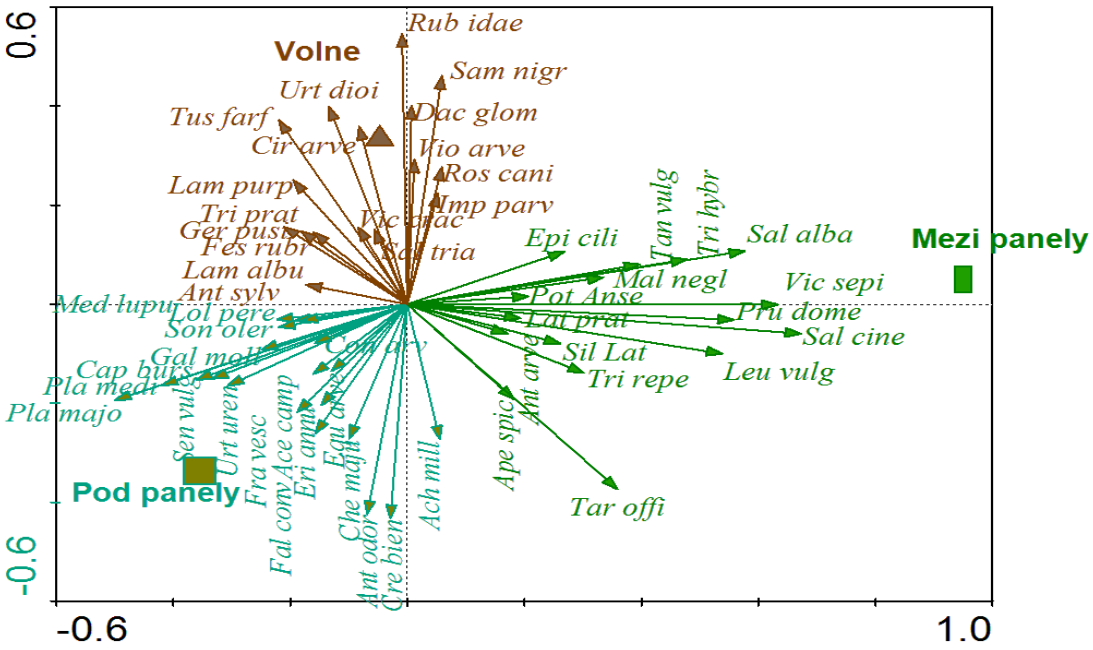
The first group of plant species occurred more frequently and with higher coverage on stands called "volne", which represent open areas. Species as *Rubus idaeus, Sambucus nigra, Dactylis glomerata, Viola arvensis, Rosa canina, Impatiens parviflora, Vicia cracca, Salix triandra, Anthriscus sylvestris, Lamium album, Festuca rubra, Geranim pussilum, Trifolium pratense, Lamium purpureum, Cirsium arvense, Tussilago farfara* and *Urtica dioica* dominated on these stands.

The second group is represented by species, which occurred more frequently and with higher coverage on stand called "mezi panely". Occurring species were: *Epilobium ciliatum, Tanacetum vulgare, Trifolium hybridum, Salix alba, Vicia sepium, Prunus domestica, Salix cinerea, Leucanthemum vulgare, Taraxacum sect. ruderalia, Apera spica-venti, Anthemis arvensis, Trifoium repens, Silene latifolia, Lathyrus pratensis, Malva neglecta, Potentilla anserina.*

The third group are species identified on area called "pod panely". *Achillea millefolium, Convolvulus arvensis, Crepis biennis, Anthoxanthum odoratum, Chelidonium majus, Equisetum arvense, Erigeron annuus, Acer campestre, Fallopia convolvulus, Fragaria vesca, Urtica urens, Senecio vulgaris, Plantago major, Plantago media, Capsella bursa-pastoris, Galium mollugo, Sonchus oleraceus, Lolium perenne, Medicago lupulina.*

Vegetation of monitored photovoltaic power plant is influenced by previous land use. Part of the area was originally non-agricultural land and only a smart part was used as arable land. This factor may affect species spectrum of vegetation. Weed management may be another influencing factor. The vegetation was left to arbitrary growth till 2013. Subsequently, the vegetation was intensively regulated.

Figure 1 Ordination diagram expressing the relation of found species and different stands on land with photovoltaic power plant (RDA, Trace = 0.111, F-ratio = 4.022, P-value = 0.001)



Legend: "Pod panely" expresses the occurrence of species under photovoltaic panels. "Mezi panely" expresses alleyways, the occurrence of species between photovoltaic panels and "Volne" means the occurrence of species on an open area.

CONCLUSION

Sixty-five plant species in total were identified on area of photovoltaic power plant in Unin during three years of observation (2013–2015). It was found, that different stand affects the occurrence of individual species.

Species as *Achillea millefolium, Convolvulus arvensis* and *Plantago media* occurred most frequently on stand under photovoltaic panels. Species typical for stand in alleyway were: *Taraxacum* sect. *Ruderalia*, *Apera spica-venti* and *Trifolium repens*. Species occurred on an open area were: *Dactylis glomerata*, *Urtica dioica* and *Cirsium arvense*.

Found species as *Antryscus sylvestris, Apera spica-venti, Cirsium arvense, Dactylis glomerata, Chenopodium album, Rosa canina, Rubus idaeus, Salix alba, Salix cinerea, Sambucus nigra, Tripleurospermum inodorum* and *Urtica dioica* can be potentially problematic for working of photovoltaic power plant. Due to its excessive growth, some species may cause overshadow of photovoltaic cells and reduce a production of photovoltaic power plant.

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THE DETERMINATION OF CONTAINED COMPOUNDS IN MILK THISTLE [*SILYBUM MARIANUM* L. (GAERTN.)] BY THE MEANS OF FT-NIR

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Abstract: The aim of our work was to verify the possibility of using Fourier transform near infrared spectroscopy (FT-NIR) for the determination of the content of individual silymarin complex components in the achenes of milk thistle [*Silybum marianum* L. (Gaernt.)] and to evaluate the possibility of distinguishing between the two milk thistle varieties (Silyb and Mirel) by the means of this spectroscopic analysis. The compounds were extracted for the determination of the silymarin complex components and determined by the high performance liquid chromatography (HPLC). The results obtained from analytical methods were used to generate the calibration model for the FT-NIR spectrometer using the partial least square method (PLS). According to correlation coefficients of the calibration models obtained for individual ingredients of the silymarin complex (0.725–0.987) these models are suitable for the preliminary determination of silymarin complex constituents. The varieties Silyb and Mirel have different ratio of the main silymarin complex components. The method of discriminant analysis (DA) was used to distinguish the varieties. Calibration model that can classify these varieties was created.

Key Words: milk thistle, quality, quantity, NIR, varieties

INTRODUCTION

Silymarin complex as a substance contained in milk thistle [*Silybum marianum* L. (Gaertn.)] achenes is a mixture of flavonolignans (silybin A, silybin B, isosilybin A, isosilybin B, silychristin and silydianin) used from the dawn of time to prevent and treat damage and disorders of the liver, spleen and gallbladder (Gažák et al. 2007). Milk thistle achenes contain also 25–30% of high-quality oil with a high content of vitamin E and a high ratio of unsaturated fatty acids such as oleic and linoleic (Keshavarz Afshar et al. 2014).

The reference method for the determination of silymarin complex content – according to the Pharmacopea Bohemica (2009) – is the liquid chromatography, which must be preceded by a continuous Soxhlet extraction to remove fat from the sample. Methanol extraction follows after the fat removal step. Then the sample is evaporated and ready for the HPLC analysis. This method is time consuming, expensive and therefore it would be appropriate to develop and use an alternative quick and easy-to use method, like FT-NIR.

The FT-NIR spectroscopy is a mathematical method based on the analysis of spectra signals (Bradáčová et al. 2014). This branch of spectroscopy uses the area between visible and medium infrared spectrum of the electromagnetic field, commonly referred to the wavelength range of 800–2.500 nm, and is based on measuring the loss of radiation caused by the contact with the sample (Ye et al. 2016). Measurements can be performed in the transmittance or reflectance mode. Therefore, it is a measurement of the radiation reflected and absorbed by the sample, directly related to the transformation of rotational or vibrational state of the molecules. This process consequently provides detailed structural information about the behaviour of O-H, N-H and S-H bonds (Wu et al. 2008, Ingle et al. 2016). This is a promising alternative, fast, non-destructive method for the analysis of samples without the need of sample pre-treatment and other instrumentation, which can be used for analysis and quality examination of food and agricultural products. The most studied parameters include the determination of major components such as moisture, oil content, the content of nitrogenous substances, but also minor and trace compounds. The method is widely used in petrochemical, textile and pharmaceutical industries (Chen et al. 2008,

Gaspardo et al. 2012, Koláčková and Šišperová 2013, Kaur et al. 2016, Sunoj et al. 2016). This type of analysis provides not only qualitative but also quantitative information about the sample, both from the chemical and the physical viewpoint (Blažek et al. 2005). The FT-NIR spectroscopy is useful for a wide range of samples from the solutionsto suspensions, emulsions, powders or solid samples with rough or irregular surface and strongly absorbing materials (Muselík 2012).

Before the actual measurement can be done, it is necessary to create so-called calibration model using the widest possible portfolio of samples. The necessary prerequisite for creating a precise calibration model is the as-much-accurate-as-possible determination of the monitored parameter of quality obtained from the reference analytical method. Accuracy and precision of the reference method affects the correlation coefficient between the values obtained from the analytical method and from the FT-NIR spectrometer. To create a functional method, it is necessary to get the most possible accurate data from the reference determination (Tenkl et al. 2009). The calibration model can be created using the partial least squares method that allows to convert the spectral matrix to a much smaller number of so-called PLS factors. The matrix of quantitative data is also modified in the same way and a regression dependence between the two matrices is found out. The output of this method is a linear regression between the given quantitative values and the values calculated by the calibration model (Šustová 2007). The discriminant analysis method (DA) is a qualitative method that allows to enter so-called classes described by any number of standards into the calibration model. The output of this method is the name of the class that is most similar to the sample under study and the Mahalanobis distance, i.e. the distance of the evaluated sample from the centroid of each class (Dvořák et al. 2013, Králová et al. 2014).

MATERIAL AND METHODS

Plant material origin–sources of the samples

The samples of Silyb, Mirel varieties and other materials of different origin and harvest years obtained from growers, research organisations and from genetic resources were used for the experiment.

Analytical methods and the creation of calibration model

Spectra of the samples were measured by the means of the Nicolet Magna FT-NIR spectrometer 550 (Thermo Fisher Scientific Inc., USA) with the detector InGaAs and beamsplitter CaF_2. Spectral data were gathered in the range of 11.500–4.000 cm^{-1} at the resolution 8 cm^{-1} and 64 scans in the reflectance mode. Each sample was measured triplicate in a rotation cuvette on the integration sphere of the instrument using the Omnic 8 programme.

The same milk thistle samples were analysed also by the means of HPLC. The achenes were ground to fine powder, 20 mg of sample was further homogenised using mortar and pestle with sea sand and addition of 0.5 ml isooctane and 0.5 ml methanol. 1.5 ml of methanol and 1 ml of isooctane were added after the homogenisaton and 2 ml aliquot was centrifuged for 5 min. at 14.000 rpm. Then 0.75 ml of lower phase was taken from each sample and analysed by the means of Dionex Ultimate 3000 HPLC instrument. The silymarin complex was separated using the Hypersil GOLD column 150 × 4.6 mm at 30 ºC. 5 µl of the sample was injected to the column. The analysis was performed at the mobile phase flow rate 1 ml/min and the UV detection at 288 nm. The analytes were eluated at isocratic conditions with 65% of mobile phase A (0.1% formic acid) and 35% of mobile phase B (100% methanol). Time of one analysis was 45 minutes.

Data obtained from HPLC analyses were used for the creation of the calibration models for the FT-NIR Nicolet instrument using the TQ Analyst software.

RESULTS AND DISCUSSION

Calibration model of the silymarin complex

The calibration models for the determination of silymarin complex components were created from the spectra of 90 samples and validated using the independent set of validation values. The range and average values of samples provided by HPLC are given in the Table 1. The calibration and validation linear regression equations for the individual components of silymarin complex and the correlation coefficients of the calibration equations (see Table 2) are similar to the results of Šustová

et al. 2007 (correlation coefficients 0.943 and 0.964) and the results indicate that our created models are suitable for the detection of silymarin complex. Also Koláčková and Šišperová (2013) obtained similar value of the correlation coefficient, 0.980. The value of the correlation coefficient for isosilybin A is lower (0.725), probably due to the low representation of the isomer and its probable dependencies on other isomers – isosilybin B, silybin A and silybin B. Because of the low content of this isomer, the effect of a decreased correlation coefficient to the determination of silymarin complex is smaller than would be total elimination of the isomer from the determination and is therefore suitable to be kept in the method as it is. Future addition of more standards may increase the value of this coefficient.

Bias, or the systematic error of calibration, is the difference between the average values predicted via NIR and the reference method values. If significantly different from zero, it indicates that the calibration is burdened with systematic errors. However, as apparent from Table 2, bias values are close to zero for all calibration equation (see Table 2, Figure 1–6).

Table 1 The range of values and average values of the samples used to create the calibration models

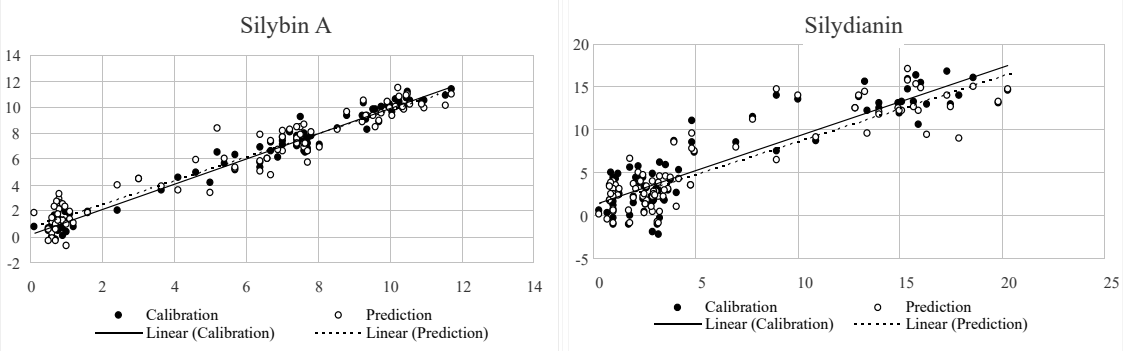
Silymarin complex	Range of values [g/kg]	Average value [g/kg]
Silybin A	0.10–11.73	5.63
Silybin B	0.10–17.73	8.72
Isosilybin A	1.00–3.90	2.81
sosilybin B	0.05–2.50	1.14
Silychristin	1.00–13.38	6.22
Silydianin	0.30–20.30	6.06

Table 2 The correlation coefficients r, calibration equations and bias of the calibration models for the determination of silymarin complex components

Silymarin complex	Correlation coefficient	Regression equation of the calibration	Regression equation of the prediction	Bias
Silybin A	0.9873	$y_1 = 0.9748x + 0.1417$	$y_2 = 0.9077x + 0.6574$	-0.0004
Silybin B	0.9685	$y_1 = 0.9379x + 0.5423$	$y_2 = 0.9235x + 0.6383$	0.0004
Isosilybin A	0.7248	$y_1 = 0.5246x + 1.3353$	$y_2 = 0.4992x + 1.3974$	-0.0004
Isosilybin B	0.9575	$y_1 = 0.9167x + 0.0948$	$y_2 = 0.8991x + 0.1437$	-0.0001
Silychristin	0.9487	$y_1 = 0.9000x + 0.6223$	$y_2 = 0.9191x + 0.6197$	0.0003
Silydianin	0.8986	$y_1 = 0.8074x + 1.1672$	$y_2 = 0.7631x + 1.1481$	0.0367

The calibration model for distinguishing between the milk thistle varieties Silyb and Mirel was created from the data on 23 samples using discriminant analysis. Distribution of samples is clearly seen in the Figure 7. This model allows to enter an unknown sample of one or the other variety, and can be extended to other varieties in the future.

Figures 1–6 The relation between calibration and prediction results of the content of individual sylimarin complex components (silybin A, silydianin, silychristin, isosilybin A, isosilybin B, silybin B)



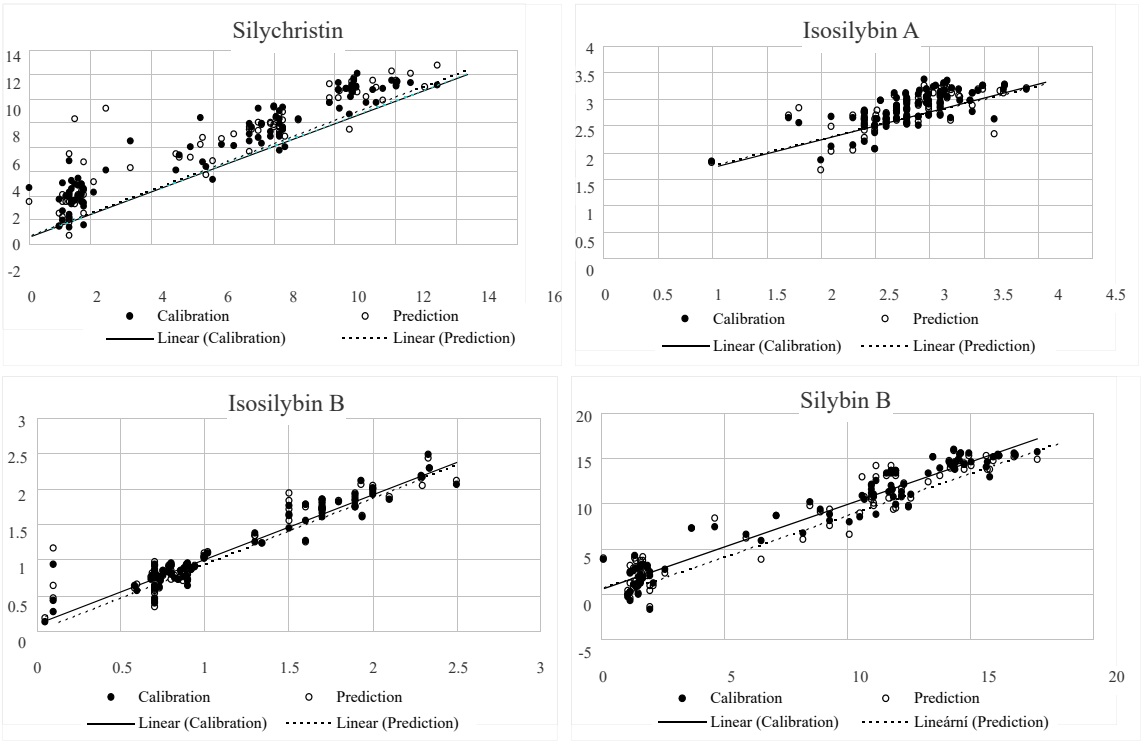
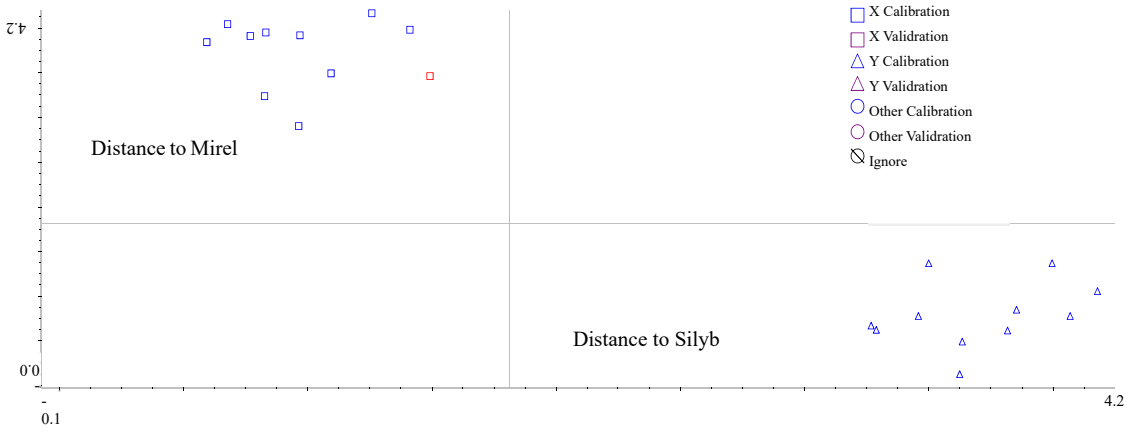


Figure 7 The calibration model for the classification of Silyb and Mirel varieties



CONCLUSION

The calibration models developed using the partial least squares method based on the given values for samples with known silymarin complex content and composition can be used for the determination of silybin A, silybin B, isosilybin B, silychristin and silydianin. To determine the entire silymarin complex, it is recommended to include also isosilybin A that has lower correlation coefficient value (0.7248). It was also proven that the calibration model for the determination of varieties (Silyb and Mirel) created by the means of discriminant analysis can be used to classify an unknown sample as one of the varieties. All created calibration models can be enlarged with additional calibration standards to enhance the stability of the calibration model (the calibration model predictive value).

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THE INFLUENCE OF CULTIVATION ENVIRONMENT TO THE PHENOLOGICAL PHASE, THE YIELD-PRODUCING ELEMENTS AND THE YIELD OF MILK THISTLE [*SILYBUM MARIANUM* L. (GAERTN.)]

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Abstract: The interest in milk thistle cultivation has increased in recent years due to higher demand for this product both in the pharmaceutical industry and in cosmetic or feed industry. Phenological characteristics of milk thistle related to the yield-producing elements are very important from the viewpoint of cultivation. In this paper, samples of the milk thistle variety Mirel grown in two different localities (Vanovice–Drválovice and Šumperk) in pilot conditions and in randomized block plots were investigated. Two variants of the experiment, control and treatment with the preparation Stomp 400 SC, the dose 2.5 l/ha, were monitored. Plant height and number of anthodia were found higher on the site Šumperk (178.1 to 183.5 cm and 9.67 to 10.6, resp.). As for the yield-producing elements, higher yield was found for samples from the location Šumperk (1.61 t/ha). Higher average yield was observed at both studied sites for the variants treated with Stomp 400 SC, 2.5 l/ha. However, the weight of thousand seeds was found to be higher in pilot conditions (Vanovice–Drválovice), the average value was 25.83 g.

Key Words: milk thistle, yield, achenes, plant height, anthodia

INTRODUCTION

Milk thistle [*Silybum marianum* (L.) Gaertn.] is a plant specie belonging to the family *Compositae,* newly *Asteraceae*. It is originally from the Mediterranean where it grows in the wild as a biennial herb, but culturally it is grown as an annual plant with characteristic morphology (Zhelev et al. 2014). The basic determination characteristic of milk thistle are distinctive, spiny pinnatilobate leaves (0.5–0.6 m long and 0.2–0.3 m wide) with white spots that forms a rich leaf rosette at the bottom of the stem; the stem is 0.4–2 m long, straight, the upper part is branched. Individual branches are ended by inflorescences, which are ovoid-shaped anthodia of purple colour with distinctive spines on their basis (Karkanis et al. 2011, Alemardan et al. 2013, Qavami et al. 2013, Nasrabadi et al. 2014). Milk thistle fruit is the achene 5–8 mm long and most often of brown to black colouring with a long white (or greyish-white) pappus. Average values of the weight of thousand seeds (WTS) are given to be 28–30 g. Dormancy is missing or very limited in milk thistle achenes (Karkanis et al. 2011, Alemardan et al. 2013). According to the latest Situation and perspective report (2014) of the Ministry of Agriculture, the area of 4.700 ha was sown with milk thistle in the Czech Republic with an average yield ranging from 0.5 to 0.8 t/ha. However, this figure is only approximate because milk thistle is not included in the survey of the Czech Statistical Office (Přibylová 2014).

Milk thistle is highly adaptable to different growing conditions (Karkanis et al. 2011). The life cycle of milk thistle can be divided into four main periods, namely: vegetative stage, stem elongation, flowering and maturing (Nasrabadi et al. 2014). The quality and quantity of the milk thistle plant production is affected by the process of growth, flowering and by transport of assimilated substances into seeds during maturation. All this is affected by both internal and external factors. The internal factors include circadian cycle, change of the growth phase and hormones. External factors are the day length, temperature, nutrition–which affects the reproductive development of the plant–and many others (Stancheva et al. 2008).

The fruits of milk thistle and the silymarin complex they contain have been already used for more than 2000 years in folk medicine to treat damage and disorders of the liver and gallbladder, including hepatitis, cirrhosis and liver protection in cases of poisoning by both chemical and environmental toxins (Flora et al. 1998, Křížová et al. 2011).

MATERIAL AND METHODS

Characteristics of the localities, experiments and samples

Milk thistle experiment plots were founded in two locations with different environmental conditions; in the company site of Agritec Šumperk (49°57′55.08″ N, 16°58′14.39″ E) at the altitude 330 m. above the sea level, where the growth was arranged as randomised blocks, and in the site of Agrospol a.d. Knínice in the township Vanovice–Drválovice (49°33′58″ N, 16°39′4″ E) at the altitude 430 m. above the sea level as a pilot experiment. The variety Mirel was sown in both localities. This variety was chosen because it is available to the growers, since milk thistle isn't a part of the National Listing of Plant Varieties. The course of individual selected phenologic phases is recorded in the Table 1.

In both localities, both the control variant (without treatment) and the variant treated with a pre-emergent application of a herbicide preparation Stomp 400 SC at the dose of 2.5 l/ha were founded. The number of plants was assessed at the stage of germination–4 true leaves.

Gathering of whole plants took place in Vanovice–Drválovice on 27 July 2016. Five plants of each variant of the experiment (treated/untreated) were collected in three repetitions. In Šumperk, the samples were taken on 4 August 2016 and 5 plants from each experimental variant (treated/untreated) were gathered here in 4 repetitions. The investigated characteristics were following: plant height, number of anthodia, number of ripe anthodia, unripe anthodia and flowering anthodia. As for the yield–producing elements, the harvest yield per unit area was evaluated after the harvest.

Table 1 The selected phonologic phases of milk thistle cultivated on two locations (Šumperk, Vanovice–Drválovice)

	Drválovice	Šumperk
Sowing	16. 4. 2016	21. 4. 2016
Emerging	5. 5. 2016	12. 5. 2016
Leaf rosette	3. 6. 2016	15. 6. 2016
Beginning of the extensive growth	20. 6. 2016	30. 6. 2016
Beginning of the ripening	20. 7. 2016	4. 8. 2016
Harvest ripeness	5. 8. 2016	29. 8. 2016

Statistics

The results were statistically analysed by the variance analysis (ANOVA) and simple correlation methods using STATISTICA (data analysis software system), version 12 by StatSoft, Inc. (2013), www.statsoft.com. The lowest significant difference (LSD) for the achene yield and other features was computed based on Fisher's test at a significance level of $P = 0.05$.

RESULTS AND DISCUSSION

The evaluation of milk thistle phenologic characteristics

Gathering of whole plants in both localities (Vanovice–Drválovice, Šumperk) for the determination of selected phenologic characteristic (plant height, number of anthodia, number of ripe anthodia, unripe anthodia and flowering anthodia) was performed before harvest. The analysis of variance in Table 2 shows a statistically very highly significant effect of the treatment variant, locality and mutual interaction of both factors to the plant height. At the same time, a statistically very highly

significant effect of the locality to the number of unripe anthodia was observed. The locality statistically influenced also the number of milk thistle anthodia.

Table 2 The analysis of variance for the monitoring of milk thistle phenologic characteristics

Source of variance	d.f.	Plant height	Anthodia	Ripe	Unripe	Flowering
		MS				
Variant	1	3861***	1.719	0.6857	4.8762	5.6679
Locality	1	17236***	70.876*	6.5190	86.7857***	1.8107
Variant*Locality	1	7175***	6.519	0.0000	0.0762	3.0964
Error	66	168	15.328	2.5965	5.1056	6.1144

*Note: * - $p \leq 0.05$; ** - $p \leq 0.01$; *** - $p \leq 0.001$; [g/kg]; (plant height, number of anthodia, number of ripe anthodia, unripe anthodia and flowering anthodia)*

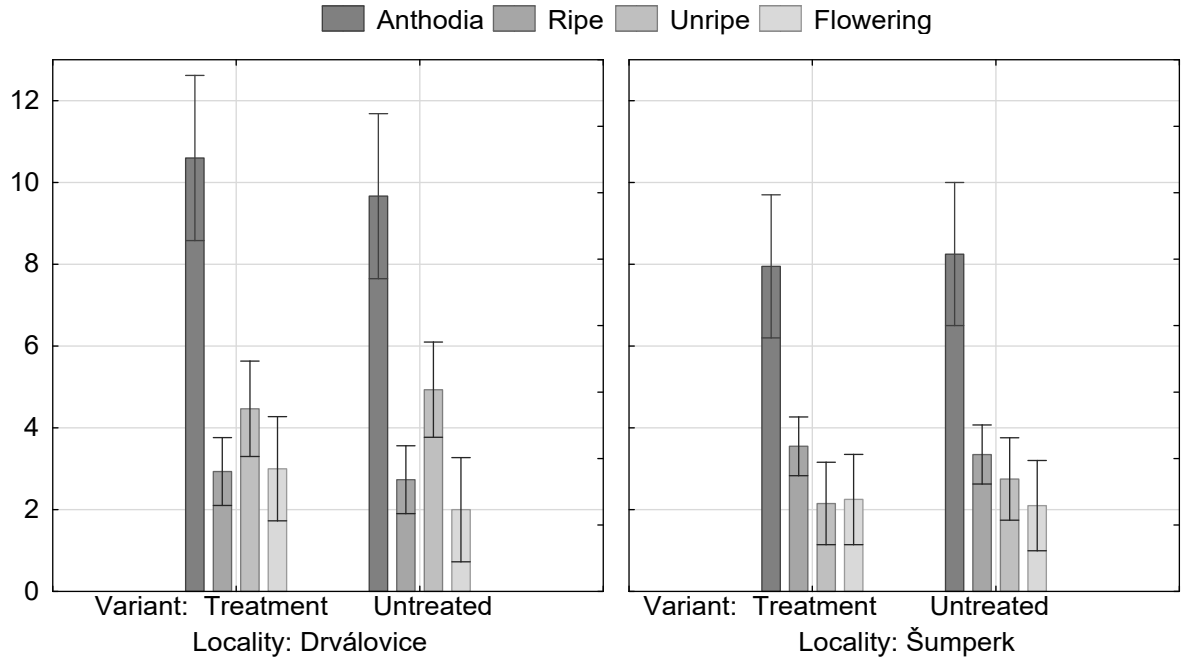
The plant height was studied in the stands at various locations and for samples gained using different methods of cultivation (see Table 3). It was found out that the plant height is statistically significantly higher for crops from Šumperk, both for the treated and the untreated variants of experiment (183.5 cm and 178.1 cm, resp.). No statistically significant differences were found for the number of anthodia, number of ripe anthodia and number of flowering anthodia, although in average a higher number of anthodia, number of ripe anthodia and number of flowering anthodia was observed in randomised block sites in Šumperk (see Table 3, see Figure 1). However, statistically significant differences were found between the sites in the number of unripe anthodia. The average number of unripe anthodia was more than twice higher in the samples from the locality Šumperk.

Table 3 Average values of milk thistle phenologic characteristics

Locality	Variant	Plant height	Anthodia	Ripe	Unripe	Flowering
Drválovice	Untreated	166.8 b	8.25 a	2.93 a	2.75 a	2.10 a
	Treatment	131.3 a	7.95 a	2.73 a	2.15 a	2.00 a
Šumperk	Untreated	178.1 c	9.67 a	3.35 a	4.47 b	2.25 a
	Treatment	183.5 c	10.60 a	3.55 a	4.93 b	3.00 a

Note: Average values marked with different letters in columns vary on a statistically significant level at P = 0.05; [g/kg]; (plant height, number of anthodia, number of ripe anthodia, unripe anthodia and flowering anthodia)

Figure 1 Average values of monitored phenologic characteristics of milk thistle cultivated in two variants of treatment in different localities



Evaluation of milk thistle yield-producing elements

The obtained results indicate that the average yields were found higher in the growths sprayed pre-emergently (see Table 4). The experiment in the locality Vanovice–Drválovice gave the average yield of 1.07 t/ha from the untreated variant, while the average yield of the treated variant was higher by 0.24 t/ha. The average yield in Šumperk from the untreated variant was 1.44 t/ha and similar to Vanovice–Drválovice the average yield of the treated blocks was higher by 0.34 t/ha.

Table 4 The average yield of milk thistle and the weight of thousand seeds (WTS) in both monitored localities

Locality	Variant	Yield [t/ha]	WTS [g]
Drválovice	Untreated	1.07	25.52
	Treatment	1.31	26.13
Šumperk	Untreated	1.44	25.33
	Treatment	1.78	25.03

However, the weight of thousand seeds was higher from the experiments in the Vanovice–Drválovice locality. The untreated variant had the WTS 25.52 g, the treated growth shown higher WTS 26.13 g. Lower average WTS was found for the experiments in the Šumperk locality; untreated milk thistle blocks had average WTS of 25.33 g while the average WTS from the treated blocks was found to be 25.03 g.

The plant height was studied as a phenological characteristic also by Andrzejewska et al. 2011; the average plant height during the years 2004–2006 was found to be 96.7 cm at the sowing density of the growth 12 kg/ha. Thus, the plant height not only from the Vanovice–Drválovice locality, but also from Šumperk (164.93 cm) was higher at the sowing density of the growth 9 kg/ha.

Stancheva et al. (2008) also studied the milk thistle growing in randomised blocks during the years 2004–2006 and tested various types of fertilisation. Phenological observation were performed in the blocks for the characterisation of the number of not yet flowering, flowering and ripe anthodia; results were given only in graphic form. The plant height in the experiments of Stancheva et al. (2008) was found to vary in the range from 66.7 ± 4.5 cm to 78.6 ± 5.0 cm. Thus, even in comparison with this authors was the height of our monitored plants significantly higher (131.3–183.5 cm). The number of anthodia per m^2 in the experiments of Stancheva et al. (2008) during the three years period varied in average in the range of 31.70–48.75 and according to the experimental methodology there were 5 plants per m^2. It means that the number of anthodia per one plant ranged from 6.34 to 9.75 and that are values comparable with the Mirel variety.

Different milk thistle genotypes cultivated in Iran were investigated by Shokrpour et al. (2011). This authors monitored 25 varieties. The WTS of studied genotypes varied in the range of 15.13–22.74 g. Compared to the Mirel variety we can say that this variety belongs to the genotypes with higher WTS. The height of the plants grown in Iran was found to be in the range 129.3 to 166.5 cm. The variety Mirel had grown to comparable height in various cultivation conditions, in randomised growths in Šumperk up to 183.5 cm. The yields of monitored genotypes were higher in some cases, but only for 3 from 25 varieties, namely the Tatar variety with 2.42 t/ha, Parsabad variety with 2.24 t/ha and Ramhormoz variety with 1.87 t/ha. The yield of other genotypes varied in the range of 0.89–1.79 t/ha. The yield of the Mirel variety was rather high in comparison with genotypes studied by Shokrpour et al. (2011).

CONCLUSION

The aim of this work was to determine of difference of phenologic characteristic and yield-producting elements of Mirel variety sown in two different localities (Vanovice–Drválovice; Šumperk) with different environmental conditions and experiment design (pilot conditions; randomised blocks; untreated; treatment). Milk thistle is known as a highly adaptable plant and this work suggests that the locality, growth treatment and also the interaction of both factors had statistically very highly significant impact on the plant height. The site had very highly statistically significant effect on the number of unripe anthodia.

The results indicate that the yields were higher in Šumperk (1.61 t/ha), the lover altitude locality. In both localities, higher average yield was obtained from the variant treated with the preparation Stomp 400 SC. However, the WTS was found to be higher in Vanovice–Drválovice, the average value was 25.83 g.

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THE VARIABILITY OF CONTAINED COMPOUNDS IN SELECTED MILK THISTLE [*SILYBUM MARIANUM* L. (GAERTN.)] VARIETIES CULTIVATED IN 2010–2015

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Abstract: The aim of this work was to demonstrate the variability of silymarin complex isolated from milk thistle [*Silybum marianum* (L.) Gaertn.] achenes. The silymarin complex was determined in 63 different samples of two home-grown varieties (Silyb and Mirel) originating from various environmental conditions and different years of harvest. The samples were analysed by the means of a reference method using HPLC (high performance liquid chromatography). The obtained results indicate that the year of harvest had statistically significant influence to both the amount and the composition of silymarin complex in the samples of the Mirel variety. The situation was similar for the samples of the Silyb variety, except of the isosilybin B component.

Key Words: silymarin, quality, flavonolignans, milk thistle

INTRODUCTION

The silymarin complex is a group of flavonolignan compounds contained in the pericarp and seed of milk thistle [*Silybum marianum* (L.) Gaertn.] achenes. It contains ca. 70–80% of silymarin flavonolignans and ca. 20–30% of chemically non-specified compounds, mostly polymeric and oxidised polyphenolic compounds (Gažák et al. 2007, Andrzejewska et al. 2011). According to the Pharmacopea Bohemica (2009) the official drug is a ripe fruit without pappus that have a minimal silymarin content of 1.5%, expressed as silybinin ($C_{25}H_{22}O_{10}$; M_r 482.4). The achenes usually contain 1–3% of silymarin in the dry weight, but the silymarin content can be also more than 8% (Karkanis et al. 2011). The base components of the silymarin complex are silybin A, silybin B, isosilybin A, isosilybin B, silychristin, silydianin and the flavonoid taxifolin (AbouZid 2012). According to Šeršeň et al. (2006) the silymarin complex has following distribution of individual components: 36.3% silybin, 15.9% silychristin, 5.9% silydianin, 5.1% isosilybin and 1.9% taxifolin. According to Nasrabadi et al. (2014) whole plant can be used for medicinal purposes (for phytopharmacy), but it is the achenes that have the highest content of active compounds.

Milk thistle has been known as a medicinal plant since the ancient times; 2000 years ago it was used as a treatment for liver dysfunctions. Nowadays it is a part of preparations for the treatment of diseases and disorders of liver, gallbladder and spleen, including in particular viral hepatitis (B, C), cirrhosis, hepatitis, gall colic. It also serves as protection against damage caused by chemical toxins and environmental pollutants like snake bites, insect bites, swallowed poisonous mushrooms and especially against liver damage caused by alcohol (Gažák et al. 2007, Kroll et al. 2007, Cardile et al. 2013). The basic effect of silybin on the human body is its ability to destroy free radicals and stabilize the cell walls, i.e. the cytoprotective effect, but it has also chemoprotective effects which inhibit carcinogenic effects of many chemicals. Silymarin complex has anti-allergic and anti-inflammatory effects, lowers the concentration of cholesterol in blood, it has anticancer effect, especially in the case of prostate cancer, supports and protects the liver during the treatment of HIV, helps against stress-induced gastric ulcers etc. (Flora et al. 1998, Šeršeň et al. 2006, Gažák et al. 2007, Stancheva et al. 2008, Cacho et al. 2013).

MATERIAL AND METHODS

Plant material sources

In the framework of this research, 63 samples of milk thistle achenes of two varieties – Silyb and Mirel – cultivated in the Czech Republic were investigated. The samples were grown in different provenances and years of harvest and were provided by growers and research organizations. Samples of the Mirel variety come from the harvest years 2011 and 2013–2015 and total 27 samples were evaluated. Samples of the Silyb variety come from the harvest years 2010–2014 and the evaluation of 36 samples was performed and included to the paper.

The silymarin complex and its basic components (silychristin, silydianin, silybin A, silybin B, isosilybin A, isosilybin B) were determined by the reference method.

The reference method for the determination of contained compound

For the sample processing, 20 mg of ground seeds was weighted and homogenised by the means of mortar and pestle with the addition of 0.5 ml isooctane and 0.5 ml methanol. 1.5 ml methanol and 1 ml isooctane were added to the sample after the homogenisation and 2 ml aliquot was centrifuged for 5 minutes at 14 000 rpm. 0.75 ml of lower phase was taken from each sample and analysed by the means of HPLC.

The analysis was performed by the means of the liquid chromatograph Dionex Ultimate 3000. The silymarin complex was separated using the Hypersil GOLD column 150 × 4.6 mm at 30 °C. 5 µl of the sample was injected to the column. The analysis was performed at the mobile phase flow rate 1 ml/min and the UV detection at 288 nm. The analytes were eluated at isocratic conditions with 65% of mobile phase A (0.1% formic acid) and 35% of mobile phase B (100% methanol). Time of one analysis was 45 minutes.

RESULTS AND DISCUSSION

The silymarin complex in milk thistle variety Mirel

27 samples of achenes of milk thistle variety Mirel of different provenances from the years of harvest 2011 and 2013–2015 were investigated.

Table 1 Variance analysis for the contained compounds of milk thistle variety Mirel in monitored harvest years 2011 and 2013–2015

Source of variance	d.f.	Silychristin	Silydianin	Silybin A	Silybin B	Isosilybin A	Isosilybin B
		MS					
Year	3	8.182***	0.7367***	5.368***	15.23***	0.5286***	0.089***
Error	80	0.696	0.1458	0.499	1.26	0.0684	0.005

*Note: * - $p \leq 0.05$; ** - $p \leq 0.01$; *** - $p \leq 0.001$; [g/kg]*

The analysis of variance in Table 1 shows a statistically very highly significant effect of the harvest year on the contents of all monitored components of the silymarin complex in the Mirel variety samples.

Table 2 The variability of contained compounds of milk thistle variety Mirel (g/kg) in monitored harvest years 2011 and 2013–2015

Year	Silychristin	Silydianin	Silybin A	Silybin B	Isosilybin A	Isosilybin B
2011	9.05 a	3.23 ab	8.20 a	12.19 a	2.79 a	0.65 a
2013	10.45 b	3.39 b	9.45 b	14.21 b	3.20 b	0.71 b
2014	10.25 b	2.98 a	9.52 b	14.10 b	3.06 b	0.70 ab
2015	10.78 b	3.08 a	9.39 b	14.47 b	3.15 b	0.81 c

Note: Average values marked with different letters in columns vary on a statistically significant level at p=0.05; [g/kg]

The samples of milk thistle variety Mirel were monitored in harvest years 2011 and 2013–2015. The lowest content of the flavonolignan silychristin that is a part of silymarin complex was found out in the harvest year 2011 (9.05 g/kg). The average content of silychristin in the year 2011 was statistically

significantly different from the samples investigated in harvest years 2013–2015 (10.45 g/kg, 10.25 g/kg and 10.78 g/kg, resp.). The lowest content of the silydianin component was found in the samples from the harvest year 2014 (2.98 g/kg), but the values were not statistically significantly different from the samples from the harvest year 2015 (3.08 g/kg). On the other hand, the highest content of silydianin was found in the samples from the harvest year 2013 (3.39 g/kg). In the harvest year 2011 the lowest content of not only the silychristin component, but also of silybin A (8.20 g/kg), silybin B (12.19 g/kg), isosilybin A (2.79 g/kg) and isosilybin B (0.65 g/kg) was found out in the comparison with other monitored years of harvest. The highest content of silybin A was found in the harvest year 2014 (9.52 g/kg), but the values from these samples were not statistically significantly different from the samples taken in the harvest year 2013 (9.45 g/kg) and 2015 (9.39 g/kg). The content of silybin B, another component of the silymarin complex, was found higher in the years 2013–2015, in the range of 14.10–14.47 g/kg, in comparison with above-given harvest year 2011. The highest content of isosilybin A was found in the harvest year 2013 (3.20 g/kg).

The silymarin complex in milk thistle variety Silyb

The samples of the Silyb variety were monitored in the harvest years 2010–2014. 36 samples of milk thistle achenes of the variety Silyb were used for the analyse.

Table 3 Variance analysis for the contained compounds of milk thistle variety Silyb in monitored harvest years 2010–2014

Source of variance	d.f.	Silychristin	Silydianin	Silybin A	Silybin B	Isosilybin A	Isosilybin B
		MS					
Year	4	8.339***	1.8880***	14.064***	14.250***	1.3354***	0.24320*
Error	113	0.487	0.0673	0.449	0.892	0.0586	0.09977

*Note: * - $p \leq 0.05$; ** - $p \leq 0.01$; *** - $p \leq 0.001$; [g/kg]*

As shown in the Table 3, the components of the silymarin complex in monitored samples of the Silyb variety were very highly significantly influenced by the year of harvest, except of the isosilybin B that was only influenced.

Table 4 The variability of contained compounds of milk thistle variety Silyb in monitored harvest years 2010–2014

Year	Silychristin	Silydianin	Silybin A	Silybin B	Isosilybin A	Isosilybin B
2010	9.51 a	2.59 a	8.32 a	12.87 a	2.80 a	0.55 a
2011	10.34 b	2.91 b	9.13 b	13.82 b	3.08 b	0.82 b
2012	10.21 abc	2.74 abcd	8.89 abc	14.00 abc	2.97 ab	0.85 ab
2013	10.88 c	3.06 d	10.02 d	14.67 c	3.34 c	0.66 ab
2014	10.10 b	2.42 c	9.84 cd	14.08 b	3.02 b	0.70 ab

Note: Average values marked with different letters in columns vary on a statistically significant level at p=0.05; [g/kg]

The content of silychristin found in the samples of the Silyb variety investigated in the years 2010–2014 varied in the range of 9.51–10.88 g/kg (see Table 4). The contents of silydianin, silybin A, silybin B and isosilybin A components were found to be statistically significantly highest in the year 2013 (3.06 g/kg, 10.02 g/kg, 14.67 g/kg and 3.34 g/kg, resp.). Thus, the obtained results indicate that the harvest year 2013 was the most favourable for investigated samples of the Silyb variety. On the other hand, the lowest values were found for the harvest year 2010.

The silymarin complex of milk thistle varieties Mirel and Silyb in comparable harvest years 2011, 2013 and 2014

Milk thistle samples of selected varieties Mirel and Silyb could be compared in harvest years 2011, 2013 and 2014. As shown in Table 5, the components silychristin, silydianin and silybin A+B were very highly significantly influenced both by the variety and by the year of harvest, but only silychristin and silybin B were very highly significantly influenced by the mutual interaction of both investigated factors.

Table 5 Variance analysis for the contained compounds of selected milk thistle varieties (Mirel and Silyb) in monitored harvest years 2011, 2013 and 2014

Source of variance	d.f.	Silychristin	Silydianin	Silybin A	Silybin B	Isosilybin A	Isosilybin B
		MS					
Variety	1	8.20***	5.136***	11.23***	14.02***	0.536**	0.047
Year	2	10.99***	3.263***	13.63***	23.15***	1.503***	0.031
Variety * Year	2	4.45***	0.230	0.83	6.23***	0.224*	0.136
Error	146	0.51	0.109	0.43	0.94	0.057	0.078

*Note: * - $p \leq 0.05$; ** - $p \leq 0.01$; *** - $p \leq 0.001$; [g/kg]*

Table 6 The variability of contained compounds of selected milk thistle varieties (Mirel and Silyb) in monitored harvest years 2011, 2013 and 2014

Variety	Year	Silychristin	Silydianin	Silybin A	Silybin B	Isosilybin A	Isosilybin B
Silyb	2011	10.34 b	2.91 b	9.13 b	13.82 b	3.08 bc	0.82 b
	2013	10.88 c	3.06 bc	10.02 d	14.67 c	3.34 d	0.66 ab
	2014	10.10 b	2.42 a	9.84 cd	14.08 b	3.02 b	0.70 ab
	Average	**10.44**	**2.80**	**9.66**	**14.19**	**3.15**	**0.72**
Mirel	2011	9.05 a	3.23 cd	8.20 a	12.19 a	2.79 a	0.65 a
	2013	10.45 b	3.39 d	9.45 b	14.21 b	3.20 c	0.71 ab
	2014	10.27 b	3.00 bc	9.54 bc	14.14 bc	3.06 b	0.70 ab
	Average	**9.93**	**3.21**	**9.06**	**13.51**	**3.02**	**0.69**

Note: Average values marked with different letters in columns vary on a statistically significant level at p=0.05; [g/kg]

The comparison of Silyb and Mirel varieties in the Table 6 shows that the most represented component of the silymarin complex was silybin B with the content in the range of 12.19–14.67 g/kg. Both varieties gave the lowest content of silybin B in the harvest year 2011. The second most represented component was silychristin that was in average found more in the samples of the Silyb variety. The Mirel variety on the other hand showed higher content of the silydianin component.

The average content of individual components of the silymarin complex in the investigated samples is in good accordance with the results of Andrzejewska et al. (2011) that states the content of silychristin to be in the range of 0.58–0.88%, silydianin of 0.27–0.37%, silybinin A+B of 0.6–0.97% and isosilybin A+B of 0.21–0.28%. Thus, it is obvious that the monitored samples of Silyb and Mirel varieties cultivated and filed for legal protection in the Czech Republic are comparable with foreign varieties and in some harvest years can give even higher than average values of the silymarin complex.

Stancheva et al. (2008) states the content of silydianin+silychristin to vary in the range of 0.74–0.95% and silybin+isolybinin in the range of 1.09–1.45%.

Results obtained in this work give the average content of silydianin+silychristin in the range of 1.23–1.39% and that of silybin+isolybinin in the range of 2.38–2.87%. Thus, the content of silydianin+silychristin is comparable. The difference is evident in the content of silydianin+silychristin where higher values were found in the monitored samples of the Silyb and Mirel varieties; it could be caused by the oiliness of the achenes.

CONCLUSION

The silymarin complex was investigated in two different milk thistle varieties (Mirel and Silyb), which were grown in different provenances and in the harvest years 2010–2015. The average content of individual components of the silymarin complex in the studied samples was very highly statistically significantly affected by the cultivation year. Most represented ingredient of the silymarin complex was silybin B, its content in compared varieties ranged from 12.19 to 14.67 g/kg.

These results were compared with data published by several other authors. The comparison of the results shows that in some cases the silybin content was higher in monitored varieties. From that it can be concluded that the investigated varieties (Mirel, Silyb) are a high level one in terms of the quality of silymarin complex.

Analyses by HPLC reference method are quite time-consuming and costly. Therefore, it would be a benefit to verify and expand the possibilities of using the FT-NIR spectroscopy calibration model for rapid, non-destructive detection of silymarin complex as well as other important substances in milk thistle (oiliness, vitamin E, oleic acid and linoleic acid, etc.).

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GROWING WINTER WHEAT VARIETIES AND THEIR MIXTURES ON DIFFERENT SITES IN TERMS OF YIELDS, QUALITY, AND ECONOMY

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Abstract: Winter wheat is currently the most important cereal in the Czech Republic. Growers are trying to achieve high yields in bread quality. Based on these requirements, the currently cultivated varieties in the Czech Republic include those with different quality and yield potential. One approach that is being experimentally verified in the field is the planting of stands of two varieties cultivated on a single plot of land. We can speak about mixtures of two varieties, which should complement each other in their properties so as to achieve an increase in grain yield in the conditions of climate change. The aim of the field trial was to evaluate the varieties of winter wheat (Bohemia and Tobak), grown in mixture in terms of grain yield, quality (grain bulk density, protein content) and the economy (using gross margin) compared to traditional planting of one variety at two different sites (South Moravia, Czech Republic) in the year 2014/2015 and 2015/2016. The impact of year and location was found out. The advantage growing of winter wheat varieties in mixtures was found out in year 2015 at location with heavy soil, where better values of all assessed parameters were obtained in comparison with average of monoculture of two varieties. The yield was increased 0.2 t/ha and gross margin higher 489 CZK per hectare. The results from year 2016 showed, that year in general is one the most unpredictable factor, which can cause different results. Also location, in our case heavy and sandy soil can play important role in growing of varieties and bringing various results. The yield decrease of Tobak (to the lower level than Bohemia) in location of sandy soil (Žabčice-Písky) is typical example of unsuitable variety to the condition with higher environmental stress.

Key Words: winter wheat, varieties, mixtures, yield, grain quality

INTRODUCTION

The significance of bread wheat (*Triticum aestivum* L.) in the Czech Republic is the result of its dominant position in the structure of cereals and other crops grown on arable land, where it occupies about 30% of the area. Yet there are interannual variations in sown areas and due to fluctuations of annual conditions, variations of total grain volume as well (Prugar 2008). The aim of agricultural practice is to achieve bread quality, which is then implemented at a higher purchase price, on a greater part of sowing areas of wheat. When not used in the food or feed industries, there is a subsequent effort to find other applications of wheat, for example in non-food use, such as in the production of bioethanol.

The winter wheat form in the Czech Republic is a key cereal and its production is essential for the formation of optimal proportions between crop and livestock production and food supply to the population. Winter wheat has among all kinds of cereals the best preconditions for intensification of production. A favourable level of growing profitability is achieved primarily in field conditions of sugar beet and corn production areas (Hůla and Procházková 2008).

In recent years, there is expansion of requirements from both farmers and processors (millers) to increase yields and quality of winter wheat in bread quality. Especially in dry areas, there are increasing efforts to cultivate suitable varieties of winter wheat, which should have sufficient yield potential, but also a reasonable level of tolerance to drought, while achieving adequate quality of production. Particularly for wheat, alternative procedures for establishing growth with more varieties are being verified. The idea of using mixtures of varieties for stand planting is not new, since as early as in 1970,

mixtures of varieties, especially for wheat and barley have been used in the UK. Unfortunately, the acreage of these mixtures of cereals was not very big, because it strongly discouraged brewers and millers who did not want to purchase mixed varieties, despite the fact that these mixtures may have additional quality characteristics. But despite this negative attitude of processors, there was a gradual, further testing and cultivation of various mixtures of wheat varieties. An example might be the year 1981, when one of the tested mixtures of wheat varieties achieved a world record in yield (13.99 t/ha) in the mixture grown in Scotland (Finckh et al. 2000).

For cereal crops such as wheat, some mixtures of varieties were partly grown on small plots in several European countries. In recent years, there has been a growing interest in the concept of mixtures of crops and varieties in many countries, which led to the founding of COST (European Cooperation in Science and Technology), working group for the protection of varieties of cereals grown in mixtures (Finckh et al. 2000). The main advantages include particularly the stabilization of yield in different environments, increased tolerance to fungal pathogens, as well as restricting the occurrence and spread of new kinds of important pathogens (Wolfe 1985). Another advantage that should not be ignored is environmental friendliness. Mixtures create a greater diversity compared to varieties grown in a monoculture (Procházka 2015). Moreover, the parallel sowing of two varieties of winter wheat, which complement each other in their properties, such as high and lower resistance to frost, drought, diseases, lodging, with excellent and less stable values of grain quality, increases the interannual stability of yields and quality of production (FARMET 2014).

Despite the advantages mentioned above, the cultivation of mixtures of varieties is connected with some problems. The selection of suitable components for the mixture is essential, as it requires knowledge of the varieties in terms of earliness, and the essence of the foundation of yield or quality. These facts are important for proper planting, growing and harvesting. Mundt (2002) states that the practical difficulties associated with the mixtures of varieties were often overestimated and it is expected that mixtures are likely to play an increasingly important role.

MATERIAL AND METHODS

For the field trial, currently widely grown varieties of winter wheat were selected, namely Bohemia and Tobak. The Tobak variety is characterized by high yield, while Bohemia has a stable quality due to the higher content of proteins in grain. Furthermore, we can emphasize the tolerance to infection by yellow rust and powdery mildew on the leaf in the Tobak variety. In contrast, the Bohemia variety has an increased grain bulk density, thousand grain weight (TGW) and tolerance to freezing.

The field trial was set up on the experimental field station of the Mendel University in Žabčice, which is 25 km south of the town of Brno. The station is located in a maize production area, at an altitude of 179 m. The average annual air temperature reaches 9.2 °C, while the thirty-year average annual rainfall is 480 mm. This site is thus among the warmest and driest areas in the Czech Republic, with more frequent occurrence of prolonged drought in recent years. The trial was set up on two sites with different soil properties. The first site Obora has a fluvisol soil type, with higher content of clay particles and with good access to groundwater level, which is influenced by the fluctuating level of the Svratka River that flows near the test site. In contrast, the second station at Písky is a site with light sandy soils, where during the growing season often comes to negative phenomena of drought due to lack of water available for plants.

Planting of winter wheat was carried out by small-plot seeder, made by the FARMET company, which allows for precise seeding. In the case of planting a stand with a mixture of different varieties, the varieties were sown into odd and even lines. At both sites and in both years, ploughing and seedbed preparation of soil were carried out. Sowing of both varieties and mixture of varieties in the 2014/2015 year at the Obora site took place on October 15th, 2014. In the year 2015/2016, the sowing took place at a later date, namely on November 2nd and 3rd, 2015. In both years, there has been sowing to depths of 3 cm in seed rates of 3 MGS/ha and 4 MGS/ha (millions of germinating seeds per hectare) in 4 repetitions. At the Žabčice-Obora site, the total applied dose of nitrogen was 160 kg/ha N, but at the Žabčice-Písky site it was 80 kg/ha N, corresponding with lower yield level of location. Other applications included phosphorous and potassium mineral fertilizers, 1x herbicide, 1x insecticide, 1x fungicide, and 1x growth regulator. The harvest at the Žabčice-Obora site in the year 2014/2015 took place on July 20th, 2015.

The harvest in the year 2015/2016 at the Žabčice-Písky site took place on July 11th, 2016 and at Žabčice-Obora site on July 25th, 2016. Grain harvest was done by a small combine machine SAMPO 2010. The achieved yields from the harvested plots (the size of 10.5 square meters) were recalculated per hectare. The results were statistically analysed using ANOVA (analysis of variance) using the statistical software Statistica and Tukey post-hoc test.

For the economic evaluation of two cultivated varieties and mixture of varieties of winter wheat, gross margin was used. Gross margin was calculated as the difference between the earned sales and incurred variable costs. Variable costs included the costs for seeds, mineral fertilizers, pesticides, and mechanized field work. Prices of inputs, such as seeds, fertilizers, and pesticides, were determined according to the price lists of the NAVOS company for 2015 and 2016, while the prices for mechanized field work were taken from the "Standards for agricultural and food production". Product price (grains of bread wheat) was determined according to data from the Czech Statistical Office (CSO) for the month of June 2015 and 2016. The later prices for the months of July or August, 2016, were at the time of preparation of this article not yet available on the CSO website. Therefore, we have used the prices for the month of June, which were published for both years.

The parameter of the protein content in grain was used as the criterion for calculating the average revenues for the individual variants for the winter wheat trial, to which the average purchase price (Table 1) was assigned, according to data from the Czech Statistical Office. Minimum content of proteins of 12.0 % was determined for the category of bread wheat**.** For wheat with a higher content of proteins an extra surcharge was added when for every percentage content of proteins above this limit, the price increased by CZK 200/t. The limit for food grade wheat in terms of bulk density was then set at 720 g/l.

Table 1 Purchase price (CZK/t) for the category of quality according to the content of proteins in grain

Content of proteins (%)	Purchase price (CZK/t) June 2015	Purchase price (CZK/t) June 2016
12.0	4 382	3 631

RESULTS AND DISCUSSION

Tables 2, 3, and 4 show the average results for four repetitions of the Bohemia and Tobak varieties as well as their mixtures with subsequent gross margin (GM) and statistical evaluation using ANOVA (analysis of variance) for both sites and years.

Tables 2 and 3 show that at Žabčice-Obora with heavy fluvisol soil type, at a seed rate of 4 MGS/ha, that a lower yield has been achieved than at 3 MGS/ha. The only exception was the mixture in the year 2014/2015, at a seed rate of 4 MGS/ha, which has achieved a higher yield by 0.41 t/ha. In the year 2014/2015, a statistically significant difference in the yield was observed not only in the varieties of Tobak and Bohemia, but also in mixtures with seed rate of 3 MGS/ha. In the following year, there was a statistically significant difference in the yields between the Tobak variety in both seed rates, as well as in the Bohemia variety at a seed rate of 4 MGS/ha. Furthermore, we can say that in the protein content in the year 2015/2016, there was no conclusive statistical difference between varieties and mixture. In contrast, in the year 2014/2015, there were statistically significant differences between the Tobak variety, at a seed rate of 3 MGS/ha, and the Bohemia variety at 4 MGS/ha. The content of protein content at the 12.0% limit was not reached only in the Tobak variety at 4 MGS/ha in the year 2014/2015, as it reached only 11.9%. The bulk density in the year 2014/2015, was no statistically significantly different when compared to the year 2015/2016 , which had a statistically significant difference between the Tobak variety in both seed rates, mixture with 4 MGS/ha, and the Bohemia variety with 3 MGS/ha. In the year 2015/2016, the limit of 720 g/l for grain bulk density was not reached in the Tobak variety in both seed rates. Values of bulk density were here only 719 g/l and 715 g/l. In the heavier fluvisol soil type in Žabčice-Obora, there were recorded differences between the years 2014/2015 and 2015/2016, between the average grain yields and protein content in both varieties and mixture. In general, the grain yield level at this location was from 10 to 13 t/ha, except for Bohemia variety in the year 2015/2016 and seed rate of 4 MGS/ha, where grain yield was only 9.82 t/ha. In the result of the economic evaluation, it meant a higher gross margin in the year 2014/2015 (in average 34 271 CZK/ha) in comparison with 19 089 CZK/ha in 2015/2016. It is caused mainly by higher yields in 2014/2015.

Table 2 Average values and their evaluation by Tukey post-hoc test for varieties/mixture at Žabčice-Obora for the year 2014/2015

Variety	Seed rate (MGS/ha)	Yield (t/ha)	Protein content (%)	Bulk density (g/l)	Gross margin - GM (CZK/ha)
Tobak	3	13.33 c	12.2 a	821 a	40 405
	4	12.03 c	11.9 ab	817 a	34 415
Average of variety Tobak		12.68	12.0	819	37 410
Bohemia	3	10.60 a	13.3 bc	810 a	30 562
	4	10.32 a	14.0 c	810 a	31 049
Average of variety Bohemia		10.46	13.7	810	30 806
Mixture (Bohemia + Tobak)	3	11.56 b	12.7 abc	817 a	32 649
	4	11.97 bc	13.2 ab	818 a	36 546
Average of mixture		11.77	13.0	818	34 597
Average of both varieties		11.57	12.9	815	34 108

Different letters indicate statistically significant difference at p <0.95.

Table 3 Average values of four repetitions and their evaluation by Tukey post-hoc test for varieties/mixture at Žabčice-Obora for the year 2015/2016

Variety	Seed rate (MGS/ha)	Yield (t/ha)	Protein content (%)	Bulk density (g/l)	Gross margin - GM (CZK/ha)
Tobak	3	11.55 c	13.0 a	719 a	23 219
	4	10.62 b	13.2 a	715 a	19 376
Average of variety Tobak		11.9	13.1	717	21 297
Bohemia	3	10.32 ab	13.3 a	760 c	18 505
	4	9.82 a	13.2 a	757 bc	16 310
Average of variety Bohemia		10.07	13.2	759	17 407
Mixture (Bohemia + Tobak)	3	10.52 ab	13.3 a	747 bc	19 274
	4	10.23 ab	13.0 a	743 b	17 853
Average of mixture		10.37	13.1	745	18 564
Average of both varieties		10.99	13.2	738	19 352

Different letters indicate statistically significant difference at p <0.95.

At the Žabčice-Písky site, with light sandy soil, there was a statistically significant difference in yields between the mixture at a seed rate of 3 MGS/ha and the two varieties of the same seed rate. The highest yield was achieved at a seed rate of 3 MGS/ha (except for a mixture of varieties, where the higher yield was achieved at a seed rate of 4 MGS/ha, by 0.46 t/ha (Table 4). For the content of N-substances, there was a statistically significant difference between the Tobak variety at a seed rate of 3 MGS/ha and the mixture at a seed rate of 4 MGS/ha. The 12.0% limit of protein content was achieved in both varieties and mixture in both seed rates. The values of N-substances were significantly higher than at Žabčice-Obora.

In terms of bulk density, the limit of 720 g/l was reached only in the Bohemia variety at a seed rate of 3 MGS/ha, when bulk density was 723 g/l. Statistically significant difference in terms of bulk density was detected at a seed rate of 4 MGS/ha for the Tobak variety, in the mixture at a seed rate of 3 MGS/ha, as well as in the Bohemia variety at seed rates of both 3 and 4 MGS/ha. The very low yield played a major role for the amount of economic evaluation (gross margin). In contrast, due to the high content of proteins, there was a higher purchase price per tonne, due to the increased surcharge for each per cent of content of proteins by CZK 200/t, which helped to increase the gross margin.

Table 4 Average values and their evaluation by Tukey post-hoc test for varieties/mixture at Žabčice-Písky for the year 2015/2016

Variety	Seed rate (MGS/ha)	Yield (t/ha)	Protein content (%)	Bulk density (g/l)	Gross margin - GM (CZK/ha)
Tobak	3	5.16 b	17.7 a	681 ab	14 435
	4	4.87 ab	18.5 ab	674 a	13 789
Average of variety Tobak		5.01	18.1	678	14 112
Bohemia	3	5.15 b	18.5 ab	723 d	15 422
	4	4.95 ab	18.5 ab	718 d	14 197
Average of variety Bohemia		5.05	18.5	720	14 809
Mixture (Bohemia + Tobak)	3	4.51 a	18.7 ab	700 c	12 330
	4	4.97 ab	19.2 b	690 bc	15 259
Average of mixture		4.74	18.9	695	13 794
Average of both varieties		5.03	18.3	699	14 461

Different letters indicate statistically significant difference at p <0.95.

The influence of the year was evident at the Obora site. In 2015, there were higher grain yields than in 2016, especially in the Tobak variety, which was associated with a decrease in protein content. In 2016, the amounts of protein content in all varieties were very similar, while differences were detected in bulk density, which showed the influence of varieties, where the Bohemia variety had the higher values. The use of mixture of varieties in 2015 had a positive effect by increasing the content of proteins in grain and in 2016 an increase in bulk density compared to the Tobak variety, which yielded a lower bread quality compared with the Bohemia variety.

The advantage growing of winter wheat varieties in mixtures was found out in year 2015 at location Žabčice-Obora, where better values of all assessed parameters were obtained (table 2; written in bold; yield, protein content, grain bulk density and gross margin). The results from this year are an example of benefits for mixtures, when we can increase yield in comparison with so called monoculture of two varieties. Similar conclusion described other authors, for instance Tilman (1996), Yachi and Loreau (1999). They conclude, that varietal mixtures enhanced spatial yield stability compared to the mean of the component monocultures, supporting the hypothesis that biodiversity increases ecological stability i.e. the ability of an ecological system to maintain or quickly regain productivity despite diverse environmental stresses. The positive impact of barley mixtures confirmed Newton et al. (2008), when grain consistency and grain quality has been shown to be equal to and even better than the sum of the mixture components.

The results from year 2016 showed, that year in general is one the most unpredictable factor, which can cause different results. Also location, in our case heavy and sandy soil can play important role in growing of varieties and bringing various results. The yield decrease of Tobak (to the lower level than Bohemia) in location of sandy soil (Žabčice-Písky) is typical example of unsuitable variety to the condition with higher environmental stress.

The potential to exploit ecological processes that generate beneficial plant–plant interactions therefore depends on the presence of suitable varieties as mixture components. Field trials are necessary for accurate mixture assessment as it is often difficult to predict the performance of a variety in mixture from its monoculture yield due to the complexity of ecological interactions taking place within the crop and the variability of field environments (Lopez and Mundt 2000, Mille et al. 2006).

CONCLUSION

The results suggest that growing a mixture of varieties on one plot can combine the advantages of varieties characterized by high yield with varieties characterized by high bread quality of winter wheat. In order to obtain generally valid conclusions, experience gained over several years is needed in field trials to verify the combinations of different varieties.

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ASSESSING THE IMPACT OF DROUGHT STRESS ON WINTER WHEAT CANOPY BY HERMES CROP GROWTH MODEL

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Abstract: The main aim of this study was evaluate a drought stress effect on winter wheat development, growth (leaf area index), soil moisture and yields. Simultaneously, the ability of Hermes crop growth model to simulate drought stress response was tested. The field trial was established at Domanínek station (Bystřice nad Pernštejnem district, Czech Republic) in 2014. Mobile rain-out shelters for precipitation reduction were installed on the plots of winter wheat in May 2015. Results of this study showed that model is able to reproduce well a soil moisture content and to certain extent the drought stress for grain yields of winter wheat. Using the rain-out shelters (from 19 May to harvest on 6 August 2015), real winter wheat yields were reduced by 1.7 t/ha. The model was able to estimate the average yield with a deviation of 0.15 t/ha (6%) for no stressed variant. Model underestimated the yields for sheltered variant with a difference 0.67 t/ha (71%) on average against observed yields.

Key Words: leaf area index, rain-out shelters, soil moisture, water balance, yields

INTRODUCTION

Winter wheat is the most grown cereal in the Czech Republic. In the years 2014–2015 wheat exceeded the area of 830 000 ha and it was produced 5 274 000 tons of grain yield (ČSÚ 2016, Mikulášová 2015). Drought that hit the Czech Republic in 2015 belong to the most serious historical drought episodes. The occurrence of more frequent droughts may become a major problem in the coming years (Daňhelka et al. 2015). If the temperature rises by 2 °C or more (above late 20th century levels), for the major crops is excepted adverse climate change which will have a negative impact on production increased annual yields variability in many areas (IPCC 2014).

There are a lot of crop growth models used to assessing the impact of a future climate change. Each the crop growth model is unique in architecture, complexity, algorithms and parameterization (Palosuo et al. 2011). Hermes crop growth model belong between wide used, easily accessible and well-documented crop growth simulation model (i.g. Palosuo et al. 2011). It is used for example to cropping systems, soil nitrogen dynamics, to estimate irrigation water demand and predicting yield response to nitrogen fertilization with satisfactory results (e.g. Salo et al. 2015, Graß et al. 2015, Hlavinka et al. 2015).

The main aim of this study was evaluate the drought stress effect on winter wheat using the Hermes crop growth model. Realistically measured parameters of development, growth, soil

moisture and yields were compared with the simulated results of model Hermes within sheltered and unsheltered variants.

MATERIAL AND METHODS

Field experiment

Field experiment was established at Domanínek experimental station (49°31'42"N, 16°14'13"E, altitude 560 m) in the season 2014–2015. Mean annual temperature is 7.2 °C and mean precipitation is 609.3 mm within period 1981–2010. This area is characterized by a low soil quality (the soil type dystric cambisol). Soil properties are shown in Table 1.

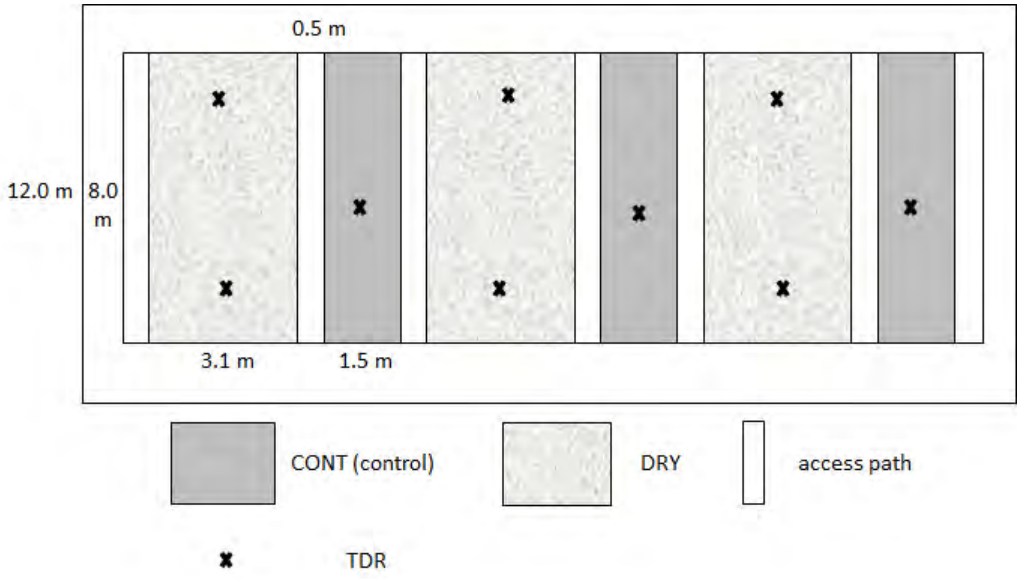
Table 1 Basic soil properties at the experimental site of Domanínek

Borderline [m]	WP [%]	Field water capacity [%]	Soil porosity [%]	Soil type	Soil texture		
					Clay [%]	Silt [%]	Sand [%]
0.0–0.3	8	24	44	Silty-loamy sand	8–17	40–50	33–52
0.3–0.4	6	23	39	Silty sand	0–8	25–40	52–75
0.4–0.5	6	23	39	Loamy sand	8–12	10–40	48–82
0.5–2	2	6	17	Loamy sand	8–12	10–40	48–82

Legend: WP – wilting point

The experiment with winter wheat variety Bohemia was set up in 2 variants on 30 September in 2014. The first variant was conducted under natural climatic conditions (abbreviated as CONT, plot size 1.5 × 8.0 m). In the second variant, the drought stress was induced using rain-out shelters (abbreviated as DRY, plot size 3.1 × 8.0 m). Mobile rain-out shelters (size 3.1 × 8.0 m) were installed on the plots of winter wheat on 19 May 2015 and were removed in the harvest day (6 August 2015). To production the shelters a corrugated material (Suntuf CS – clear polycarbonate with two-sided UV filter; trapezius 76/16, thickness 0.8 mm) was used. Each variant was repeated three times (Figure 1).

Figure 1 Field trial map with the position of rain-out shelters (DRY) and TDR sensors for soil moisture measurements



As a basal fertilization 20 kg/ha N (NPK) was applied before sowing (September 2014). 35 kg/ha N regenerative fertilization DASA (nitrogen fertilizer containing sulfur) and 25 kg/ha N regenerative fertilization LAV (ammonium nitrate with limestone) was applied in March 2015 and 60 kg/ha N

production fertilizer LAV was introduced into the soil in April 2015. CONT and DRY variants included the same dose of fertilizations.

The leaf area development (leaf area index - LAI) was measured by SunScan (Delta-T Devices, Cambridge, UK) at intervals of 6–21 days during June to August. In harvest parcels were installed TDR sensors (time domain reflectometry, CS 616, Campbell Scientific Inc., Shepshed, UK) to measure soil moisture content (depth 0.3 m) and two TDR sensors were always placed under each of the roofs and another (one sensor) was outside of the roof (control). See Figure 1.

Crop growth simulation model

Assessment of the drought stress was carried out by the Hermes crop growth model (e.g. Kersebaum 2008). It is a process-oriented model for estimating development and growth of the field crops, soil water balance and the dynamics of nitrogen for arable land. The benefit of using Hermes is the ability to work with a relatively small amount of input data sets that are ordinarily available at the farm level (Kersebaum 2011).

Crop growth is capped by water and nitrogen stress. Drought stress is indicated by the ratio of actual and potential transpiration. For this study was selected the Penman-Monteith approach to estimate reference evapotranspiration (Allen et al. 1998, Monteith 1965). Dynamics of soil water is derived from a simple capacity approach (Kersebaum 2011).

Input data sets are divided into three parts: daily weather data (average, minimum and maximum temperature, air humidity, wind speed, precipitation), soil properties (soil nitrogen content, soil moisture, field capacity, wilting point and porosity) and agrotechnical (management) data (tillage, pre-crop, fertilizing, sowing and harvesting). These data were obtained from Domanínek experimental station for the period 2013–2015.

RESULTS AND DISCUSSION

The total rainfall was 93 mm in the Domanínek experimental station from rain-out shelters installation to the harvest. These total rainfall was reduced under drought stress experiment.

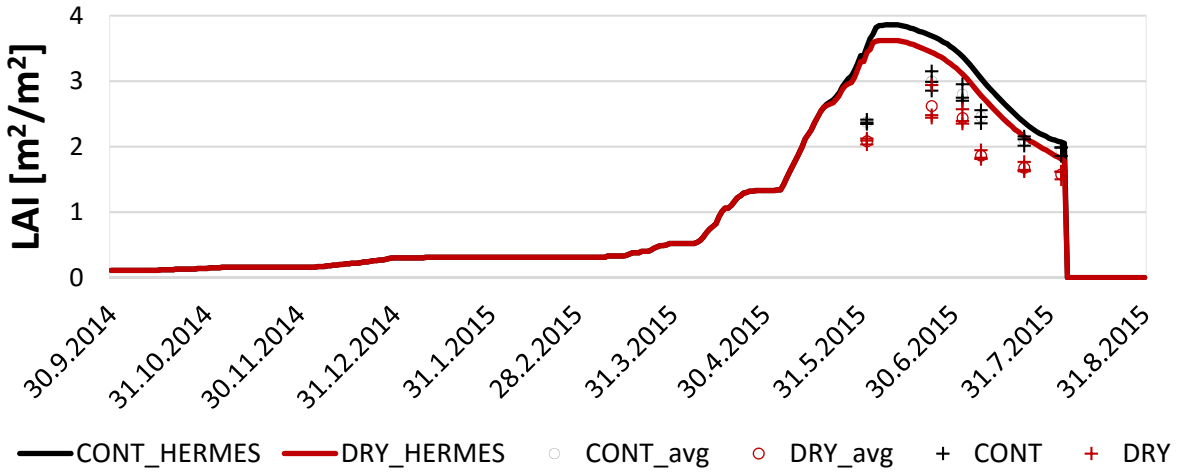
Calibration

The first step to the model adaptation for a variety Bohemia was calibration for crop phenology (emergence, tillering, heading, flowering and maturity). Model was calibrated on the basis of measured and observed data from field experiments. Successive alteration temperature sums leaded to corresponding onset of phenological phases. After calibration, the model showed almost the same results of the phenological phases duration as measured values. On the other hand for phenological phase of maturity model underestimated DRY variant by 11 days.

Leaf area development

From the point of view of leaf area development, crop growth model overestimated that development (Figure 2). It is necessary to mention that the Hermes model simulates only the leaf area, while measuring with SunScan covers a total area of above-ground of plants. Therefore, the measurement points should be above simulated curves. It can be explained by the fact that the year 2015 was considerably dry and wheat canopy was low and had also sparse participation. Otherwise, it is necessary to recalibrate the model data from ongoing measurements to obtain more precise data from the Hermes model. Within Palosuo et al. (2011) study dealing with the comparison between models for winter wheat, the Hermes model led the average. In current study, the crop growth model Hermes is able to evaluate a little bit better the CONT option then DRY variant. However, the differences between CONT and DRY variants are almost similar within the simulated and measured leaf area index. On the other hand, the model captured the growth dynamics of leaf area at similar level as in the Pohanková et al. (2013) study.

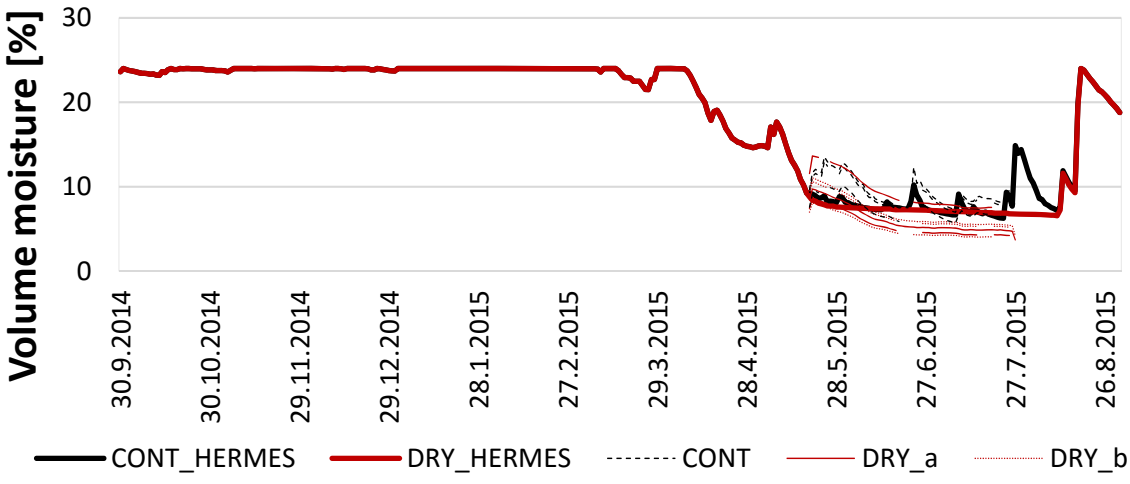
Figure 2 SunScan measurements compared with simulated LAI. The average measured values are indicated with circles and measured values are indicated with crosses.



Soil moisture

Evaluation of the soil moisture estimates using TDR sensors was very accurate. Within DRY variant, modeled data were evaluated as nearly flat curve which confirms waterproof of roofs (Figure 3). Modeled CONT variant depict changes in the soil moisture under the influence of precipitation with good precision as it is compared with the curves of controls. Simulated curves for CONT and DRY variants were sufficiently corresponded to the shape of curve measured values by TDR sensors. Pohanková et al. (2013) study confirmed the accuracy of the Hermes model using in Domanínek locality. Within crop model inter-comparison (e.g. Palosuo et al. 2011 or Rötter et al. 2012), the Hermes model estimated the soil moisture with a pinpoint accuracy.

Figure 3 Comparisons between the simulated and measured (under roof and outside) soil water content from 0.0–0.3 m. Control (dashed line) represent measurement outside the roof (CONT). DRY_a and DRY_b depicted TDR sensors which were placed under one roof. Period from sowing to the end of August.

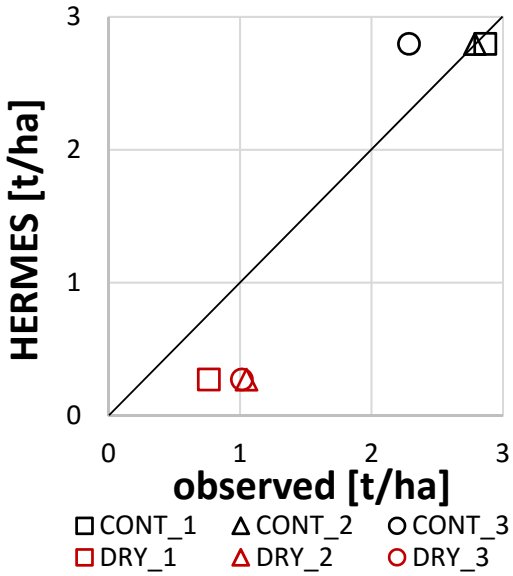


Yields

The impact of a moisture shortage was reflected to the values winter wheat yield by the Hermes model (Figure 4). Model slightly overestimated the yields in an uncovered variant (CONT) in average of 0.15 t/ha (6%) and underestimated the yields in a rain-out shelters variant (DRY) in average of 0.67 t/ha (71%). It may be caused by UV filter presence or by the fact, that model is not able to simulate all kind of stresses (like effect of pest, disease etc.). For example Feng et al. (2007), Kataria and Guruprasad (2012) or Lizana et al. (2009) studies showed the UV radiation which has a significant

effect on the wheat yield. Kataria and Guruprasad (2012) study demonstrated the increase yields while the UV-B and UV-A radiation were reduced. Study Lizana et al. (2009) examined again the effect of increased UV-B radiation leading to reducing the yield of 12–20%. In the current study using the rain-out shelters real winter wheat yields were reduced by 1.7 t/ha. For CONT variant, especially first and second parcels, the Hermes model assessed excellently winter wheat yields.

Figure 4 Comparison observed and modeled winter wheat yields for roof versions (DRY) and rainfed parcels (CONT) and repetitions 1 to 3.



CONCLUSION

This study assessed the impact of drought stress on winter wheat canopy by Hermes crop growth model. Under field trial, the winter wheat was exposed drought stress using rain-out shelters. These results were subsequently compared with simulated values.

First, model was calibrated to set a model for the Bohemia variety. Additional step, observed and simulated values of selected parameters were compared to assessing the ability of the model to capture the lack of soil moisture and the resulting effects. Investigated parameters were leaf area index (LAI), soil moisture and yields. Under the evaluation of the leaf area development, the modeled values were overestimated against the measured values. However, curve dynamics was depicted relatively correctly. As far as the measured and simulated soil moisture, results were very similar. Within comparing the yields was found, that the model Hermes overestimated mean yields about 0.15 t/ha for ambient climate conditions. In case of field crops stressed by drought, the model Hermes underestimated mean yield about 0.67 t/ha. This may be implication of two-sided UV filter which the roofs contain since the model does not take into consideration lower of UV radiation due to using Suntuf. The impact of roofs (especially solar UV exclusion effect) on canopy is investigated in a separate experiment in 2016.

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Section – Animal Production

DEGRADABILITY OF MYCOTOXINS USING IN VITRO METHOD

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Abstract: Barley is classified as one of the most important cereals in the Czech Republic. *Fusarium* Head Blight (FHB) is a worldwide grain disease caused by microscopic filamentous *Fusarium* fungi, infecting crops in the course of vegetation. The following mycotoxin levels such as nivalenol, deoxynivalenol, 3-acyl-deoxynivalenol, 15-acetyldeoxynivalenol, deoxynivalenol-3-glukosid, beta-zearalenon, zearalenon, alternariol-methylether, enniatin (B, B1, A, A1), HT-2 toxin were determined in this study. It was based on the hypothesis that the enzyme preparations can eliminate the occurrence of mycotoxins. *In vitro* method called Daisy II was used in this study. All monitored parameters were significantly reduced. The aim was to create an alternative method for mycotoxin degradation using *in vitro* method.

Key Words: barley, Fusarium, mycotoxin, Daisy II

INTRODUCTION

Barley is classified as one of the most important cereals in the Czech Republic. It was used for livestock feed and food industry – especially malting (Běláková et al. 2014, Horký et al. 2012a) nevertheless barley can be a source of mycotoxins. Mycotoxins are metabolites of fungi that after consumption or absorption can cause health problems. Fusarium mycotoxins in foods are the most frequent type of contamination. Deoxynivalenol mycotoxins, zearalenon and T2-toxin are responsible for the extensive damage to both feed and food. They directly threaten the health of consumers (Horký 2014a, Maul et al. 2014). Fusarium Head Blight (FHB) is a worldwide disease attacking cereal crops already in the course of vegetation (Hooker et al. 2002).

In terms of temperate climate zones, the most commonly occurring and most monitored mycotoxins are trichothecenes, zearalenone and fumonisin. Exposure by trichothecenes may cause nausea, vomiting, as well as liver damage, endocrine and nervous systems. Zearalenone has strong estrogenic effects (Creppe 2002). From reason to protect consumers from the exposure to various chemical pollutants, the European Commission issued Regulation (EC) no. 1126/2007 of 28 September complementing Regulation 1886/2006 of 19 December. These Regulations indicate the maximum levels of various contaminants in food. Currently, the maximum limits of fusarium mycotoxins – deoxynivalenol (DON), zearalenone (ZON) and fumonisin are legislatively determined (European Commission 2007).

In addition to "free" mycotoxins in cereals, "disguised" form can also occur. The first (indirect) account of the existence of the hidden sources of toxic secondary metabolites of filamentous fungi invading cereals were already found out in the mid-80s of the 20th century currently with the animal mycotoxicoses occurrence. Clinical trial results did not confirm the levels of mycotoxins in fixed feeding feed. These "masked" forms of mycotoxins likely to arise in detoxification processes in cereals, as demonstrated and subsequently carried out studies on the transformations of mycotoxins in plants (Kostelanská et al. 2009). Plant metabolites of DON, ZON and ochratoxin A have been identified (Engelhards et al. 1999).

Ruminants are considered to be less sensitive towards mycotoxins than monogastric animals because rumen macrobiotics prove to have mycotoxin-detoxifying capacities. Therefore, the effect

of mycotoxins towards ruminants has been studied to a lesser extent compared with monogastric animals (Wambacq et al. 2016). Of course the negative effects of health stay.

This study was conducted using the method called Daisy II – incubators with special solution to simulate setting of digestive system (like ruminant). Daisy II method allows *in vitro* research that is respectful of animal welfare. But it can never provide the accurate results as *in vivo* methods. However Daisy II provides a potential for the research of mycotoxins degradability in the rumen. The aim was to develop a method that can be useful with regard to the duration and financial demands.

MATERIALS AND METHODS

The total of 14 mycotoxins (nivalenol, deoxynivalenol, beta-zearalenol, zearalenon, 3-acetyl-deoxynivalenol, deoxynivalenol-3-glukoside, enniatin B, enniatin B1, enniatin A, enniatin A1, 15-acetyl-deoxynivalenol, HT- 2 toxin, 15-acetyldeoxynivalenol, beauvericin) was analyzed in nine different barley samples.

Precise sampling coming from Libcany area (the Czech Republic) were included in the experiment from the harvest in 2012. The barley was artificially treated with *Fusarium culmorum* (WGSm. Sacc. Strain KM16902; DON chemotype). The inoculation with a conidia suspension of the pathogenic isolate of *F. culmorum* (concentration 0.5 mil. conidia/1 ml of inoculum; spray dose of 200 l/ha) was performed in the optimal vegetative phase according to the methodology of Tvarůžek et al. (2012).

Before the incubation (in Daisy II), all barley samples were analyzed for the content of individual mycotoxins. From each group, the three samples were collected and analyzed. The results of average concentrations of mycotoxins are listed in Table 1. The barley samples were grounded on the laboratory mill with mesh size of 1 mm. The machine Daisy II Incubator – Ankom Technology, New York was used for the incubation. A 4 g milled sample was taken for the incubation divided into the incubation bags – F57 (Ankom, Macedonia) in the amount of 0.25 g per incubation bag.

Preparation of Solutions

A 1.5 liter of solution was used for one incubation. For preparation of pepsin solution, 3 g of pepsin (Pepsin from porcine gastric mucosa 800–2500 units/mg protein – Sigma-Aldrich, Germany) dissolved in 1.5 liters of 0.1 M HCl then heated to 40 °C. Immediately, it was put in the incubation. Acetate buffer (pH 4.6): 10.2 g sodium acetate (3 H_2O) was dissolved in 1.5 l of distilled water. The pH value was modified using acetic acid or NaOH. In the preparation of cellulase solution, 1.5 g of cellulase was dissolved (Cellulase *Trichoderma viride*, 3–10 units/mg solid – Sigma Aldrich, Germany) in 1.5 liters of acetate buffer heated to 40 °C then the incubation could start. The incubation was carried out for 24 hours at 37 °C. The obtained cultured fluids were analyzed on the concentration of mycotoxins.

Solid Samples – Extraction

A 2 g barley sample was weighed to PTFE centrifuge tubes (50 ml) followed by the addition of 10 ml of distilled water acidified (0.2% formic acid). Then the sample was shaken, closed and left for 30 minutes due to the wetting of the matrix. A 10 ml of acetonitrile was added in the sample with water followed by the extraction on the laboratory mixer for 30 minutes (240 RPM). The 4 g of $MgSO_4$ and 1 g of NaCl were put in the cuvette and shaken vigorously for 1 minute. The prepared sample was centrifuged for 5 minutes (10,000 RPM). After centrifuging, the sample was taken (approx. 1.5 ml) for purification using a microfilter with a porosity of 0.2 µm (centrifugation for 2 min, 5000 RPM). The sample was transferred to the vials and prepared for analysis. The samples were stored at -18 °C in glass vials before the analysis.

Liquid Samples

Liquid samples were purified using a microfilter with a porosity of 0.2 µm (centrifugation for 2 min, 5000 RPM) before the instrumental analysis.

Determination of Mycotoxins

The total of 14 mycotoxins of microscopic filamentous fungi of the genus *Fusarium*, *Aspergillus, Alternaria* were set such as nivalenol, deoxynivalenol, beta-zearalenol, zearalenon, 3-acetyl-

deoxynivalenol, deoxynivalenol-3-glukoside, enniatin B, enniatin B1, enniatin A, enniatin A1, 15-acetyl-deoxynivalenol, HT- 2 toxin, 15-acetyldeoxynivalenol, beauvericin. For the identification and quantitative determination of the mycotoxins, Acquity UPLC® System (Waters, Milford, MS, USA) in a connection with tandem mass spectrometer QTRAP® (AB Sciex, Toronto, ON, Kanada) was used for the instrumentation of ultra-efficient liquid chromatograph Acquity UPLC® System (Waters, Milford, MS, USA). The program Analyst ® (Thermo Fisher Scientific) was used for data processing.

Statistics

The data were statistically processed using STATISTICA.CZ, version 10.0 (the Czech Republic). The results were expressed as average values with standard deviation (SD). Statistical significance was determined by the examining the basic differences between groups by ANOVA and Scheffé's test (one-way analysis). The samples were analyzed in five repetitions. The differences with $P<0.05$ were considered to be significant.

RESULTS AND DISCUSSION

The following mycotoxins were analyzed after the incubation: deoxynivalenol, zearalenone, deoxynivalenol-3-glucoside, 3-acetyl-deoxynivalenol. Some mycotoxins (beta-zearalenol, alternariol, alternariol-methylether, enniatin B, enniatin A, enniatin A1), analyzed in barley samples inoculated by *Fusarium,* were found out below the detection limit that was indicated after the incubation. The values of concentrations were so low that the device could not measure the values. It demonstrates the fact, that the mycotoxins were largely eliminated by digestive enzymes. All concentrations of mycotoxins were distinctly reduced. The average values of concentrations are listed in Table 1.

Table 1 Average concentration of mycotoxins

Mycotoxins	Barley samples inoculated by *Fusarium*			Cultured fluids of inoculated barley samples		
	Average values [µg/kg]	SD	Coefficient of variation [%]	Average values [µg/l]	SD	Coefficient of variation [%]
Nivalenol	137.4	± 195.8	142.5	0	± 0	–
Deoxynivalenol (DON)	39207.8	± 12810.2	32.7	30.3*	± 11.2	36.8
Beta-zearalenon	183.6	± 57.9	31.5	0	± 0	–
Zearalenon	3454.6	± 1337.5	38.7	4.7*	± 1.8	39.4
3-acyl-deoxynivalenol	5728.5	± 2128.2	37.1	3.9*	± 2.4	59.5
Alternariol-methylether	4.4	± 2.5	57	0	± 0	–
Deoxynivalenon-3-glukosid	8350.8	± 2850.1	34.1	14.3*	± 8.5	59
Enniatin B	397.8	± 239	60	0	± 0	–
Enniatin B1	228.1	± 144.6	63.4	0	± 0	–
Enniatin A	107.1	± 104.9	98	0	± 0	–
Enniatin A1	94.4	± 67.4	71.3	0	± 0	–
HT-2 toxin	16.7	± 47.3	282.8	0	± 0	–
15-acetyldeoxynivalenol	244.7	± 152	62.1	0	± 0	–
beauvericin	12.1	± 11.1	91.8	0	± 0	–

** The mean difference is significant at the P<0.05 level*

Deoxynivalenol (DON) from group B trichothecenes occurs frequently and is usually found out in higher concentrations compared to other mycotoxins from this group. It is mainly produced by *Fusarium graminearum* and *F. culmorum* (Obst et al. 2000). In our case. the content of deoxynivalenol also showed the highest values (39207.8 µg/kg±12810.2) than other concentrations

of mycotoxins. The experiment. which studied the activity levels of the mycotoxin in barley harvest. detected a maximum value for deoxynivalenol 7050 mg/kg. and the lowest value 227 µg/kg (Mätthaus et. al. 2004).

The interesting results were obtained from the studies of Edwards (2009). summarizing the occurrence of mycotoxins in conventionally and organically grown barley in 2002–2005. From the 446 samples. DON was detected in 57% of samples. Only in one sample. the concentration of DON exceeded the maximum limit for unprocessed cereals intended for human consumption (1250 µg/kg) and acetylated form of DON (3-and 15-ADON) were also detected. In this study. the average concentration of DON was exceeded this limit. so our barley samples had high values of DON.

HT-2 and T-2 were determined in 36% and 12% of all samples with maximum levels of 105 and 138 µg/kg. The average level of HT-2 toxin was found out of 16.7 µg/kg ± 47 in our study. On the 6th forum focused on Fusarium toxins. which were summarized in the study by (Bouxin 2009) and represented an average of 60% compound feed in the EU produced in 2004–2008. The results showed that the contamination by T-2 toxin was low in raw materials. The amounts of T-2 toxin in feed mixtures were found out in general less than 0.1 mg/kg.

Generally, it is believed that the population of protozoa in the rumen proves the highest ability to detoxify the ingested mycotoxins. Various mycotoxins are nevertheless capable of modifying the rumen flora (Escoula 1992, Tapia et al. 2002). Morgavi et al. (2003) showed that certain types of mycotoxins significantly reduce the degradation of alfalfa hay and thus arrived at the opinion that the cellulose especially inhibited due to the antimicrobial activity in the rumen (Morgavi et al. 2003). Theory that mycotoxins deteriorate rumen microflora corresponds to the observations in clinical practice. in which cows had the decreased rumen content. poor feed conversion and mild diarrhea after a period of feeding silage contaminated with fungus. The latter symptoms. accompanied by lowered milk production and an increased incidence of subclinical mastitis with increased somatic cell. contribute to the loss of milk (Hadley et al. 2006, Wenz et al. 2007).

CONCLUSION

A significant influence of the incubate medium. containing digestive enzymes. resulted in a lower occurrence of mycotoxins in barley samples infected by *Fusarium culmorum.* These results have confirmed that the Daisy II method is suitable for the research of mycotoxins degradability in the rumen. Therefore. it can be possible to focus on the technology of processing barley as a feed for cattle or the percentage of barley in rations or even the suitability of different barley varieties.

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EFFECT OF DIFFERENT PHYSICAL FORMS OF STARTER ON FEED INTAKE AND PERFORMANCE OF CALVES

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Abstract: The objective of this study was to determine the effect of different types of starter on starter intake and growth performance of calves in the period of milk nutrition. The experiment was performed with Czech Fleckvieh calves (n = 28). Calves were housed in outdoor individual boxes. The calves were fed by colostrum (for the first 5 days of life) or milk feed mixture (from the 6th day of live) and starter. For the experiment the calves were divided into 4 groups (fed various types of starter: A – pelleted starter with 20% of oats; B – completely pelleted starter, C – textured starter, D – starter with chopped straw) per 7 calves. The mean age of calves in group was balanced (reached in all groups an average of 13 days) at the start of experiment. The experiment lasted for 32 days. The mean intakes of individual types of starters were relatively balanced (differences between groups are statistically insignificant, P > 0.05). The average daily gain of calves in individual groups for the period was statistically insignificant too (the range is from 0.59 to 0.69 kg/head/day). Based on the evaluated parameters we can conclude that the type (physical form) of starter fundamentally not affects the palatability or attractiveness of starter for calves during milk nutrition.

Key Words: calf, growth performance, pelleted starter, textured starter, starter with chopped straw

INTRODUCTION

Calves entering the feeding phase are adjusting to nutritional and environmental changes, placing them at risk for diseases that negatively affect performance. The owner immediately before this transition is in a unique position to reduce these disease risk factors (Anderson et al. 2009). Calves are born with a physically and metabolically underdeveloped rumen and initially rely on milk to meet nutrient demands for maintenance and growth. Initiation of solid feed consumption, acquisition of anaerobic microbes, establishment of rumen fermentation, expansion of rumen in volume, differentiation and growth of papillae, development of absorption and metabolic pathways, maturation of salivary apparatus and development of rumination behavior are all needed as the calf shifts from dependence on milk to solid feed. In nature and some production systems (e.g., most beef calves), young ruminants obtain nutrients from milk and fresh forages. In intensive dairying, calves are typically fed restricted amounts of milk and weaned onto starter feeds (Khan et al. 2016).

Modern starters are composed mainly of mashed cereal, corn, oats, barley, eventually soybeans and components comprising a structural fiber and have structure of muesli (Mudřík et al. 2006). Starter feeds contain easily fermentable carbohydrates. It is thought, that starter feeds stimulate rumen development, including changes in the epithelium of the forestomach (Khan et al. 2016). The composition and ratios of feed ingredients have a significant impact on the development of forestomach and body weight. Calves fed large amounts of milk replacer (MR) gain more body weight preweaning than calves fed less MR; however, postweaning growth may be reduced because of impaired digestion of nutrients (Hill et al. 2016), but provision of chopped hay to calves fed high volumes of milk can promote solid feed dry matter intake and rumen development without affecting body weight gain (Khan et al. 2011). The provision of high-starch and low-fiber starter feeds may negatively affect rumen development and that forage supplementation is beneficial for promoting development of the gut and rumination behavior in young calves. It is important to note that both the physical form of starter diets and their nutritional composition affect various aspects of development

in calves. Further research is warranted to identify an optimal balance between physically effective fiber and readily degradable carbohydrates in starter diets to support development of a healthy gut and rumen, rumination behavior, and growth in young calves (Khan et al. 2016).

Starters are composed of different types of cereals (corn, oats, barley etc.). Regarding oat, under the conditions of study (Suarez-Mena et al. 2015), greater rumen weight and papillae length in calves fed pelleted oats starter may be the result of greater nutrient availability of oats. Grain, mainly corn, has traditionally played a major role in the cattle feeding industry because of its higher energy content when compared to roughages (Hill 2012). Calves on a corn diet have greater ruminal capacity to accommodate feed bulk. More physically and metabolically functional rumens in calves on corn and wheat diets probably resulted in greater feed consumption and nitrogen retention (Khan et al. 2008). Calves on corn diet consumed more solid feed and gained greater body weight than those fed barley, oat, and wheat diets (Khan et al. 2007). In turn it is important that nutrition calves contains a structural fiber (forage). In conclusion of study (Castells et al. 2013), calves supplemented starter with oat hay have a better rumen environment than calves offered no forage and do not have an increased gut fill. The results of experiments about physical forms of starters are inconsistently (Franklin et al. 2003, Bach at al. 2010, Terre et al. 2015) and next research is necessary.

MATERIAL AND METHODS

The experiment was performed with Czech Fleckvieh calves (n = 28). Calves were housed in outdoor individual boxes with straw bedding. The calves were fed by colostrum for the first 5 days of life. Milk feed mixture calves receiving from the 6th day of live. Milk was offered in three doses in a total quantity of 7 liters per day (2.5 liters in the morning, 2 liters in noon and 2 liters in the evening). Access to drinking water and starter was *ad-libitum* from first day of live. The calves were divided into 4 groups (A, B, C, D) per 7 calves, for the experiment. The mean age of calves in group at the start of experiment was balanced (reached in all groups an average of 13 days). The A group had available to complete pelleted starter which was mixed with 20% of oats. Group B calves had access to completely pelleted starter without addition of any components. To calves from C group had presented textured starter contains pellets, oats, maize grain and maize flakes. The last group (D group) had access to mixture of ground cereals, extruded maize grain, protein-energy concentrate and chopped what straw. The starters intake was evaluated daily. The calves were also weighted at regular intervals in order to evaluate their body weight gain. The experiment lasted for 32 days from August to September. The average age of the calves in each groups was 45 days. Based on the records and evaluation of daily starter consumption by calves were calculated the average intake of starter between day 20–23, 30–33 and between days 39–42 of calves age in each group. The average daily gain of calves it was also calculated.

Data has been processed by Microsoft Excel (USA). To ensure evidential differences Student´s t-test was applied and $P < 0.05$ was regarded as statistically significant difference.

RESULTS AND DISCUSSION

Development of intake of individual types of starters is shown in Figure 1. The intakes of individual types of starters were relatively balanced. It is not possible to do conclusion on different palatability of individual starters. In the first reporting period between day 20 to 23 of calves age was daily intake of starters averaging between 180–305 g/head/day. Between calves were big differences. While some calves didn't eat starters in that age, some calves taken starters to 850 g/day. When evaluating the daily consumption of starter during the one month of calves age (30th–33rd day of age) we can see the mean intake of starters ranged from 505 to 723 g/head/day in this period. Certain tendency to the highest intake of starter is in the C group, which received the textured starter (pellets, whole grains and flakes). However, differences between groups were statistically insignificant ($P > 0.05$). A similar trend continues until the end of the period between 39th–42nd day of age of calf. The mean intake of starter in each group ranged from 835 to 1241 g/head/day. The highest intake persisted in Group C. The differences were not statistically significant as well. We can say that palatability of receiving various types of starters (pelleted, pelleted with whole oats grain, textured, and starter with chopped straw) was balanced. The average starter intake by calves about the 40th day of age moving around 1 kg corresponds

to the results of experiments by other authors (Bach et al. 2010, Hosseini et al. 2015) in their experiments starter intake by calves at the same age was around 0.8 kg/day.

The results of consumption of different starters are consistent with the average daily gain of calves (Figure 2). The figure shows that the average daily gain of calves in individual groups for the period (an average age of calves in all groups for 45 days) was non-significantly different. The range is from 0.59 to 0.69 kg/head/day. The average daily gain of calves under the age of 45 days moving about 0.6 kg is slightly higher than the gain in publication of Terré et al. 2015, where the growth of calves was about 0.5 kg.

Figure 1 Mean intake (g/day) of individual types of starters (A – pelleted starter with 20% of oats; B - completely pelleted starter, C - textured starter, D - starter with chopped straw)

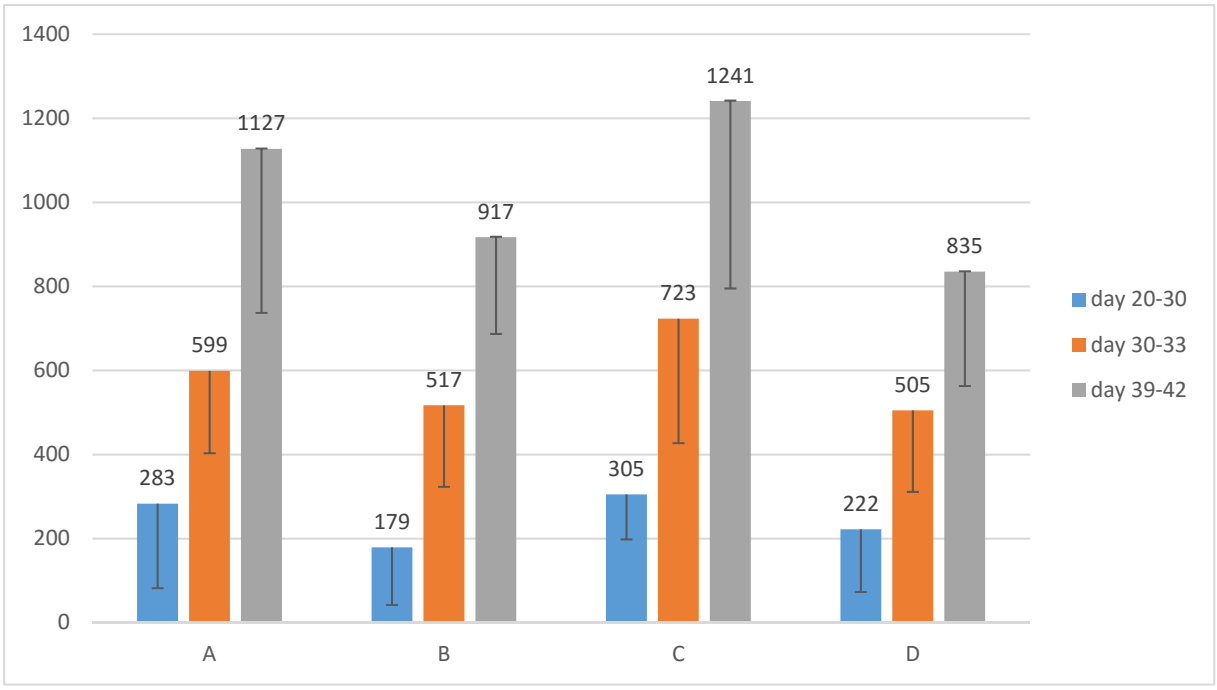
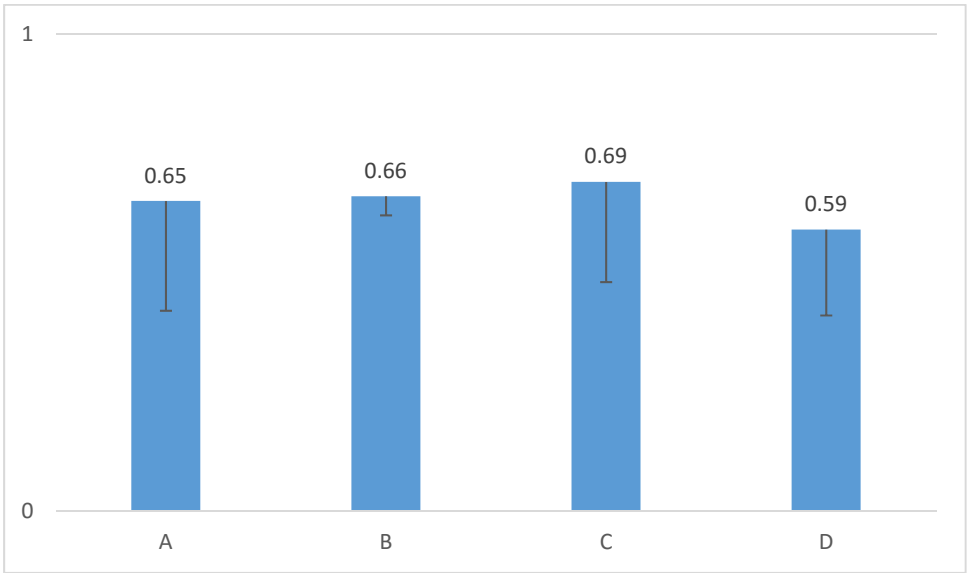


Figure 2 The mean average daily gain (kg) of calves consumed different starter (A – pelleted starter with 20% of oats; B - completely pelleted starter, C - textured starter, D - starter with chopped straw)



CONCLUSION

Based on the evaluated parameters we can conclude that the type (physical form) of starter fundamentally not affects the palatability or attractiveness of starter for calves during milk nutrition.

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EFFECTS OF MONENSIN ON MILK PRODUCTION AND METABOLISM OF DAIRY COWS

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Abstract: The effect of monensin (intraruminal bolus, 32.4 g) was observed in Holstein cows (milk yield of 10,200 litres) in three experiments. Blood and milk was examined during 4 to 8 weeks after parturition. The positive effect of monensin resulted in lower concentration of beta-hydroxybutyrate in blood (0.60–1.31 mmol/l) and milk (0.075–0.137 mmol/l). Milk yield increased by 3.4–11.2% for the first 100 days of lactation, fat yield by 6.9–12.0%, and protein yield by 1.81–5.4%. No significant differences were found in plasma glucose, triglycerides, and urea.

Key Words: ketosis, milk yield, metabolic parameters, thyroxine, triiodothyronine

INTRODUCTION

In the relation to the energy deficit in high-production milking cows during 2nd to 6th week of lactation after parturition the occurrence of ketosis is found. The illness is connected to the increase of nonesterified fatty acids (NEFA) production, which are the cause of higher hepatic ketogenesis and rise of ketone bodies in body fluids, including milk. The result of such is a decrease in milk production and changes in qualitative parameters of milk (Duffield 2000; Litherland et al. 2011). The detection of ketosis, already in subclinical level, leads to precocious protective measures and it also preventing the clinical symptoms of diseases (Hanuš et al. 2013). The diagnosis lays in the detection of the increased keto bodies in body liquids. In blood, the increase reaches values above 1.0 mmol/l (Hofírek et al. 2004) or even 1.2 mmol/l (Šlosárková et al. 2015). In milk, the acetone level increases above 0.40 mmol/l and beta-hydroxybutyric acid (BHB) increases above 0.20 mmol/l (Geishausr et al. 2000) or 0.25 mmol/l (Hofírek 2004). From the effectivity aspect of anti-ketogenetic profylaxation the most commonly used is monensin ionophore in the form of intraruminal boluses, from which the monensin is gradually released (Šlosárková et al. 2015). Monensin positively affects ruminous fermentation for the bacteria producing propionic acid, which is a necessary substrate of gluconeogenesis.

MATERIAL AND METHODS

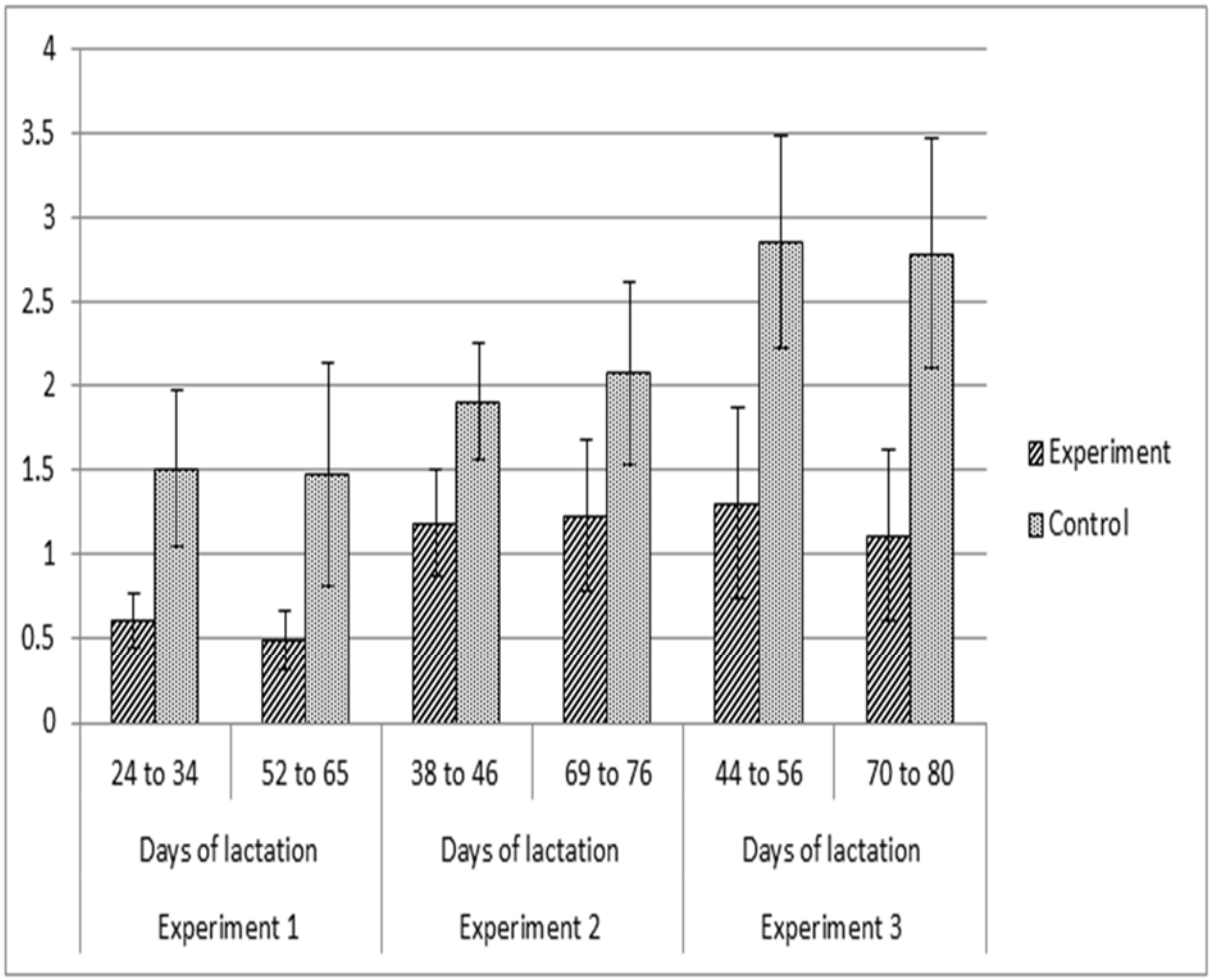
The effect of intraruminal bolus (Kexxtone) containing monensin (32.4 g) was validated in 3 experiments using milking cows of Holstein breed with average production of 10,200 litres of milk during lactation. Experimental group E was used in each experiment (n = 8, monensin was applied 3 weeks before parturition) as well as a control group C (n = 8). Blood and milk was examined twice during 2nd to 6th week after parturition, when the energy deficit is highest (see Introduction). The presence of BHB in milk will be determined by infrared spectroscopy (FT-MIR) method. The milk components were determined by infrared absorption analyzer Combi Foss. Production of milk was validated by yield control. Metabolic parameters and BHB of blood plasma were measured by using a metabolic analyzer Dialab. Thyroxine (T4) and Triiodothyronine (T3) in blood serum were determined by RIA methods (kits Immunotech Praha).

All data were analysed using program Microsoft Excel. Both graphs are shown in a column planar type with expressing standard deviations. In this program were determined also P – values, on tables averages with standard deviations. In the same manner was performed recalculating the absolut values to the percentage expression. Lactation persistence was expressed in percentage of index P 2:1 (2nd to 1st days of lactation).

RESULTS AND DISCUSSION

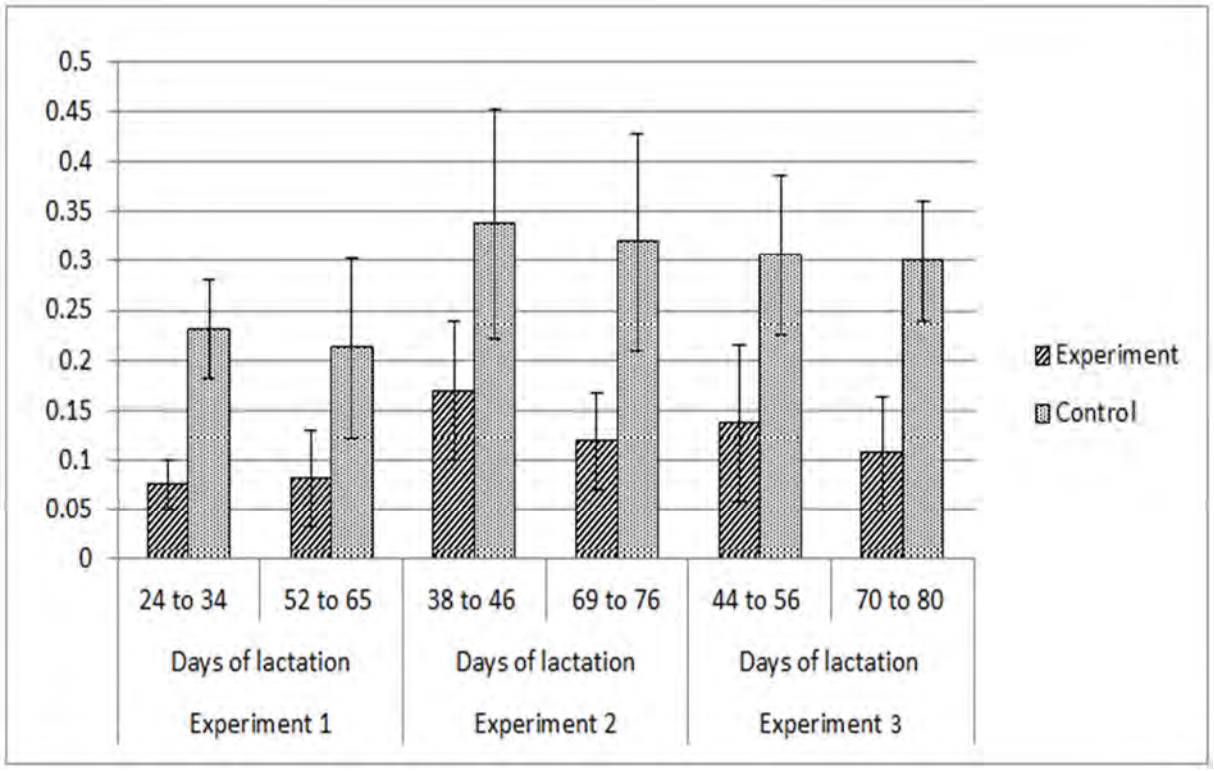
The application of monensin in the form of intraruminal bolus had in, all three experiments, positive effect on statistically significant lower content ($P < 0.01$ and $P < 0.05$) of BHB acid in milk and also in blood plasma (Figure 1, 2). Correlational coefficients between the contents of BHB in milk and blood plasma were in trial group in the range of 0.541 to 0.894 and in control group from 0.718 to 0.932. The average content of BHB in milk of trial group was in the range of 0.075 to 0.179 mmol/l and in control group (not treated by monensin) from 0.213 to 0.338 mmol/l. Concentration of BHB in milk above 0.200 mmol/l corresponding to the subclinical ketosis (Geishauser et al., 2000) was in milking cows in experimental groups from 6% (1st experiment) to 25% (2nd and 3rd experiment) and in control group from 68% to 94%.

Figure 1 Concentration of beta-hydroxybutyric acid in milk



Data are expressed with +/- standard deviation

Figure 2 Concentration of beta-hydroxybutyric acid in blood plasma



Data are expressed with +/- standard deviation

The importance of antiketogenetic profylaxation used for stabilizing the milk production is obvious in table 1. Trial group of milking cows reached higher production of milk in the first 100 days of lactation (in 1st experiment increase of 3.4%, in 2nd experiments increase of 4.9% and in 3rd experiment increase of 11.2%) also relating to higher milk fat and proteins content (fat increase of 6.9% to 12.0% and protein increase of 1.81% to 5.4%). The increase in milk production corresponds to favorable lactation persistence. Lactation persistence was in trial group 88.9% to 96.9% and in experimental group 81.8% to 89.8%. In milking cows of control group, the increase of milk fat was also noted (trial group 3.62 to 3.77%) being linked to an increase in lipo-mobility (Hanuš et al. 2013). The average values of milk protein and lactose did not show significant differences in the first 8 weeks of lactation.

Table 1 Milk, fat and protein yield for the first 100 days lactation

Experiment	Group	Milk yield (kg)	Fat yield (kg)	Protein yield (kg)
1.	E	3902.5±892.3	157.8±40.1	119.6±22.9
	C	3772.8±845.3	140.8±31.6	117.5±24.6
2.	E	3777.2±618.3	147.5±16.9	116.4±15.4
	C	3601.5±795.2	137.8±25.6	113.8±22.7
3.	E	3629.6±486.3	142.6±21.6	116.4±15.7
	C	3264.6±518.9	130.8±18.9	110.0±18.1

Data are expressed with +/- standard deviation

Table 2 Metabolic parameters of the blood plasma

Experiment	Group	Glucose (mmol/l)	Urea (mmol/l)	Total protein (mmol/l)	TG (mmol/l)	T3 (mmol/l)	T4 (nmol/l)
1.	E	2.90±0.38	7.54±1.84	79.91±2.43	0.29±0.01	1.78±0.22	51.43±3.1
	C	2.87±0.44	6.52±1.42	81.24±1.83	0.28±0.03	1.79±0.09	49.93±5.1
2.	E	3.39±0.29	3.30±0.65	74.30±5.94	0.46±0.03	2.30±0.51	54.05±9.9
	C	3.57±0.34	3.84±0.69	70.30±3.11	0.45±0.01	1.96±0.47	49.59±8.6
3.	E	2.82±0.53	4.37±0.96	69.69±2.69	0.49±0.06	2.37±0.65	51.04±9.7
	C	2.60±0.39	4.09±0.82	63.29±6.86	0.51±0.02	2.48±0.28	47.43±8.4

Data are expressed with +/- standard deviation

Legend: TG - Triglycerides, T3 – Triiodothyronine, T4 – Thyroxine

The effect of monensin on selected metabolic parameters is stated in table 2. There were no statistically significant differences between the trial and the control group. Lower content of glucose and plasma protein (1st and 3rd experiment) and higher content of urea (1st experiment) relates to the energy deficit (Hofirek et al. 2004). In all three experiments the control group showed lower thyroxine concentration in blood plasma, which had no statistical significance. Lower levels of thyroxine in cows with ketosis onset are claimed by Ropstad et al. (1989). Lower content of ketone bodies (BHB) in milk and blood, or even a decrease in numbers of status corresponding to subclinical ketosis supports the significance of used anti-ketogenic profylaxation. The positive production effect of monensin in the first period of lactation was also confirmed in experiments by Antanaitise et al. (2015), or by work of Duffield et al. (2008), Slosarkova et al. (2015) and others.

CONCLUSION

The intraruminal aplication of monensin (32.4 g) 3 weeks before parturition had a positive effect on the reduction of subclinical ketosis and on higher the milk production in the first 100 days of lactation.

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MULTIPLE PREGNANCY IN MARES

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Abstract: Mare is in terms of reproduction described as uniparous, it means that she has one foal. More embryos, fetuses or foals represents non – physiological phenomenon and as such it brings a lot of complications. In terms of the etiology is discussed as a predisposing factor mare's breed, the most common is a higher incidence associated with Thoroughbreds. Although in context of multiple pregnancies often mentioned is mare's age. The aim of this study was to evaluate the incidence and results of multiple pregnancy in Thoroughbred and Old Kladruber Horse mares. It was found 323 records of multiple pregnancies in Thoroughbred and 48 in Old Kladruber Horse mares and the result of the multiple pregnancy was in Thoroughbreds in 258 cases twin abortion, in 45 cases parturition of 2 dead foals, 15 records of parturition 1 live and 1 dead foal and in 5 cases it was the parturition of 2 live foals. In Old Kladruber Horse twin abortion was recorded 36 times, in 7 cases 2 dead foals and in 5 cases 2 live foals.

Key Words: horse, breeding, reproduction, pregnancy, twins

INTRODUCTION

The reproduction standard in horse breeding is the fundamental informative factor affecting its success and profitability. One of the factors that can decide about the reproduction outcome is also multiple pregnancy.

Multiple pregnancy basically may occur naturally either by spontaneous division of embryos, or by fertilization of two oocytes after multiple ovulation. Specifically horses in the vast majority of cases have dizygotic twins, thus originating from two separate embryos. In terms of double ovulation, which has the potential to result in a multiple pregnancy, can be distinguished according to the localization of an unilateral (two ovulations in the same ovary) and bilateral (one ovulation per each ovary). In the case of the time determination it may be a synchronous (usually during the 24 hours), and asynchronous ovulation. Multiple pregnancy as such can also be classified as unilateral and bilateral, depending on whether at the stage of fixation one embryo is attached in each uterine horn, or both together in one.

All these factors, with maternal and environmental influences together, and especially management of selected twins from the breeder's side affect the outcome of multiple gestation, and thus how its impact is reflected on mare's reproduction.

The incidence of multiple pregnancy in mares generally, for example, as Bílek et al. say (1957) between 2–2.5% of cases, Hutton and Meacham (1986) at 1.1% and Doležel et al. (2000) from 1.5 to 2.5%.

As predisposing factors for multiple gestation are most frequently mentioned – breed, mare's age, reproductive history and heredity. Górecka and Jezierski (2003), Bresińska et al. (2004), McCue (2009), Sheerin (2014) and others state that a breed with the highest incidence of multiple pregnancy is Thoroughbreds. Specifically, in Thoroughbreds Hutton and Meacham (1968) illustrate the incidence of multiple pregnancies at 2.4%, Doležel et al. (2000) at 2.57%, and Bresińska et al. (2004) and Wolc et al. (2006) at 3.5%. Regarding the age Davies Morel (2012) reported that incidence of multiple pregnancy increases in the older mares. Davies Morel and O'Sullivan (2001), Górecka and Jezierski (2003) and McCue (2009) argues that the mares, in which multiple ovulation or pregnancy already occurred once, have a greater likelihood of recurrence than mares in which this phenomenon has not been recorded. With respect to the inheritance factor from the work of Pawlak et al. (2000), Górecka and Jezierski (2003) and Davies Morel (2008) arises that predisposition for the multiple pregnancy is inheritable.

In case of occurrence of multiple pregnancy, the most of the authors are united that only in the low percentage it results in one or two foal births. For example, Larson (2011) says that the chance of a mare having full term of two foals and their successful parturition is 1:10 000. Davies Morel (2008) says that 9% of twin pregnancies survives to full term and the parturition results in the death of both foals in 64.5% cases.

The aim of this study was to evaluate characteristics of multiple pregnancy occurrence in the Thoroughbred population and for comparison in the Old Kladruber Horse, to find out in how many cases the result of multiple pregnancy is successful parturition of one or two foals and to evaluate the interrelationships between the result of multiple pregnancy and mare's breed or age.

MATERIAL AND METHODS

In the research, selected reproduction characteristics of Thoroughbred and Old Kladruber Horse breed mares were evaluated. Required data were obtained from the Czechoslovak Thoroughbred Studbooks (Volume XI.–XIII.), Czech Thoroughbred Studbooks (Volume 1. to 4.) and the Old Kladruber Horse Studbook. In the Thoroughbred Studbooks records of mares born in the years 1965–2001 and active in breeding in 1985–2008 were found. In the Old Kladruber Horse Studbook they found records of mares born between the years 1978 to 2001 and active in breeding in the years 1981–2009.

The Thoroughbred breed was chosen because of increasing incidence of multiple pregnancy within the breed and recorded by many authors and also because of the fact that Thoroughbred can only come of the natural breeding, so the factors like insemination, embryo transport and more cannot influence the evaluation. Moreover, Thoroughbred Studbooks contains exact and comprehensive records about reproduction including evidence of multiple pregnancy for many years.

Old Kladruber Horse breed was chosen as a representative of warmblood for comparison with Thoroughbred. Another reason also was that it is possible to find records needed for evaluation in Old Kladruber Horse Studbooks.

In the mentioned volumes of Studbooks all the records about reproduction of all the listed mares were reviewed and detailed appraisal was done just in those mares which had the multiple pregnancy during the reproductive life.

For evaluating of the founded data and their relationships were used to the statistic programme STATISTICA.12 (© Statsoft, CZ version). The outputs of the programme were rounded to two decimal places or where it was appropriate (eg. an evaluation of the particular year) to integers. Testing took place at a significance level $\alpha = 0.05$.

RESULTS AND DISCUSSION

Table 1 Multiple pregnancy in Thoroughbred mares

Variable	N valid	Average	Median	Mode	Frequency	Minimum	Maximum
The age of the multiple pregnancy	323	10.21	10	Multiple	35	4	20

Table 1 states elementary descriptive statistics about the Thoroughbred mare's age in the multiple pregnancy age. It was recorded in 323 cases of multiple pregnancies. The average age of the mares in the year of such gravidity was 10 years old. The middle value was also 10 years. The most common value was multiple–exactly the 7 and 9 age with the frequency of 35 times. The minimum age of mares in the multiple pregnancy was 4 years, the maximum 20 years.

Table 2 Multiple pregnancy in Old Kladruber Horse mares

Variable	N valid	Average	Median	Mode	Frequency	Minimum	Maximum
The age of the multiple pregnancy	48	10.65	11	11	8	4	20

For the characteristics of the age of Old Kladruber Horse mares in the time of multiple pregnancy 48 records were found. The average age was 11 years and also the median. The most common had the

multiple pregnancy mares in the 11 years old with the frequency 8 cases. The lowest age was 4 and the highest 20 years old.

The observed data regarding the age of the mares do not correspond with the assertion of Reef (1998), which says that the older mares show a multiple pregnancy more likely, since the oldest mare with such pregnancies recorded were twenty years old in both breeds, it can be assumed that the age of seven and nine-year-old in the Thoroughbreds and eleven-year-old in Old Kladruber Horse does not fall into those categories. On the other hand, Bresińska et al. (2004) are indicated as the most predisposed mares between 5 and 10 years of age and Doležel et al. (2000) say the same thing about mares aged 6-10 years, which would match the data found in the Thoroughbreds.

Figure 1 Repeated occurrence of multiple pregnancy in Thoroughbred mares

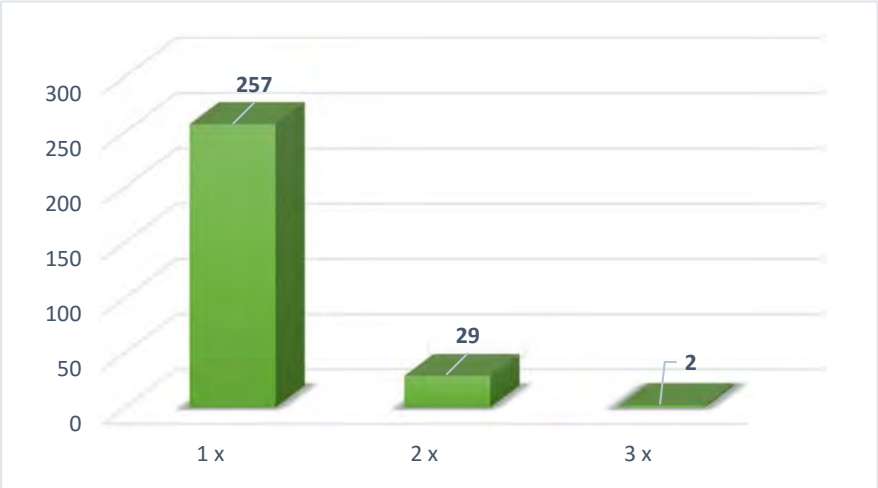
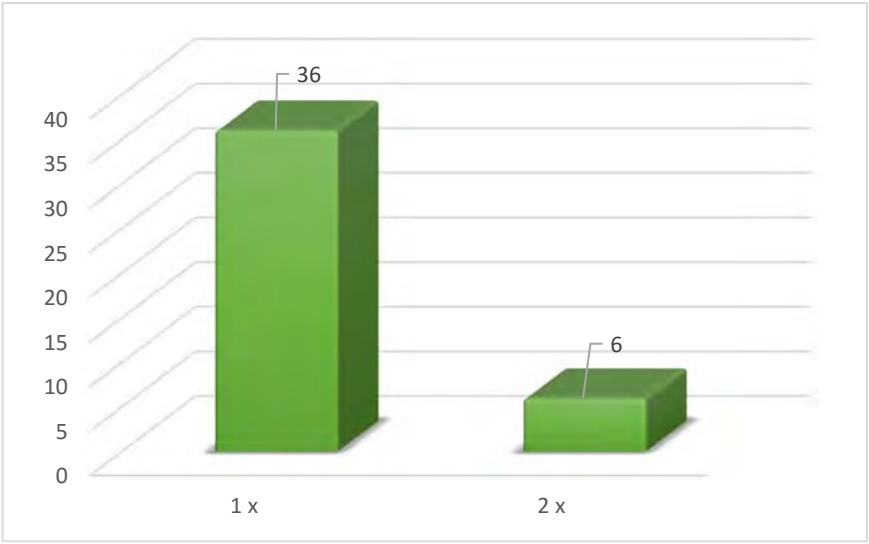


Figure 1 shows the frequency of recurrence of multiple pregnancy. First column shows in how many mares from the observed file of multiple pregnancies occurred once in recorded reproductive history (257 mares), i.e. 89.24% frequency phenomenon. Second column describes which of the number of mares were multiple pregnancies reported in this sense, twice, with 29 mares (10.07%) and similarly in category 3 describes it in 2 mares (0.70%).

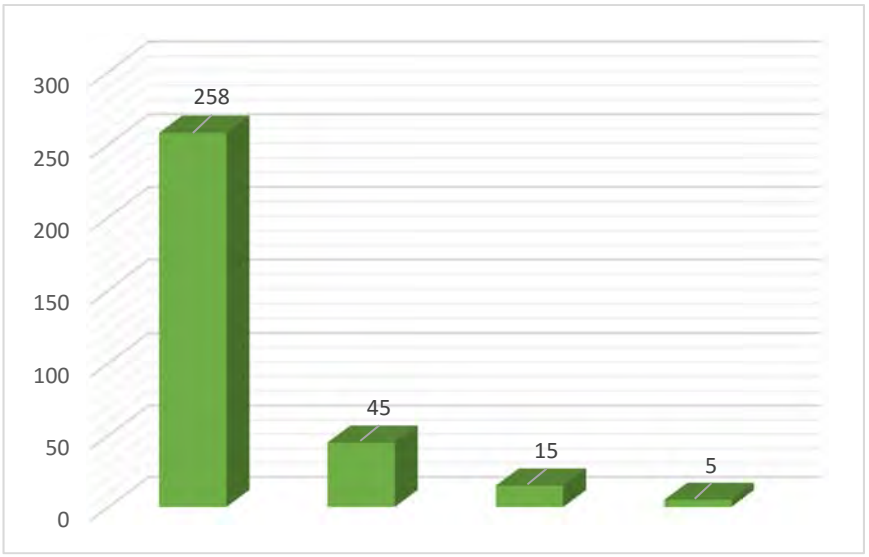
Figure 2 Repeated occurrence of multiple pregnancy in Old Kladruber Horse mares



As documented in Figure 2, in mares of Old Kladruber Horse breed was found only one variant of repeating multiple pregnancy during the reproductive history of mares, and that in 36 cases (85.71%) and the option of two repetitions in six cases (14.29%).

With the results of repeating occurrence of multiple pregnancies in mares generally correspond with McCue's (2009) assertions that mares which had twins previously have greater probability of their occurrence in future. The phenomenon of recurrence is also confirmed by Bresińska et al. (2004).

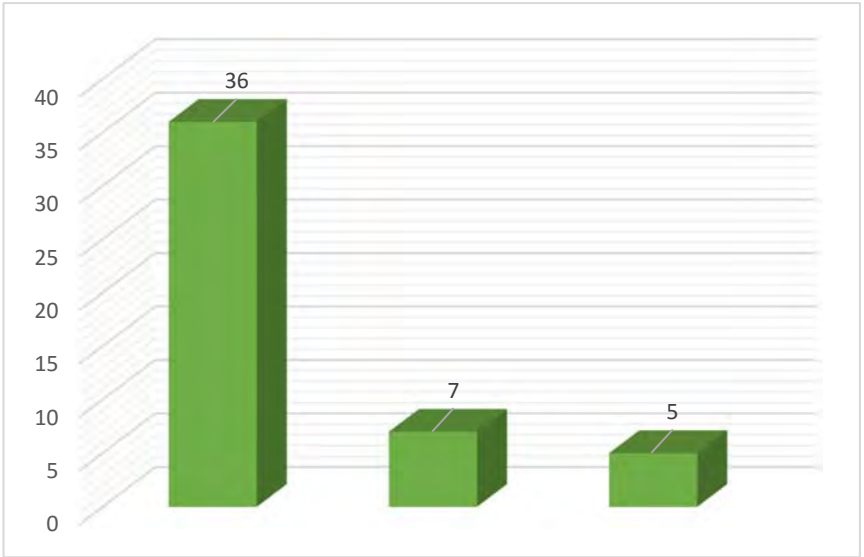
Figure 3 The result of multiple pregnancy in Thoroughbred mares



Legend: 1. Column – twin's abortion, 2. Column – 2 dead foals, 3. Column – 1 live and 1 dead foal, 4. Column – 2 live foals

Figure 3 describes the frequency of individual reproductive results of Thoroughbred mares with recorded multiple pregnancies. 258 cases were found (79.88%) when the mare aborted twins, followed by 45 cases (13.93%) births of two dead foals, 15 cases (4.64%) when there was a birth of one living and one dead foal and 5 cases (1.55%) of births of two live foals.

Figure 4 The result of multiple pregnancy in Old Kladruber Horse mares



Legend: 1. Column – twins abortion, 2. Column – 2 dead foals, 3. Column – 1 live and 1 dead foal, 4. Column – 2 live foals

The same factors as in the previous Figure were evaluated for reproductive outcomes of Old Kladruber Horse mares in Figure 4. There were 36 cases (75.00%) of abortion of twins, seven cases

(14.58%) of births of two dead foals and 5 cases (10.42%) of births of two live foals. The records of the birth of one living and one dead foal were not found.

Table 3 Relationship between breed and the pregnancy result

Variable	Chi-square	df	p
Pearson's chi-square test	14.56158	df = 3	p = 0.00223
Cramér, V	0.1981149		

Dependence between the mare's breed and result of a multiple pregnancy was evaluated by Pearson's chi-square test, where the null hypothesis was the assumption of independence of assessed characters. Since the p-value < α, the null hypothesis was refuted, therefore between mare's breed and result of multiple pregnancy exists a statistically significant correlation. However, given the values of Cramér factor described dependence is assessed as weak.

Table 4 Relationship between pregnancy result and the age

Variable	Chi-square	df	p
Pearson's chi-square test	49.31532	df = 48	p = 0.42035
Cramér, V	0.2104958		

Dependence between mare's age and multiple pregnancy result was also evaluated by using the chi-square test in which the null hypothesis was the assumption of independence of assessed characters. Given that p > α, the null hypothesis cannot be rejected, and therefore between mare's age and resulting multiple pregnancy there is no statistically significant correlation.

Table 5 The proportion of multiple pregnancies from the total number of admitted Thoroughbred and Old Kladruber Horse mares

Pregnancy result	The average of the total number of mated mares A1/1	The average of the total number of mated Old Kladruber Horse mares
Multiple pregnancies	1.53%	0.96%
Twins abortion	1.22%	0.72%
1 life and 1 dead foal	0.07%	0.00%
2 dead foals	0.23%	0.15%
2 life foals	0.01%	0.09%

In the Table 5 there are records of multiple pregnancy shown in Thoroughbred and Old Kladruber Horse mares. Data obtained from individual years of reproductive records were evaluated and were filled their average values here. From the total number of admitted mares were recorded incidence of multiple pregnancies in 1.53% of cases in the Thoroughbred. This result is contrary to the research of Hutton and Meacham (1968) which claims the incidence of multiple pregnancies in Thoroughbred mares, specifically at 2.4% and also Doležel et al. (2000) reporting 2.57% frequency. Wolc et al. (2006) also indicate a higher incidence - namely 3.5%. In Old Kladruber Horse was recorded the incidence of multiple pregnancy in 0.96, which approximates to the results of Doležel et al. (2000) who indicates the incidence in mares generally between 1.5 and 2.5%. Přibyl (1952) documents the rate of 1%-almost the same as found. In the case of twin's abortion specifically it was in the 1.22% and 0.72% of the cases, 1 alive and 1 dead foal recorded at 0.07% of the Thoroughbreds, in Old Kladruber Horse such result was not detected, 2 dead foals at 0.23% and 0.15% of cases and 2 live foals at 0.01% and 0.09%.

The average incidence of multiple pregnancy in the assessment of both breeds together was detected in 1.25%.

CONCLUSION

Based on the goals of this work the incidence of multiple pregnancy at 1.53% for the Thoroughbred and 0.96% for Old Kladruber Horse mares was evaluated. The successful birth of one or two foals were 20 cases of multiple pregnancies for 323 Thoroughbreds (6.19%) and 5 cases out of 48 in Old Kladruber Horse.

Based on the information gathered from available literature and my own work, it is possible to argue that multiple pregnancy is a persistent problem in horse breeding. Due to the development of effective reduction techniques already incurred multiparie ceased to be a mare with such a predisposition excluded from reproduction and this may result in an extension of that predisposition in the population. It offers the chance of a multiple pregnancy prevention rather than solving the problem already in place and recommendations on the prudent management of such mares and preferences of their exclusion from breeding, especially because of the probability of transmitting the character to offspring and thus also the indispensability of human intervention in equine reproduction.

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THE GROWTH INTENSITY OF ABERDEEN ANGUS IN ORGANIC FARMING

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Abstract: This studies was focus on compare growing abilities of aberdeen angus which are bred on four organic farms. All these organic farms are situated in region Zlin in area The White Carpathians. Compared were weights of animals at birth and weight in 120, 210 and 365 days. Weights of animals at birth were in all breeds quite balanced and reached 38.38 ± 3.57 kg. The average weight of calves in 120 days was during the years conclusively the lowest on the C farm and it was 177.93 ± 25.06 kg. The same situation happened ($p < 0.05$) at weighing in 210 days when distinctly ($p < 0.05$) the lowest weight in watched breeds was on the farm C. On annual weighing were the heaviest animals which were bred at farm C 416.28 ± 97.29 kg. Distinctly ($p < 0.01$) the lowest annual weight had animals from farm D 336.13 ± 47.12 kg although in 120 days animals had the highest weight most of all farms. Interesting was weight progression at farm C which as just the only one farm applied winter calving and animals could fully utilize pasturage. On this farm was supplemented feeding by concentrates.

Key Words: aberdeen angus, organic farming, growth, birth weight, 120, 210 and 365 days

INTRODUCTION

Aberdeen angus breed is second the most widespread in Czech republic. In 2013 were bred in our country 3719 cows of this beef cattle type (Kvapilík et al. 2015). Undemanding breeding conditions are one of the reasons why popularity of this breed is growing for beginning breeders. Aberdeen angus is very often bred in organic breeding.

Aberdeen angus is typical for his good growth abilities and daily average gain of male is 1000–1100 g/day and dam gain of weight is 850–950 g/day (Louda et al. 2000). Zahrádková et al. (2009) described this breed as undemanding, constitutionally solid with possibility breeding outside during whole year without stabling. Meat of Aberdeen angus breed is for his really specific properties high valuable (fragility, juiciness, marbling and typical flavour). Aberdeen angus is early breed and that's why this breed store fat really early.

Growth potential of cattle has big influence on economic efficiency of breeding. Šiler et al. (1980) consider growth as a basic measure of profitability. Large number of aspects have a big influence on growth. Nutrition and feeding technology in individually period of breeding play the most important role from external factors. The main internal factors include genetic predisposition of individual (breed, genotype, sex, mother, father, etc.). Ruminants have the slowest growth intensity but by modern genetic techniques is possible to achieve an increase of growth on valuable parts of cattle (Šubrt and Hrouz 2009). On the other hand increasing level of growth intensity can cause negatively affect on quality of beef (Mlynek et al. 2014).

MATERIAL AND METHODS

Farm descriptions

Animals have been reared on four organic farms. All the farms are situated in the Zlin region relatively near each other. Climatic and geographic conditions are almost similar. On farm A, B and D are used natural breeding. On the farm C was used natural breeding and artificial insemination. The Individual farms are marked by letters A - D, because breeders wanted to stay anonymous.

Beef cattle is breeding on the farm A since 1997. Animals are during whole year on pasture even in winter. In summer matter ration composed of pasture vegetation, water and mineral block. During the winter are animals fed by hay, water and mineral block. Calving are realized on pastures from late March to middle of June.

The farm B is situated near to town Zlin. This farm began breeding beef cattle in 2003. Animals are during whole year on pasture. Summer matter ration is same as on the farm A. Winter matter ration composed of hay, haylage, water and mineral block.

The farm C is situated in the White Carphathians. This farm is breeding aberdeen angus for 12 years. Calving of cows are realized on wintering ground from January to middle of April. During the winter is cattle fed by hay, haylage, concentrates (mixture of wheat and oats), mineral block and water. Summer matter ration is same as on the farm A and B.

Last farm D is the youngest. Beef cattle is breeding on the farm D since 2008. Breeding was set up buying heifers from farm A. Summer and winter matter ration are same as on the farm B. On this farm strive to increase number of animals.

Methodology of experiment

On farms were compared weight of animals at birth and weight in 120, 210 and 365 days. Data were obtained from yeild control of cows without market production of milk which is provided according to the Methodology yeild control of cattle without market production of milk. This methodology was published by Czech beef breedrs association. To analyse weights were used data from set up the breedings until 2015.

RESULTS AND DISCUSSION

Figure number 1 displayed average weights of animals on individual farms at birth, 120, 210 and 365 days. The final weights are average weights of heifers and bulls obtained during the whole time of farming. Weighing animals in year of life is little bit distorted because of number of checked animals in this period and this is in table number one. Birth weight of calves in organic breeding farms was balanced. An interesting fact was progress of average birth weight at all farms during the last 10 years. Figure number 2 shows an increase of birth weight of calves almost up 4.5 kg during 10 years. How is it show in table number 2 increases of birth weight in latest years could be caused by higher number of dams with more than 5 births. Koch and Clark (1955) published that cow's older than six years usually have calves with higher birth weight.

On the farm C was significantly ($p < 0.05$) the lowest weight of progeny in 120 days. This result was not expected because this farm exercised winter calving. Animals have while moving to pasture fully developed proventriculi and can take advantage of young pasture vegetation. The animals are supplementary feeding by concentrates. The highest weight in fourth month of calves life was on farm D 192.36 ± 36 kg. Weighing in 210 days was the lowest ($p < 0.05$) on the farm C 265.04 ± 39.55 kg. On the farm A in this control period animals reached an average weight 92.65 ± 39.16 kg. Animals which were breeding on the farm B had weight of 3.5 lower than animals from farm A. On the farm D was difference almost 6.5 kilograms.

The annual weighting of animals shows that significantly ($p < 0.01$) the lowest weight of breed was noted on the farm D (336.13 kg). According to my opinion this fact was caused because the fast growing animals was sold too early and before checkweighing in one year of life. Most of the animals which were weighted in life were heifers to renewal of herd. Heifers have lower intensity of growth than bulls. On the farm D was in year of life weihgted a low number of animals and it could caused distortion of annual weight. In rest of the farms were weights in 365 days more than 400 kg (A – 404.09 kg, B – 416.18 kg, C – 416.28 kg). On the farm C is interesting increase of weights where animals in 120 and 210 days reach the lowest weights from all farms. Increase of weights in year of life could be caused by regular suplementary feeding by concentrates.

From results is obviously that all farms realize selection of animals with appropriate level of growth. Heifers have good growth properties and constitution properties and that is reason why are kept on the farm to recover herd. From gained weights during the last 10 years is obviously that aim of

breeders is choose heifers with bigger body frame. This aim of breeders has positive influence on increasing weights of posterity in individual years of breeding.

Figure 1 The average weights of animals in breeds

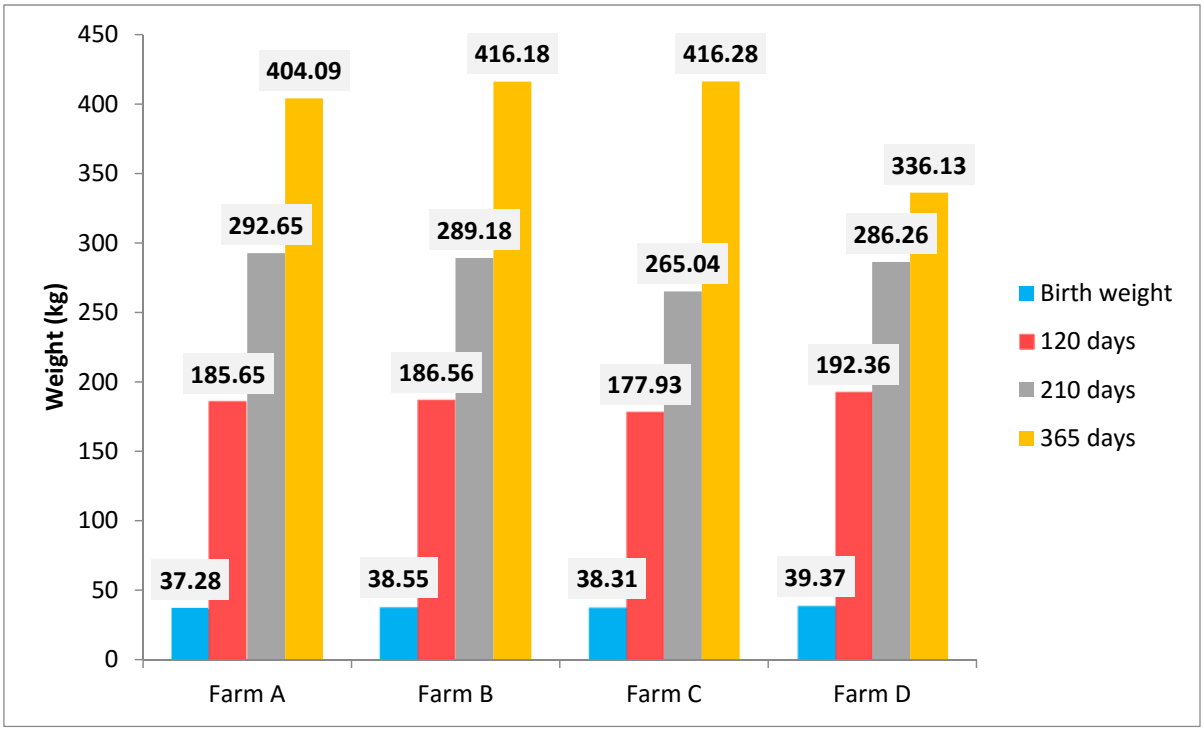

Figure 2 The progress of birth weight

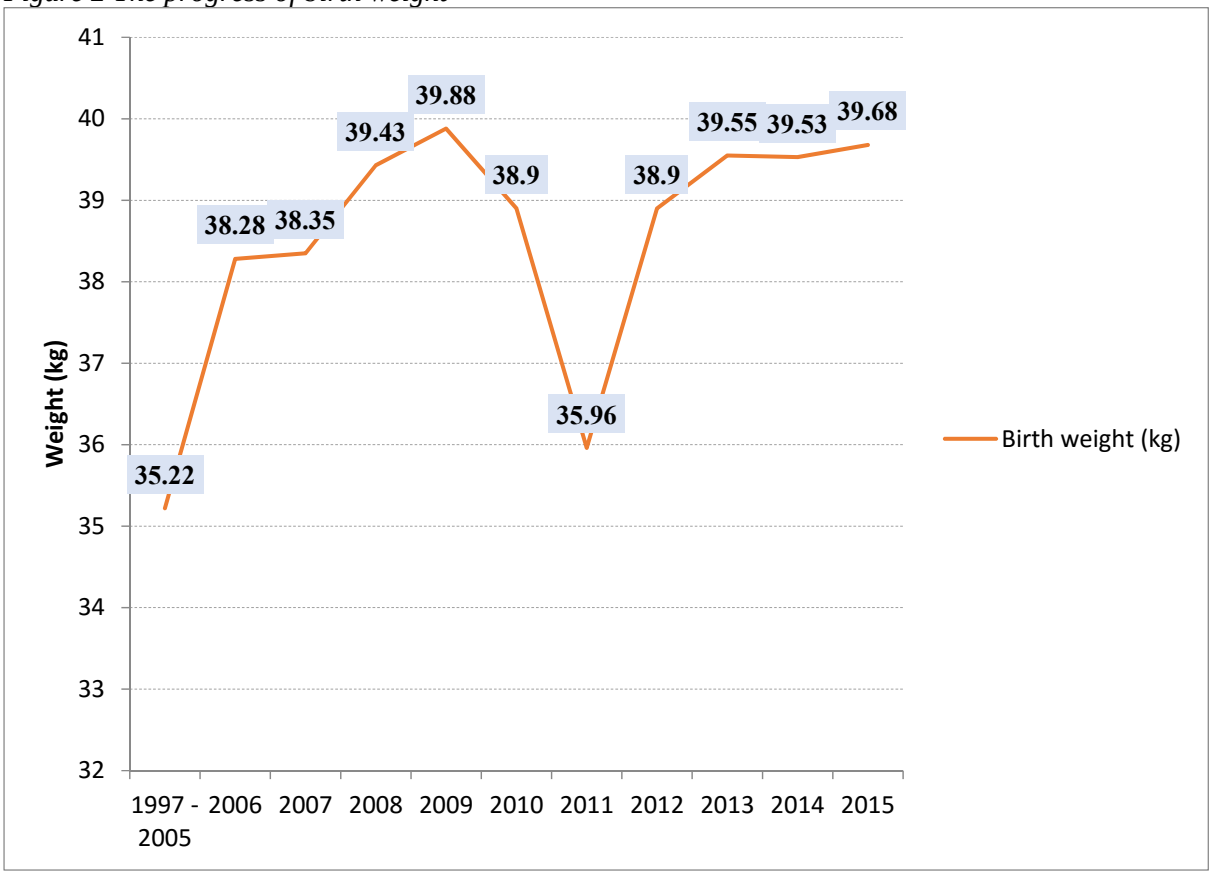

Table 1 The average weights of the animals on farms

Farm	Weight of animals (kg)											
	Count N	Birthweight		N	120 days		N	210 days		N	365 days	
		$\bar{x}$	s_x		$\bar{x}$	s_x		$\bar{x}$	s_x		$\bar{x}$	s_x
A	664	37.28	4.41	549	185.65	24.96	439	292.65^{a}	39.16	106	404.09^{A}	103.07
B	565	38.55	2.57	407	186.56	24.61	298	289.18^{a}	35.34	65	416.18^{A}	101.15
C	415	38.31	4.15	301	177.93^{a}	25.06	138	265.04^{b}	39.55	47	416.28^{A}	97.29
D	527	39.37	2.26	466	192.36^{b}	24.05	274	286.26^{a}	35.48	38	336.13^{B}	47.12
Total	2171	38.38	3.57	1723	185.63	25.08	1149	283.28	38.28	256	393.17	98.49

Different letters between the levels of each of these factors mean statistically significant difference (a, b = $p < 0.05$; A, B, = $p < 0.01$).

Table 2 The number of cows depending on the number of calves born

Categories of cows	Farm A	Farm B	Farm C	Farm D
To 5 calves	75	51	77	97
6–10 calves	37	33	30	34
Over 10 calves	15	12	0	0
Total	127	96	107	131

CONCLUSION

The results show that in organic farming is possible to achive very good weight gains without concentrated feedingstuffs. On farms A, B and D calves weighed at 120 and 210 days more than calves from farm C although there were feeding provided by concentrates. On farms A and B are the cows which had more than 10 calves during their life. This testifies to the longevity of cows and very good level of breeding on these farms. Their progeny can have better growth predispositions than dams with less than 5 calves. Farm D is the youngest farm and there is a lot of cows with less than 5 cows but young cattle has a very good intensity of growth.

Zlin region is suitable for grazing method of livestock because there is an optimum altitude and amount of precipitation. This region is very typical for breeding of beef cattle as for breeding a sheep.

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PRODUCTION AND QUALITY OF SPERMATIC FLUID OF BOARS DEPENDING ON BREED GROUPS

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Abstract: The aim of the present paper was to prove the influence of the breed on the qualitative and quantitative parameters of spermatic fluid of boars present at the insemination station. The research material consisted of 265 boars of breeds Czech Large White, Czech Landrace, Duroc, Line 38 and Line 48. The breed influence was identified in relation to the following values: volume of spermatic fluid (ml), concentration of sperms (in thousands per mm^3), sperm motility (%) and content of pathological sperms (%). In this paper it was confirmed that there are inter-breed differences in the quality and quantity of boar's spermatic fluid, but none of the breeds achieved unique exceptional results in all indicators. The average volume of spermatic fluid of boars oscillated within the range from 220.91 (Duroc) to 333.03 ml (Czech Landrace), sperm concentration was from 330.27 (Czech Landrace) to 741.74 thousand sperms per mm^3 (Duroc), sperm motility was from 71.20 (Duroc) to 74.62% (Line 48) and the content of pathological sperms was from 5.56 (Czech Large White) to 9.04% (Duroc). For assessment of quality of spermatic fluid from a comprehensive point of view, auxiliary data was calculated for individual breeds, namely total number of sperms and corrected number of sperms in the ejaculate. The total number of sperms oscillated within the range from 91.34 (Czech Large White) to 109.99 billions (Czech Landrace) and corrected number of sperms was from 64.27 (Czech Large White) do 75.84 billions (Line 48).

Key Words: boar, breed, hybrid combination, semen production, semen quality

INTRODUCTION

The economy and profitability of pig breeding consists at present especially in reduction of costs of pork production. The rate of profit of insemination stations of boars is given by characteristics of spermatic fluid. The insemination stations usually have boars of various breed and hybrid groups. There is, however, a presumption that the indicators of spermatic fluid are different for individual breeds and lines. The aim of the paper was to prove the influence of the breed on the qualitative and quantitative parameters of spermatic fluid for the purpose of optimisation of the number of boars of individual breeds in such a way that it is possible to evenly cover the demand for insemination doses.

MATERIAL AND METHODS

The objective of the study was to assess the influence of the breed on production and quality of spermatic fluid of boars present at an insemination station. The material analysed consisted of 14,098 samples of spermatic fluid obtained during the period from January 2011 to December 2015. The samples of spermatic fluid originated from 265 boars of these breeds: Czech Large White (CLW), Czech Landrace (CL), Duroc (D), Line 38: Duroc x Pietrain and Line 48: Czech White Paternal Line x Pietrain. All boars were at the insemination station under the same conditions of animal housing, treatment, feeding, ejaculate sampling and ejaculate assessment.

Concerning the identified quantity and quality indicators of spermatic fluid, the following ones were used for the analysis: spermatic fluid volume (ml), sperm concentration (in thousands per mm^3), sperm motility (%) and content of pathological sperms (%). The volume of spermatic fluid was ascertained there with the help of a volumetric cylinder. The sperm concentration was ascertained in a photometric way, by using Spekol 11 apparatus. Motility was ascertained under the microscope by way of a subjective estimation of percentage representation of sperms with a forward straight-line motion

within 15 minutes after the sampling of the spermatic fluid. The number of abnormal sperms was ascertained on the prepared sperm count in several view fields.

The data was processed in Microsoft Excel 2016 and subsequently statistically evaluated by using the STATISTICA program, version 12.0. The provability of the differences was ascertained with the help of the HSD test for different n values (n = number of subjects in groups). The *** symbol means $P < 0.001$, the ** symbol means $P < 0.01$, the * symbol means $P < 0.05$ and NS symbol means $P > 0.05$. The SD abbreviation means standard deviation.

For the purpose of comparison of quality of the spermatic fluid of individual breeds, the total number of sperms in the ejaculate (NO_T, in billions) was ascertained, together with the corrected number of sperms in the ejaculate (NO_C, in billions) (Smital et al. 2004):

$$NO_T = \frac{VO \times CO}{1000}$$

$$NO_C = NO_T \times \frac{MO}{100} \times \left(1 - \frac{AB}{100}\right)$$

where VO is the spermatic fluid volume (ml), CO is the sperm concentration (in thousands per mm^3), MO is the sperm motility (%) and AB is the content of pathological sperms (%).

RESULTS

Table 1 shows the ascertained results of the volume of spermatic fluid of boars according to individual breeds and lines. The highest average volume of spermatic fluid was ascertained at the Czech Landrace breed, namely 333.03 ml ± 146.58 ml. A volume lower by 26.98 ml was achieved at the Line 38 boars, namely 306.05 ± 117.51 ml ($P < 0.001$). The volume of spermatic fluid acquired from boars of the Czech Large White breed was on average 257.87 ± 108.20 ml, which represented reduction by 48.18 ml compared to Line 38 ($P < 0.001$). In comparison to Czech Large White, the volume ascertained at Line 48 was some 9.75 ml lower 248.12 ± 90.37 ml). The difference in the values of the volume between Czech Large White and Line 48 was, however, not statistically proven ($P > 0.05$). The lowest volume of spermatic fluid was obtained from the Duroc breed boars, with an average value of 220.91 ± 88.89 ml, which is a volume 27.21 ml lower in comparison to Line 48 ($P < 0.001$).

Table 1 Basic statistical characteristics of volume of spermatic fluid

Breed	n of samples	Semen volume (ml)	Statistical significance					
		Mean ± SD	Breed	CL	CLW	D	L38	L48
CL	2097	333.03 ± 146.58	CL		***	***	***	***
CLW	821	257.87 ± 108.20	CLW	***		***	***	NS
D	9791	220.91 ± 88.89	D	***	***		***	***
L38	736	306.05 ± 117.51	L38	***	***	***		***
L48	653	248.12 ± 90.37	L48	***	NS	***	***	

*** = $P < 0.001$; NS = $P > 0.05$*

The results of sperm concentration for individual breeds and lines are provided for in Table 2. The largest values of concentration of sperms were achieved for the Duroc breed sperms, namely 471.74 ± 187.01 thousand per mm^3. Spermatic fluid of the Line 48 boars contained a lower amount (by 33.06), compared to the Duroc breed, namely 438.68 ± 157.34 thousand per mm^3. For other breeds, the concentration of sperms oscillated below the value of 400. Particularly for the Czech Large White breed, the average concentration was 355.29 ± 157.82 thousand per mm^3, compared to the previous line (L 48) this is some 108.41 less ($P < 0.001$). Compared to the Czech Large White breed concentration, the value achieved at Line 38 was lower by 2.63 thousand per mm^3, namely 352.66 ± 151.70. The lowest

concentration of sperms was contained in the spermatic fluid of boars of the Czech Landrace breed (330.27 ± 146.52 thousand per mm^3). The difference in the sperm concentration (22.39) between Czech Landrace and Line 38 was not statistically proven (P > 0.05).

Table 2 Basic statistical characteristics of sperm concentration

Breed	n of samples	Concentration (thousand/mm)	Statistical significance					
		Mean ± SD	Breed	CL	CLW	D	L38	L48
CL	2097	330.27 ± 146.52	CL		*	***	NS	***
CLW	821	355.29 ± 157.82	CLW	*		***	NS	***
D	9791	471.74 ± 187.01	D	***	***		***	**
L38	736	352.66 ± 151.70	L38	NS	NS	***		***
L48	653	438.68 ± 157.34	L48	***	***	**	***	

**** = P < 0.001; ** P < 0.01; * = P < 0.05; NS = P > 0.05*

Another evaluated parameter was the sperm motility (Table 3). The highest motility was ascertained at Line 48 (74.62 ± 5.94%), the motility lower by 0.11% was achieved at Czech Large White breed, namely 74.51 ± 5.40% (P > 0.05). For the Line 38 boars the average motility ascertained was 73.90 ± 4.94%, which is a value 0.61 % lower in comparison to Czech Large White (P > 0.05). Spermatic fluid of the Czech Landrace boars contained 73.72 ± 7.80% of sperms with a good motility. The difference between Line 38 and Czech Landrace breed was statistically non-provable and amounted to 0.18% (P > 0.05). The lowest representation of sperms with a good motility was ascertained at the Duroc breed boars (71.20 ± 7.88%). In comparison to the Czech Landrace motility, the motility of the Duroc breed is 2.52% lower (P < 0.001).

Table 3 Basic statistical characteristics of sperm motility

Breed	n of samples	Motility (%)	Statistical significance					
		Mean ± SD	Breed	CL	CLW	D	L38	L48
CL	2097	73.72 ± 7.80	CL		NS	***	NS	NS
CLW	821	74.51 ± 5.40	CLW	NS		***	NS	NS
D	9791	71.20 ± 7.88	D	***	***		***	***
L38	736	73.90 ± 4.94	L38	NS	NS	***		NS
L48	653	74.62 ± 5.94	L48	NS	NS	***	NS	

**** = P < 0.001; NS = P > 0.05*

The percentage of occurrence of pathological sperms is shown in Table 4. The highest content of pathological sperms was ascertained at the Duroc breed (9.04 ± 7.55%). Spermatic fluid of the Czech Landrace breed boars contained 7.60 ± 6.92% of pathological sperms, i.e. some 1.44% less than it was the case for the Duroc breed (P < 0.001). In comparison with Czech Landrace, the Line 38 boars had some 0.40% of pathological sperms less, i.e. 7.20 ± 5.62% (P > 0.05). Spermatic fluid of the Line 48 boars contained 6.63 ± 5.35% of pathological sperms, which is some 0.57% less compared to Line 38 (P > 0.05). The lowest representation of pathological sperms was identified at boars of the Czech Large White breed, namely 5.56 ± 4.97%. The difference in the content of pathological sperms between Czech Large White and Line 48 was 1.07% (P > 0.05).

Table 4 Basic statistical characteristics of percentage of occurrence of pathological sperms

Breed	n of samples	Pathological changes (%)	Statistical significance					
		Mean ± SD	Breed	CL	CLW	D	L38	L48
CL	2097	7.60 ± 6.92	CL		***	***	NS	NS
CLW	821	5.56 ± 4.97	CLW	***		***	***	NS
D	9791	9.04 ± 7.55	D	***	***		***	***
L38	736	7.20 ± 5.62	L38	NS	***	***		NS
L48	653	6.63 ± 5.35	L48	NS	NS	***	NS	

**** = P < 0.001; NS = P > 0.05*

In Table 5 it is possible to see the total and corrected number of sperms. As far as the total number of sperms is concerned, the highest value was achieved by the Czech Landrace breed (109.99 billion sperms), for Line 48 the number ascertained was 108.85 billion, for Line 38 it was 107.93 billion, for Duroc it was 104.21 billion and the number ascertained for Czech Large White was 91.34 billion. In the case of the corrected number of sperms, however, the order was changed. The highest content was ascertained for Line 48, namely 75.84 billion, the corrected number of sperms for Czech Landrace was 74.92 billion, for Line 38 it was 74.02 billion, at Duroc 67.49 billion and for Czech Large White this number was 64.27 billion.

Table 5 Total and corrected number of sperms by the different breeds

Breed	NO_T (billions)	NO_C (billions)
CL	109.99	74.92
CLW	91.34	64.27
D	104.21	67.49
L38	107.93	74.02
L48	108.85	75.84

DISCUSSION

The largest volume of spermatic fluid was achieved at the Czech Landrace breed, which partially corresponds to conclusions of Smital (2002) and Pinart and Piugmulé (2013), who state that the breeds excelling in this parameter are Czech Landrace together with Czech Large White. In this study it has been found out that the volume achieved for the Czech Large White boars was some 75.16 ml lower than the volume achieved for the Czech Landrace boars. The lowest volume of spermatic fluid was acquired from the Duroc breed boars, which is in conformity with conclusions of Pinart and Piugmulé (2013). Wysokińska and Kondracki (2013) state that Line 38 achieves a volume higher than the Duroc breed, which has been confirmed in the present study.

Kliment (1986) states that Duroc and its crossbreeds feature a higher concentration of sperms in comparison to the other breeds. In this study it is possible to agree only with the statement that Duroc exceeds the other breeds and crossbreeds. The Line 38 boars (Duroc x Pietrain) achieved lower concentrations than Duroc, which corresponds to the conclusions of Wysokińska and Kondracki (2013). From the results it is obvious that a low concentration was achieved also for Czech Landrace, Line 38 and Czech Large White. Lower values of concentration at Czech Large White in comparison to the other breeds are confirmed also by Ciereszko et al. (2000). Smital (2002) states that low concentrations are registered at the Czech Landrace and Czech Large White breeds.

The lowest sperm motility was found out at the Duroc breed, while the sperm motility of Line 48, Czech Large White, Line 38 and Czech Landrace achieved higher values. These results are different from the study by Smital (2002), who states that lower percentages of sperms with a good motility are contained in the spermatic fluid of boars of the Czech Landrace and Czech Large White breeds. Wysokińska and Kondracki (2013) did not register any difference between motilities of the Duroc and

Line 38 boar breeds, unlike the present study where the motility values between Duroc and Line 38 differed statistically in a highly provable way.

The breeds with a high content of pathological sperms include Smital (2002) Czech Landrace and Czech Large White. It is only possible to agree with this conclusion in the case of Czech Landrace, but the highest occurrence of pathological sperms at all was found out at the Duroc breed. Conversely, the Czech Large White breed achieved the lowest (and therefore the best) values.

Louda et al. (2001) state that for hybrid boars it is possible to observe larger testicles and therefore also associated higher production of sperms. In this paper it is not possible to unambiguously confirm such a statement. The highest corrected number of sperms was ascertained at Line 48, a high NO_c was contained also in the spermatic fluid of Czech Landrace and Line 38 boars. The highest total number of sperms was found out for the Czech Landrace breed, a little smaller quantity was contained in the spermatic fluid of the Line 48 and Line 38 boars. According to Smital (2001), the last place in the number of sperms belongs to boars of the Duroc breed. In the case of the present study it was found out that in comparison to Duroc, the Czech Large White breed features a lower number of sperms, which is confirmed also by Knecht et al. (2014). Wysokińska and Kondracki (2013) state that the total number of sperms of the Line 38 boars is higher than the one of the Duroc boars, which is in conformity with the results of this paper.

CONCULSION

In this study it was confirmed that there are differences between breeds in terms of quantity and quality of boar's spermatic fluid, but there was not any breed which would be excellent in all indicators in an unambiguous way. Also from the results of the total and corrected number of sperms it is obvious that from each breed it is possible to expect a different number of sperms in the ejaculate, and this is associated also with the different quantity of the insemination doses produced. This factor should be taken into consideration during organisation of breeding activities, e.g. during inclusion of new boars and rejection of older boars. This can help to maintain the continuity of production of insemination doses.

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THE EFFECT OF VARIOUS DIETARY MAGNESIUM LEVELS ON GROWTH PERFORMANCE AND CARCASS YIELD OF BROILER CHICKENS

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Abstract: The aim of this study was to evaluate the effect of various magnesium doses in the diet on a growth performance of broiler chickens and carcass yield. Magnesium (Mg) was added to the feed mixtures in $MgSO_4$ form. The basic feed ration contained 1.71 g of Mg per 1 kilogram dry matter. The control group received the feed with added 2.5 g/kg $MgSO_4$. The feeds for two experimental groups contained the addition of 1 g/kg $MgSO_4$ and 7.5 g/kg $MgSO_4$ (groups Exp1; Exp2; respectively). In the trial, feed intake and live weight of chickens were monitored. The experiment was conducted from day 11 to day 35 of chickens age. Experimental animals were weighed and slaughtered at the end of the trial. Feathers were removed and chickens were eviscerated. Carcass yield was calculated. Selected chickens were deboned and breast muscle and leg muscle were weighed. These parameters were expressed by the percentage of live weight of breast and leg muscle. The total magnesium levels in experimental groups (1.91 g/kg diet and 3.21 g/kg diet) had a negative effect on slaughter weight compared to the control group (2.21 g/kg Mg). This trend was noticeable in evaluation of carcass yield, as well. The differences between groups were statistically non-significant ($P > 0.05$). Statistically significant difference ($P < 0.05$) was recorded between control group (2.21 g/kg Mg of the diet) and experimental group (3.21 g/kg Mg of the diet) on deboned thigh muscle. Tested magnesium levels had non-significant effect to chicken production parameters but had a positive effect on carcass yield (thigh muscle).

Key Words: magnesium, carcass yield, poultry nutrition, live weight gain

INTRODUCTION

Minerals such as calcium (Ca) and magnesium (Mg) have important biological functions and must be provided in adequate amounts in poultry diets (Driver et al. 2005, Blair 2008). Magnesium is one of the most abundant divalent cations in living cells and plays a vital role in many cellular processes (Chakraborti 2002, Bo and Pisu 2008). Magnesium is involved in the metabolism of amino acids, fat and sugars and in bone Ca and vitamin D metabolism (Morii 2007). Magnesium acts as a cofactor or an activator of many critical enzymes for the reactions involving ATP that energize all major metabolic pathways (NRC 1994). Recommended nutrient content by Zelenka et al. (2007) indicates the delivered amount of magnesium in feed mixtures for fattening chickens 0.5 g/kg dry matter (DM). According to NRC (1994) Mg requirements for poultry do not exceed 0.6 g/kg dry matter. In the different studies was stated, that magnesium deficiency in growing poultry is accompanied by poor growth and feathering, incoordination, convulsive attacks, coma, and death (Florianczyk and Stryjecka-Zimmer 2003, Morii 2007). On the other side, high Mg level in the diet may cause diarrhoea in chickens (Lee and Britton 1987). The aim of this study was to evaluate the effect of distinct magnesium doses in the diet on a growth performance and carcass yield of broiler chickens.

MATERIAL AND METHODS

An experiment was performed with cockerels of Ross 308 hybrid (n = 96) which were fattened in cage batteries from 11 to 35 day of age. Cockerels were divided into 3 groups in four replications. There were 8 chickens per replicate pen. Prior to formulating the diets, feed components were analysed for Mg

content and the data were used to formulate the experimental diets. The basal diet contained 1.71 g Mg per kilogram DM. The composition of the basal diet is shown in Table 1. The nutrient composition of the diets is shown in Table 2.

Magnesium was added as $MgSO_4$. Control group received feed mixture with added $MgSO_4$ in the dose of 2.5 g/kg $MgSO_4$. Two experimental groups contained $MgSO_4$ in dose of 1 g/kg and 7.5 g/kg of fodder DM (groups Exp1; Exp2; respectively). See Table 3.

The crumbly feed mixture was supplied *ad-libitum* and its consumption was recorded every day. Access to drinking water was also *ad-libitum*. Weighting of chickens was carried out at the start and at the end of the trial. Microclimate and lighting regime were modified according to the technological instructions for the breed Ross 308.

The experimental animals were weighed and slaughtered. Chickens (n = 6) were selected randomly and carcasses were weighted. Breast muscle and deboned leg muscle were weighted and their percentage of live body weight was expressed.

Table 1 Composition of the basal diet (g/kg)

Component	C	Exp1	Exp2
Corn	340	340	340
Wheat	315	315	315
Soybean meal	260	260	260
Sunflower oil	40	40	40
Vitamin-mineral premix*	20	20	20
Experimental premix**	20	20	20
Chromium oxide	5	5	5

**Legend: premix content of one kg: lysine 101.65 g/kg, methionine 135.63 g/kg, threonine 51.22 g/kg,calcium 200 g/kg, phosphorus 98.19 g/kg, natrium 62.89 g/kg, magnesium 0,12 g/kg, sulphur 0.39 g/kg, chlorine 119.69 g/kg, copper 752.5 mg/kg, iron 3768.6 mg/kg, zinc 3400 mg/kg, manganese 6046.07 mg/kg, cobalt 11 mg/kg, iodine 47.95 mg/kg, selenium 8.96 mg/kg, retinol 680000 IU, cholecalciferol 250000 IU, alfatocoferol 2250 mg/kg, K3 74.8 mg/kg, B1 206.44 mg/kg, B2 344 mg/kg, B6 300.44 mg/kg, B12 1999.2 mg/kg, biotin 11 mg/kg, niacinamid 1793.4 mg/kg, calcium pantothenate 676.2 mg/kg, folic acid 82.8 mg/kg, choline chlorid 9000 mg/kg*

***Experimental premix: Content different levels of $MgSO_4$ according to Table 3*

Table 2 Nutrient composition of the basal diet (g/kg)

AME (MJ/kg)	12.54
Crude protein	191.4
Ether extract	6.08
Crude fibre	2.56
Ash	6.01

Table 3 Addition of $MgSO_4$ (g/kg) and total levels of Mg (g/kg) in the diets

	C	Exp1	Exp2
$MgSO_4$	2.5	1	7.5
Total Mg	2.21	1.91	3.21

Data has been processed by Microsoft Excel (USA) and Statistica version 12.0 software (CZ). One-way analysis (ANOVA) was used. To ensure statistical differences Sheffe´s test was applied and differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Feed consumption

The differences in the feed consumption (Table 4) were statistically insignificant (P > 0.05) among evaluated chicken groups. With higher level of magnesium in the feed mixture food intake is also higher. Van Der Hoeven-Hangoor et al. (2013) on the contrary stated decreasing food intake with increasing magnesium level in feeds. Further, they have found that the group with Mg content of 0.255 g/kg feed DM had an average feed intake 3484 g per chicken, meanwhile the group with Mg content of 2.040 g had an average feed intake of 3395 g per chicken. According to Ross Broiler Management Handbook (2014) average feed intake is 3230 g from 11 to 35 days of age. Nkukwana et al. (2014) are showing similar results, feed consumption of 3230 g per chicken (0–35 day of age).

Table 4 Average total feed consumption per chicken and trial

Group	n	Mean (g) ± standard deviation		
C	4	3588.12[a]	±	62.23
Exp1	4	3538.44[a]	±	198.11
Exp2	4	3590.00[a]	±	611.02

[a] *- Differences between groups are not statistically significant (P > 0.05)*

Body weight gain

Table 5 Mean live weight and body weight gain of chickens (n = 32 per group)

Group	C			Exp1			Exp2		
	Mean (g) ± standard deviation								
Start of the trial	261[a]	±	45.68	255[a]	±	42.88	257[a]	±	42.12
End of the trial	2103[a]	±	371.84	1888[a]	±	435.18	1866[a]	±	515.83
Body weight gain per trial	1842[a]	±	360.24	1632[a]	±	412.04	1609[a]	±	499.33

[a] *- Differences among groups are not statistically significant (P > 0.05)*

Table 5 shows that the control group achieved at the end of the experiment the highest average weight (2103 ± 371.84 g), meanwhile the experimental groups had lower weight. The differences among groups were evaluated as statistically insignificant. The results in our experiment shows that the low and the high levels of magnesium may reduce body weight of broilers.

According to the Ross Broiler Management Handbook (2014) the body weight at 35 days of age should be 2144 g. Van der Hoeven-Hangoor et al. (2013) noticed in their study that feeding with $MgSO_4$ in the dose of 0.255 g/kg of feed resulted in the chicken´s weight 2064 g at the end of the experiment (36 days of age). They also showed that with diet containing 2.040 g/kg $MgSO_4$ feed DM the weight at the end of feeding period was 1987 g. Their experiment shows that with increasing dose of Mg the chickens weight is decreasing at the end of the feeding period. These results are in accordance with values observed in our experiment. In a previous study, Lee and Britton (1980) found similar conclusions. They have demonstrated significantly lower weight at slaughter when higher Mg content was fed chickens in comparison with the control group. They experienced higher mortality rates and growth abnormalities in experimental chickens.

Carcass yield

The carcass yield evaluation is shown in Table 6. The conclusion is that the control group had higher carcass yield in comparison with the experimental groups. However, these differences were not evaluated as statistically significant. Similar trend was recorded on proportional representation of breast muscle. The control group had shown a higher proportion of thigh muscle (P < 0.05). Also Salmanzadeh et al. (2012) have not recorded statistical difference among control and experimental groups fed Mg in their study. The carcass yield of experimental chicken group was 67.2% and 67.3% of control group. Proportion of breast and thigh muscle was similar for both groups. Meanwhile Ross Broiler Management

Handbook (2014) shows that weights of deboned breast and thigh muscle should be slightly higher than in our experiment.

Table 6 Carcass yield

Group	n	Carcass			Breast meat			Leg meat without bone		
		Mean (%) ± standard deviation								
C	6	70.18[a]	±	1.04	19.33[a]	±	0.74	15.40[b]	±	0.86
Exp1	6	69.29[a]	±	1.02	18.73[a]	±	1.83	14.91[ab]	±	1.01
Exp2	6	69.76[a]	±	1.48	19.00[a]	±	0.67	13.35[a]	±	1.27

[a,b] – different letters mean statistically significant differences (P < 0.05)

CONCLUSION

The total magnesium levels in experimental groups (1.91 g/kg diet and 3.21 g/kg diet) had a negative effect on slaughter weight compared to the control group (2.21 g/kg Mg). This trend was noticeable in evaluation of carcass yield, as well. The differences between groups were statistically non-significant (P > 0.05). Statistically significant difference (P < 0.05) was recorded between control group (2.21 g/kg Mg of the diet) and experimental group (3.21 g/kg Mg of the diet) on deboned thigh muscle. Tested magnesium levels had non-significant effect to chicken production parameters but had a positive effect on carcass yield (thigh muscle).

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THE INFLUENCE OF ORGANIC OR INORGANIC SELENIUM SUPPLEMENTATION ON SELENIUM STATUS OF BEEF COWS AND THEIR CALVES

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Abstract: The aim of this experiment was to evaluate the influence of inorganic or organic selenium supplementation to pregnant beef cows (n = 12). Cows were divided into two groups per six animals and supplemented with two different forms of selenium: group A was given inorganic sodium selenite, group B was given organic selenomethionine. Before the start of supplementation cows had average glutathione peroxidase (GPx) activity in whole blood 847.07 ± 170.77 μkat/l and 791.30 ± 112.30 μkat/l (group A and B, respectively). The whole blood concentration of Se in group A was 136.70 ± 27.97 μg/l and in group B was 95.77 ± 20.05 μg/l. After parturition cows had higher mean activity of GPx by 26% and by 45% in group A and B, respectively. The mean concentration of selenium and GPx activity in whole blood of calves in group A was 155.35 ± 20.03 μg/l and 1143.08 ± 150.04 μkat/l and in group B 105.30 ± 24.26 μg/l and 1020.58 ± 304.08 μkat/l (Se and GPx, respectively). It can be concluded that both forms of selenium had analogical biological effect on focused parameters.

Key Words: sodium selenite, glutathione peroxidase, selenomethionine, Charolaise

INTRODUCTION

Selenium (Se) is a structural component of specific proteins called selenoproteins. The most important are glutathione peroxidase (GPx), thioredoxin reductase, selenophosphate synthetase, selenoprotein P, selenoprotein W and 18 kDa-selenoproteins (NRC 2007). Selenium contained in plants with bond to peptides and amino acids is present in the form of organic as selenomethionine. Selenium in this form (as the amino acid) is taken up and incorporated into tissues and becomes well usable for all kinds of animals. Unfortunately, roughages based rations do not contain the minimum amount of selenium 0.1 mg/kg (Nehasilová 2005). The most common and practical delivery of selenium in deficiency can be refilling to the mineral feed. Form of addition is particulate mineral mixtures or mineral licks with selenium either in inorganic form such as sodium selenite or in organic form as selenomethionine from *Saccharomyces cerevisiae*. The aim of this experiment was to evaluate the influence of inorganic or organic selenium supplementation to pregnant beef cows.

MATERIAL AND METHODS

Characterization of experimental design

The trial was conducted on twelve pregnant cows of Charolaise breed. Two months before expected calving cows were divided into two groups and supplemented with two different forms of selenium. Group A (n = 6) was given inorganic sodium selenite. To B group (n = 6) was given organic selenomethionine. The cows were examined to selenium status (Se concentration and GPx activity in whole blood) before the start of supplementation. Feed ration for cows during the experiment is presented in tables 1 and 2.

Next whole blood samples for the evaluation of Se status of cows and their calves were collected within 12 hours after parturition. The blood samples of cows were taken from the tail vessel and calves from the jugular vein puncture.

Table 1 Feed ration for pregnant cows (per day)

Rye silage	30 kg
Lucerne-grass hay (1 : 5)	5 kg
Barley straw	*ad-libitum*
*Supplemental mineral mix 1A/1B	*ad-libitum*

**Supplemental mineral mix composition of 1A per 1 kg: calcium 6%, phosphorus 6%, natrium 11.5%, magnesium 12% Nutritional additives: calcium iodate 120 mg, retinol 1 000 000 IU, cholekalciferol 100 000 IU, tocopherol 1,200 mg, tocopherol as alfa-tokoferol 1,080 mg, cooper as $CuSO_4$ 1,800 mg, manganesse as MnO 2,000 mg, zinc as ZnO 3,500 mg, cobalt as cobalt acetate 40 mg and selenium as sodium selenite 50 mg. Supplemental mineral mix composition of 1B per 1 kg was the same except selenium as selenomethionine from Saccharomyces cerevisiae at dose 50 mg*

Table 2 Feed ration for cows after calving (per day)

Rye silage	10 kg
Maize silage	10 kg
Sorghum silage	10 kg
Lucerne-grass hay (1 : 5)	5 kg
Barley straw	*ad-libitum*
*Supplemental mineral mix 2A/2B	*ad-libitum*

** The composition of the supplemental mineral mix 2A and 2B for cows after calving per 1 kg, was the same composition of nutritional additives such as supplemental mineral mix 1A and 1B, the difference was only the analytical aspects, specifically contained 12% calcium, compared to 6% in supplemental mineral mix 1A, 1B*

Laboratory analysis

Determination of the concentration of selenium was carried out in a biochemical laboratory by Hydride AAS techniques after high pressure microwave digestion of samples in the presence of nitric acid and hydrogen peroxide according to Pechová et al. (2005). The GPx activity was determined in a specialized biochemical laboratory by photometric method according to Paglia and Valentine (1967) using commercial set Randox on the device ELLIPSE AMS SpA, Italy.

The data were processed by Microsoft Excel (Microsoft, USA). To ensure evidential differences Student´s t-test was applied and $P < 0.05$ was regarded as statistically significant difference.

RESULTS AND DISCUSSION

The supplemental mineral mix was taken by animals in average amounts of 130 g per animal per day in Group A. In the B group was 128 g per animal per day, which is within the range of the manufacturer's instructions.

The results of selenium status of cows and their calves are presented in Table 3.

Table 3 The mean values (x) and standard deviation (sd) of selenium concentration in whole blood and the activity of GPx in whole blood of cows in both experimental groups (group A supplemented by inorganic selenium and group B supplemented by organically bound Se)

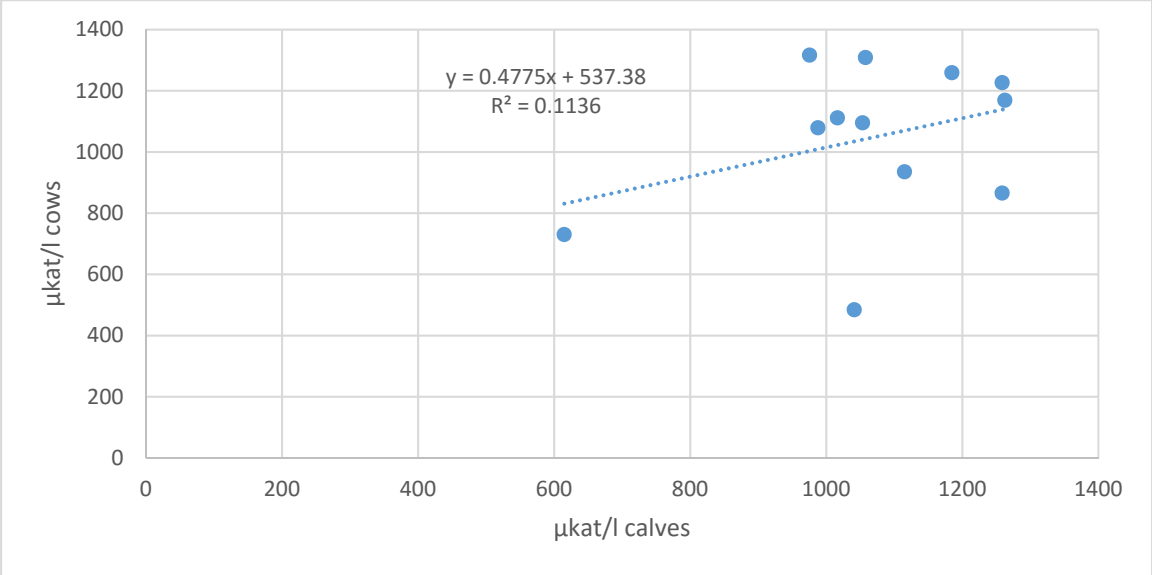
	Cows				**Calves**	
	Two months before parturition		Day of parturition		Day of birth	
	x	sd	x	sd	x	sd
Se (µg/l) - A group	136.70	27.97	140.79	23.70	155.35	20.03
Se (µg/l) - B group	95.77	20.05	100.55	19.02	105.30	24.26
GPx (µkat/l) - A group	847.07	170.77	1069.28	82.48	1143.08	150.04
GPx (µkat/l) - B group	791.30	112.30	1144.58	128.60	1020.58	304.05

According to Pavlata et al. (2002) who determined and evaluated for practical use GPx activity over 600 µkat/l as the adequate level of selenium status of organism, can rate the measured activity of GPx as sufficient at the start of trial. After an average time of 70 days from the beginning of

administration of mineral supplements the blood collected after birth again assayed GPx. Group A had the activity of GPx 1069.28 ± 82.48 μkat/l and Group B 1144.58 ± 128.60 μkat/l.

It was also determined correlations of GPx activity between cows and their calves (r = 0.337). The correlation coefficient is not statistically significant (Figure 1). The same results published Pavlata et al. (2004). In contrast, significant relationship between GPx in cows and calves determined Pavlata et al. (2003) in study on 24 mothers and calves.

Figure 1 Relationship of GPx activity between cows and their calves (n = 12)



The increase of selenium concentration during experiment in group A was 2.99% and in group B by 4.99%. The concentration of selenium in the blood of calves in group A was 155.35 ± 20.03 μg/l and in group B was 105.30 ± 24.26 μg/l. The differences between groups was statistically significant ($P < 0.05$) but this difference was significant at the beginning of trial, too. Therefore, results can´t be interpret as effect of supplementation of various forms of selenium. The similar increasing/differences of selenium status at the day of parturition in cows and their calves was recorded through supplementation of organic and inorganic forms of selenium.

CONCLUSION

Based on our results we can conclude there was no evidence of significant difference in the selenium status through supplementation of inorganic or organic selenium to pregnant cows. Both forms of selenium had similar biological effect.

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BASIC CARCASS CHARACTERISTICS OF LAMBS OF ŠUMAVSKÁ SHEEP AND ITS CROSSBREDS WITH SUFFOLK AND TEXEL

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Abstract: The main aim of the study was to evaluate basic carcass characteristics of purebred lambs of Šumavská breed (S) and its crossbreds with Suffolk (SF) and Texel (T). An integral part of the study was the evaluation of the effect of genotype on weights and proportions of kidney, kidney fat and basic non-carcass traits and composition of tissues in the left leg. The experiment was carried out on an organic farm in Proseč in 2015 and three different genotypes were included in the experiment: S 100 (n = 8), T 75 S 25 x S (n = 11) and T 75 S 25 x SF (n = 8). All lambs were males. As expected the genotype had a significant effect on most of the indicators. Lambs were slaughtered at approximately the same age, however in crossbreds (T 75 S 25) x SF the highest daily gain (0.168 g/day) and carcass yield (44.71%) were found. The best conformation score (3.87) and the highest heart and liver weights and proportions: (0.20 kg and 0.55%) and (0.53 kg and 1.46%) respectively were found in this group. Also the levels of muscle and fat in the left leg were 2.05 kg and 79.77%. In contrast, in purebred lambs of Šumavská sheep the lowest daily gain (0.124 g/day), carcass yield (38.38%) and proportion of muscle + fat in left leg (74.60%) were found. The results of the experiment indicate that use of commercial crossing of ewes of Šumavská breed with rams of meat breeds has a positive impact on growth and carcass quality of lambs. However, due to the relatively low number of lambs in our experiment it will be necessary to continue in monitoring of these genotypes.

Key Words: lamb, males, carcass characteristics, Šumavská sheep, non-carcass characteristics

INTRODUCTION

At present the main product of domestic sheep breeding is so called „heavy lamb“, which is the lamb with carcass weight higher than 13 kg. For production of these lambs mainly meat breeds are used. However for this production are also very often used lambs originating from commercial crossing of dual purpose breeds in maternal position and meat breeds in sire position. On the other hand in dairy breeds on domestic farms are produced so called „light lambs“, which are lambs with lower carcass weight than 13 kg, but this production is very limited because low number of dairy sheep is reared in the Czech Republic (Koutná et al. 2016a)

One of the most important mixte breeds reared in the Czech Republic is Šumavská sheep. Šumavská sheep is relatively resistant breed against hard climatic condition and due to this breed is above all reared in mountainous areas. In order to improve the growth and carcass value (mainly the meatiness) of lambs the ewes of this breed are very often crossed with males of meat breeds namely with Suffolk and Texel (Koutná et al. 2016b).

The carcass value of lambs is affected by a lot of different factors while the most important factors are breed, nutrition, sex, breed management and health. The effects of above mentioned factors were evaluated in the studies which were carried out by Gutiérrez et al. (2005), Teixeira et al. (2005), Shaker et al. (2002), Pérez et al. (2007) and Silva Sobrinho et al. (2003).

The main aim of our experiment was to evaluate basic carcass characteristics of male lambs of Šumavská sheep and their crossbreds with Suffolk and Texel. An integral part of the study was the

evaluation of the effect of genotype on weights and proportions of kidney, kidney fat and basic non-carcass traits and composition of tissues in the left leg.

MATERIAL AND METHODS

Assessment of the effect of genotype on basic carcass and non-carcass characteristics of purebred lambs of Šumavská breed (S) and its crossbreds with Suffolk (SF) and Texel (T) was carried out on an organic farm in Proseč in the Pardubice region (altitude 520 m, average annual temperature 6.1 °C, precipitation 800 mm). The experiment was carried out in 2015 and three different genotypes were included in the experiment: S 100 (n = 8), (T 75 S 25) x S (n = 11) and (T 75 S 25) x SF (n = 8). All lambs were males. Lambing was carried indoors, during March and April 2015. The daily feed ration (DFR) of the ewes in the period from parturition until the end of April consisted of meadow hay (*ad libitum*) and organic mineral lick (*ad libitum*). The DFR of the lambs during the same period consisted of mother's milk (*ad libitum*) and organic mineral lick (*ad libitum*); the lambs had also free access to the feedstuff of their mothers. Since May 1st until the end of the experiment the DFR of ewes consisted of grazing on permanent pasture (*ad libitum*) and mineral lick (*ad libitum*). The DFR of lambs in the same period consisted of mother's milk (*ad libitum*) until the weaning, grazing on permanent pasture (*ad libitum*) and mineral lick (*ad libitum*). The weaning of lambs was carried at the age of about 5 months. All animals were reared in one flock under identical conditions without any discernible differences regarding nutrition or management.

All lambs were weighed at birth (LW0) and before slaughter (LWS). The average live weight at the slaughter was 30.43 kg in S 100, 32.30 kg in (T 75 S 25) x S and 36.19 kg in (T 75 S 25) x SF. The average age of lambs at the slaughter were 220 in S 100, 207 in (T 75 S 25) x S and 191 days in (T 75 S 25) x SF. Daily gain (DG) was calculated in grams (g) in the interval from LW0 to LWS.

At the end of the experiment, after 24 hours of starvation, the slaughters of lambs were carried out. On the day of slaughter, live weights, age of lambs and weights of skins were recorded. After 24 hours of refrigeration (+4 °C) the evaluation of conformation and fatness of all carcasses was carried out. Simultaneously the weights of cold carcass, leg, shoulder, kidney, kidney fat and all non-carcass components (heart, lung + trachea, liver and spleen) were determined. On the same day the weights of muscle and bones from the left leg were also recorded. From the above mentioned data were subsequently calculated individual proportions. The conformation score (an extent of the scale from S = exceptional to P = poor conformation) and fatness score (the scale from 1 = very low to 5 = very high fatness) were assessed according to the S.E.U.R.O.P. (Commission Regulation EEC 461/93). For the purpose of statistical analysis (Table 1), the scale of the conformation score was quantified from the grade S = 1 to the grade P = 6.

Statistical analyses were performed using the STATISTICA software, version 12. ANOVA analysis was used to study the differences in the basic carcass characteristics, kidney, kidney fat, basic non-carcass traits and tissues in left leg in all three independent groups of genotypes. Sheffe's test was used by post-hoc analyses to identify individual significant differences between means. The differences were considered significant if $P \leq 0.05$.

RESULTS AND DISCUSSION

Evaluation of the effect of the genotype on basic carcass characteristics is presented in Table 1. As expected, genotype had an effect on daily gain in the period from birth to slaughter, while significantly higher daily gains were found in both groups of crossbreds compared to purebred lambs of Šumavská sheep. The same trend was also reported by Shaker et al. (2002) and Shaker et al (2010), however Costa et al. (2009) did not find significant effect of genotype on the growth. Genotype also had a significant effect on carcass yield, which is line with Kuchtík et al. (2011), while the highest carcass yield was found in (T 75 S 25) x SF. By contrast, Shaker et al. (2002) and Gutiérrez et al. (2005) did not find a significant effect of genotype on this indicator**.** As for carcass yield, it is necessary to point out that in all groups of lambs their levels were relatively low, however, comparable with data published by Rodrigues et al. (2006) and Teixeira et al. (2004). Genotype had further effect on the weights of leg and shoulder and on the proportion of the shoulder, which is in accordance with Cloete et al. (2004). On the other hand, Bingöl et al. (2006) did not recorded the effect of genotype on the weights of leg and

shoulder, but they found a significant effect of genotype on the proportion of the shoulder. Significantly the best conformation was observed in lambs (T 75 S 25) x SF when their conformation was comparable with data that reported in lambs of Suffolk breed Komprda et al. (2012). On the other hand, in the other two groups the conformation scores were significantly worse**.** The similar trend was recorded by Pinďák et al. (2011) and Carrasco et al. (2009).

Table 1 Effect of genotype on basic carcass characteristics

Trait	Sign.	Genotyp								
		S 100 (A)			(T 75 S 25) x S (B)			(T 75 S 25) x SF (C)		
		L.S.M.	S.E.M.	Sign.	L.S.M.	S.E.M.	Sign.	L.S.M.	S.E.M.	Sign.
LWS (kg)	**	30.43	0.72	C	32.30	1.04		36.19	1.51	A
AS (days)		220	9.13		207	8.38		191	0.00	
DG (kg)	**	0.124	0.01	C	0.137	0.01	c	0.168	0.00	A,b
CCW (kg)	**	11.68	0.70	C	13.22	0.79		16.18	1.16	A
CY (%)	*	38.38	1.35	c	40.93	1.59		44.71	1.64	a
Skin (kg)		3.61	0.12		3.81	0.19		3.28	0.21	
Skin (%)	**	11.86	0.33	C	11.80	0.59	C	9.06	0.52	A,B
Leg (kg)	*	3.77	0.19	c	4.16	0.23		5.14	0.41	a
Leg (%)		32.28	0.51		31.47	0.25		31.77	0.37	
Shoulder (kg)	**	2.26	0.12	C	2.65	0.13		3.04	0.19	A
Shoulder (%)	*	19.35	0.32		20.05	0.24	c	18.79	0.36	b
CS	*	4.87	0.13		5.00	0.27	c	3.87	0.35	b
Fatness score		2.38	0.26		3.18	0.23		3.00	0.27	

*LWS = live weight at slaughter, AS = Age at slaughter, DG = daily gains from birth to slaughter, CCW = cold carcass weight, CY = carcass yield, CS = conformation score, A, B, C - ** - P ≤ 0.01; a, b, c - * - P ≤ 0.05*

Regarding fatness score, the levels of this trait were very balanced in all groups, while its lowest value was found in purebred lambs of Šumavská breed. On the other hand, it should be noted that Komprda et al. (2012) reported lower fatness score in all genotypes in their study. At the conclusion of the Table 1, it can be stated that from the point of view of basic carcass characteristics it seems to be the best group of lambs (T 75 S 25) x SF due to its highest carcass yield, the best conformation score and relatively favourable fatness score.

Table 2 Effect of genotype on weights and proportions of kidney, kidney fat and basic non-carcass traits

Trait	Sign.	Genotyp								
		S 100 (A)			(T 75 S 25) x S (B)			(T 75 S 25) x SF (C)		
		L.S.M.	S.E.M.	Sign.	L.S.M.	S.E.M.	Sign.	L.S.M.	S.E.M.	Sign.
Kidney (kg)		0.08	0.00		0.08	0.01		0.10	0.01	
Kidney (%)		0.68	0.04		0.61	0.02		0.62	0.18	
Kidney fat (kg)		0.12	0.02		0.14	0.02		0.18	0.03	
Kidney fat (%)		1.03	0.11		1.06	0.14		1.11	0.20	
Heart (kg)	**	0.13	0.01	C	0.15	0.01	C	0.20	0.01	A,B
Heart (%)	**	0.43	0.02	C	0.46	0.02	c	0.55	0.01	A,b
Lungs + trachea (kg)		0.39	0.01		0.43	0.03		0.47	0.03	
Lungs + trachea (%)		1.28	0.03		1.33	0.06		1.30	0.06	
Liver (kg)	*	0.37	0.01	c	0.40	0.03	c	0.53	0.05	a,b
Liver (%)		1.22	0.03		1.24	0.68		1.46	0.09	
Spleen (kg)	**	0.04	0.00	C	0.05	0.00	C	0.06	0.01	A,B
Spleen (%)	**	0.13	0.00	C	0.15	0.01	c	0.17	0.01	A,b

*A, B, C - ** - P ≤ 0.01; a, b, c - * - P ≤ 0.05*

The Table 2. shows that the genotype had a significant effect on the weights of heart, liver and spleen and on the proportions of the heart and spleen. Similar trends were also recorded by Shaker et al. (2002) and Abdullah et al. (2010). By contrast, Bingöl et al. (2006) did not record the effect of genotype on the weights and proportions of basic non-carcass traits. Genotype did not have a significant effect on the weights and proportions of kidney and kidney fat. By contrast, Pérez et al. (2007) have found a significant effect of genotype on the weight of kidney. However, in our experiment the highest weight of kidney fat was found in (T 75 S 25) x SF. On the other hand, the lowest weight of kidney fat was observed in S 100, which corresponds with the lowest fatness score in this group of lambs.

Effect of genotype on composition of tissues in left leg is presented in Table 3. The genotype had a significant effect on the weights of the left leg and muscle + fat from this cut only. A similar trend was reported by Cloete et al. (2012) also. On the other hand, Bingöl et al. (2006) have not recorded significant effect of genotype on these traits. Proportions of muscle + fat and bones in all three groups of lambs were relatively balanced, nevertheless in a group of lambs (T 75 S 25) x SF the highest proportion of muscle + fat and the lowest proportion of bones were found. In contrast, in the purebred lambs of Šumavská breed the lowest proportion of muscle + fat, and the highest proportion of bones were found.

Table 3 Effect of genotype on composition of tissues in left leg

Trait	Sign.	Genotyp								
		S 100 (A)			(T 75 S 25) x S (B)			(T 75 S 25) x SF (C)		
		L.S.M.	S.E.M.	Sign.	L.S.M.	S.E.M.	Sign.	L.S.M.	S.E.M.	Sign.
Left leg (kg)	*	1.89	0.10	c	2.08	0.12		2.57	0.21	a
Muscle + fat (kg)	*	1.41	0.09	c	1.60	0.11		2.05	0.20	a
Muscle + fat (%)		74.60	1.12		76.92	1.29		79.77	1.42	
Bones (kg)		0.46	0.01		0.47	0.02		0.51	0.02	
Bones (%)		24.34	0.88		22.60	1.30		19.84	1.39	

*a, c - * - $P \leq 0.05$*

CONCLUSION

Our study shows that genotype had a significant effect on most of the monitored traits. Lambs were slaughtered at approximately the same age, however in crossbreds (T 75 S x 25) x SF the highest gain and carcass yield were found. In this group of lambs the best conformation score and the highest weights and proportions of the heart, liver and muscle + fat in the left leg were also found. These results indicate that use of commercial crossing of ewes of Šumavská breed with rams of meat breeds has a positive impact on growth and carcass quality of lambs. On the other hand, in purebred lambs of Šumavská breed the most favourable fatness score was recorded while in this breed is particularly appreciated its non-demanding nature and resistance against unfavourable environmental conditions. However, due to the relatively low number of lambs in our experiment it will be necessary to continue in monitoring of these genotypes.

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COMPARISON OF THE PERFORMANCE OF THE MOST IMPORTANT FAMILIES AND LINES SCHK – MENIK

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Abstract: The purpose of this dissertation was the evaluation of the influence of families on exterior and performance features of their offspring and the evaluation of lines which function in private breeding in Měník. Acquired data were separated into three databases and processed in Statistics 12 Soft program, through Analysis of Dispersion - ANOVA. For purpose of this dissertation were chosen 8 ongoing families and two most significant stallions - 1028 Manillon Rouge and 2626 Sahib Kubišta of the recent times. Results first database confirm positively importance of conservation of families in breeding. Long term homogeneous families demonstrably confirm the influence on evaluation of exterior indicators (height at withers , circuit chest, and circuit cannon bone) which directly relate with health and efficiency of evaluated horses. I deal with evaluation of the 1028 Manillon Rouge stallion's influence in the second database. I rate here concretely his offspring. Tukey and Scheffe tests confirm a statistically significant difference between the offspring by 1028 Manillona Rouge in the performance characteristic (basic performance test) and between (circuit chest). The mares have a basic performance test average higher than 7.9173 this result is, in my opinion, excellent and can testify to the good rideability of these descendants by 1028 Manillon Rouge. Stallions have an average below 7.5530. Averages in circuit chest are: 191.51 for mares, thus attesting to their good prerequisites to become good mothers, and for stallions these are 184.52. The third database evaluates and mutually compares 1028 Manillon Rouge's and 2626 Sahib Kubišta's daughters. Tukey and Scheffe tests confirm a statistically significant difference between the offspring by 1028 Manillon Rouge and 2626 Sahib Kubišta. Tukey and Scheffe tests confirm the difference in averages in basic performance test we see greater differences between stallions Manillon Rouge - 7.9173 and 2626 Sahib Kubišta - 7.1341.

Key Words: Czech warmblood, stallion, mares, Selle française, performance

INTRODUCTION

Targeted and systematic work with the horse starts with the selection of suitable parents who are, by their exterior, character and performance, suitable for conceiving a new child, which would reflect positive talents and the gene pool of its parents, becoming their bearer (Dušek 1992). And by selecting a mother and a father, the breeder indirectly affects the future development of the individual horse and the breed (Dušek 2007). Therefore, the knowledge of genetic dispositions of the parents, especially the families and lines, is the essence of breeding work, which aims at systematically targeted construction of a breed type, possessing characteristic utilitarian characteristics of the breed of horses in question (Jiskrová 2014).

The aim of my thesis was to analyze the long-term breeding efforts at the breeding center in Menik for the eight most important families and the offsprings of the two most prominent breeding stallions, operating in our family farming in Menik for the last 20 years. In my work, I focus on evaluating the impact of families and stallions on the exterior and performance characteristics of the offspring. For the analysis of the influence I used the statistical programs Excel and Statistics 12 Soft, using analysis of variance - ANOVA.The differences were considered as significant with $P<0.05$.

MATERIAL AND METHODS

Characterization used database A, B, C for my work

The number of horses in the database was 356. This number was divided into three databases. The first database (Database A) contained the first families and their mares, 201 mares in total, dealing with the evaluation of the impact on families, exterior and performance characteristics of their offspring. These families are dated from 1948 and up to 2012.

The second database (Database B) focused on the offspring of 1028 Manillon Rouge, and contained 68 horses that were born between 2005 and 2012. Here I deal with the evaluation of the impact of the stallion 1028 Manillon Rouge, who in 2004 was imported to the Czech Republic for the purpose of breeding in SCHK - KUBIŠTA Ltd. In this case, I assessed whether the sex of the offspring influenced the exterior and performance characteristics of the offspring by his father, 1028 Manillon Rouge.

The third database (Database C) Database contained 87 mares, and was meant to evaluate and compare the stallions Manillon Rouge in 1028 and 2626 Sahib Kubišta, on the basis of their daughters. Mares are compared with each other on the basis of exterior and performance features.

I used the statistical programs Excel and Statistics 12 Soft, using analysis of variance ANOVA. As the basis for the databases, data was drawn from in-depth records by Mr. Josef Kubista, which he led from 1960 to 1995.

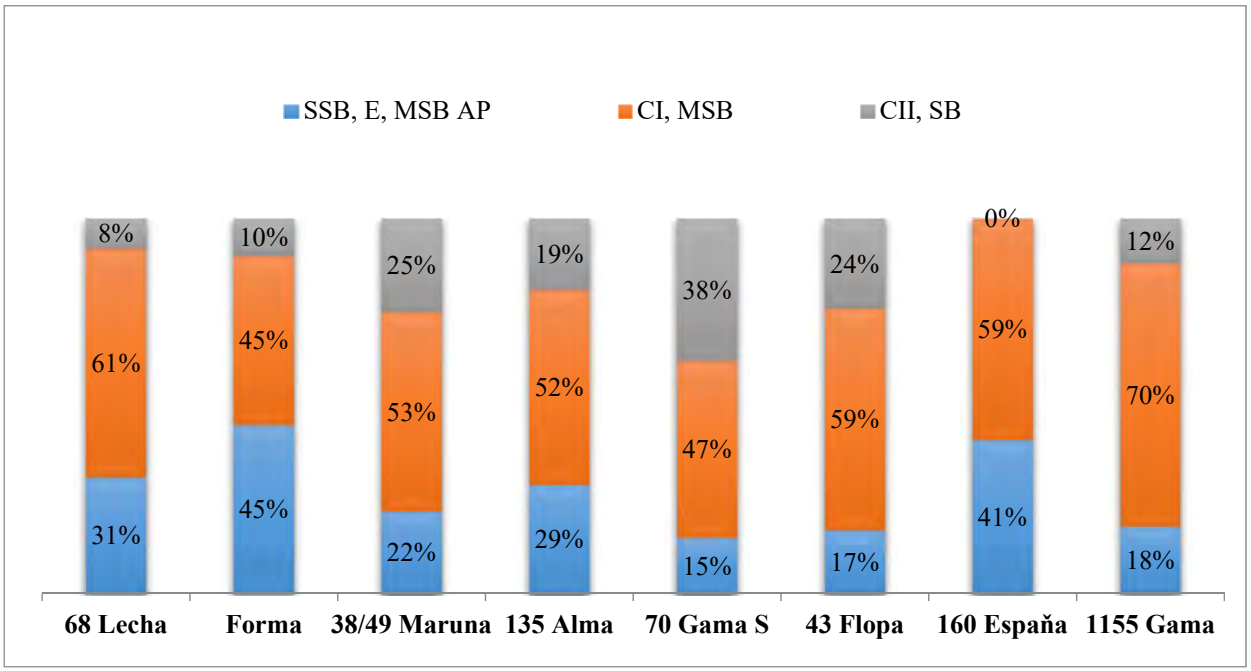
RESULTS AND DISCUSSION

Results in Database A

Single-dimension results for the families, for each dependent variable, show us highly statistically significant effect on families only in shoulder height.

According to the final inclusion in the divisions Stud-book Czech warmblood in (Figure 1) one can evaluate all the families as similar. Among the top rated families is, without a doubt, family No.1 68 Lecha no. 2 Form and family no. 7 160 Espana, in which we can classify the HW (height at withers) ratings as rather below average, but still ranking among the best-rated families. This can be judged as an excellent maneuverability at BPT (basic performance test), but in my opinion and knowledge about this family, also as a sign of good jumping potential. Relatively worst results were shown by the family of the founder 70 Gama S.

Figure 1 Offsprings of the eight most prominent families included in the Stud-book Czech warmblood



Legend: SSB, E, MSB AP = SSB - State Stud-book, E - Elite, MSB AP - Main Stud-book acceleration program; CI, MSB = CI - Class I, MSB - Main Stud-book; CII, SB = CII - Class II, SB - Stud-book.

Results in Database B

Kuřítková (2011), describes 1028 Manillon Rouge's father, the breeding stallion Papillon Rouge as excellent sport horse - the winner of many international competitions, participant of the Olympic Games in Barcelona, champion of France etc. His successes continue to farming, the best offspring includes Rochet Rouge - the participant in the Olympic Games. Papillon Rouge is successful continuer of Almaline.

Tukey and Scheffe tests confirm a statistically significant difference between the offspring by 1028 Manillona Rouge in the performance characteristic BPT (basic performance test) and between CCH (circuit chest). This results we can see in (Table 1).

Table 1 The results Scheffe tests – performance characteristic BPT, conformation characteristic CCH

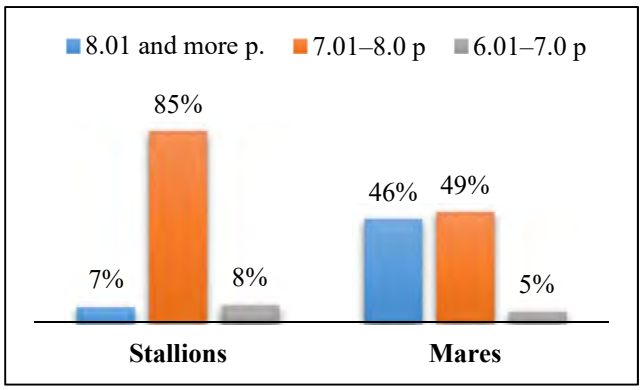
Sex	BPT		CCH	
	1 d – 7.5530	2 d – 7.9173	1 d – 184.52	2 d – 191.21
1		0.000387		0.000000
2	0.000387		0.000000	

Legend: Very statistically significant difference at level P<0.05 are highlighted in red; 1 = stallions, 2 = mare; d = diameter; BPT = basic performance test; CCH = circuit chest

The mares have a BPT average higher than 7.9173 - this result is, in my opinion, excellent and can testify to the good rideability of these descendants by 1028 Manillon Rouge. Studs have an average below 7.5530. Averages in CCH are: 191.51 for mares, thus attesting to their good prerequisites to become good mothers, and for stallions these are 184.52. In other dependent variables (EISB (enter into Stud-book), HW (height at withers), CCB (circuit cannon bone)) showed no statistical difference by sex. The mutual influence of families and offspring by 1028 Manillona Rouge has not been proven by the statistics. These results will be confirmed by the percentage comparisons in the charts below.

From (Figure 2) we can say that the mares by 1028 Manillon Rouge are very balanced in the performance characteristic BPT (basic performance test). According to Bouškova (2016) stallion 1028 Manillon Rouge has his breeding value index 145.52. In my opinion, this value corresponds to today's requirements in horse breeding.

Figure 2 BPTs by 1028 Manillona Rouge

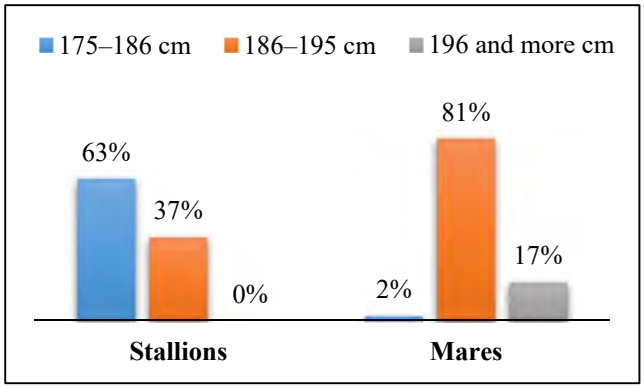


Legend: p = points

Jiskrová (2015) states that stallion 1028 Manillon Rouge demonstrably gives high quality, balanced and very good-character offspring. But his offspring is charakterized by shorter neck and this should be eliminated in future generations of 1028 Manillon Rouge and the mating plan should be also well prepared.

(Figure 3) shows that the mares' circuit chest is greater than that of the stallions. This indicator is directly related to the health and performance of horses. The results can be characterized as meeting the appropriate breeding objectives and confirm that mares by 1028 Manillon Rouge can be good mothers.

Figure 3 Comparison CCH (circuit chest) *of offsprings by 1028 Manillon Rouge*



Results in Database C

One-dimensional results for each dependent variable (EISB (enter into Stud-book), BPT (basic performance test), HW (height at withers), CCH (circuit chest), CCB (circuit cannon bone)) show that the stallion 1028 Manillon Rouge **had a very significant effect on his daughters and their performance marks**, namely EISB and BPT. In other variables (HW, CCH, CCB) there was no statistically relevant influence of the 1028 Manillon Rouge and 2626 Sahib Kubišta. Tukey and Scheffe tests confirm the difference in averages in EISB: Manillon Rouge - 7.5854, 2626 Sahib Kubišta - 7.3870. On average, BPT we see even greater differences between stallions Manillon Rouge - 7.9173 and 2626 Sahib Kubišta - 7.1341. This results we can see in (Table 2).

Table 2 The results Scheffe tests – performance characteristic EISB (enter into Stud-book), BPT (performance characteristic)

Stallions	EISB		BPT	
	1* d – 7.5854	2* d – 7.3870	1* d – 7.9173	2* d – 7.1341
1*		0.005268		0.000000
2*	0.005268		0.000000	

Legend: Very statistically significant difference at level P<0.05 are highlighted in red; 1 = stallion 1028 Manillon Rouge; 2* = 2626 Sahib Kubišta; d = diameter; BPT = basic performance test; EISB (enter into Stud-book),*

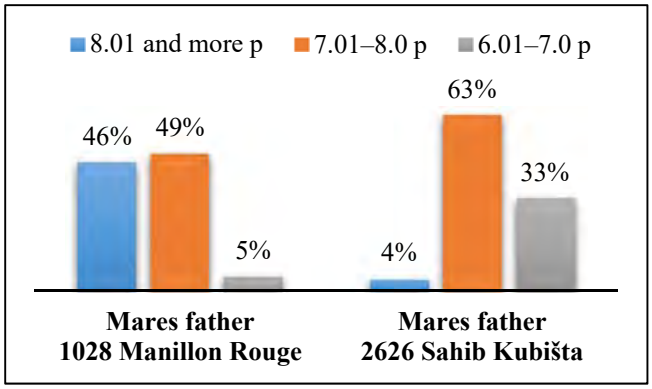
It should be noted that the averages of the EISB (enter into Stud-book), for stallion 2626 Sahib Kubišta is higher than the average BPT, which we can be judged as a sign of a worse rideability at BPT and so called "later," offsprings of 2626 Sahib Kubišta.

Staněk (2014), describes stallion Sahib Kubišta 2626 as follows: he achieved the highest jumping performance (TT), then he was a participant in the Grand Prix and he represented the Czech Republic in the Nations Cup. In 1998 he was declared as the best horse of Czech breeding in the CSIO Prague. His offspring reached the jumping performance level T, the combined driving either and the eventing as level ST. Despite of these higher than average results, the stallion 2626 Sahiba Kubišta should be considered more as the final product because the vast majority of his descendants are rather average, eventhough it can be influenced by many other factors.

Kuřitková (2011), stated that breed the Selle Français has tends to select and eliminate from breeding, horse with poor performance and wrong rideability, is important lay great emphasis on the horse's willingness to cooperate with the rider.

According to (Figure 4), stallion 1028 Manillon Rouge, who himself, due to his injury, for two years could not be tested in the sport, shows good performance in his progeny, with balanced and very good results (Evain 2009). These results also confirm my assumption and the correct decision by ŠCHK – KUBIŠTA s.r.o. to put this horse into the mating plan. The graphs below, once again, confirm these statistical results.

Figure 4 Comparison of BPTs of mares by 1028 Manillon Rouge and 2626 Sahib Kubišta

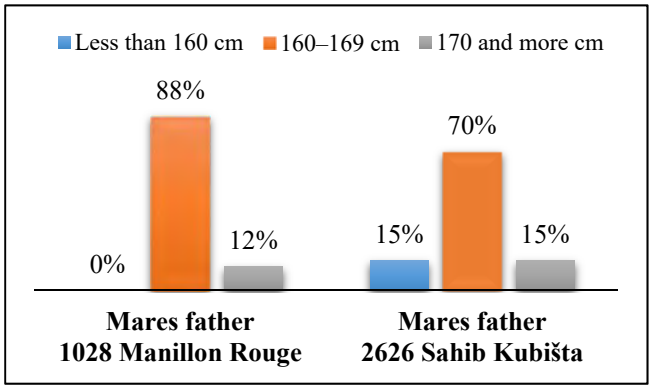


Legend: p = points

According to (Figure 5), it is clear that mares from the stallion 2626 Sahib Kubišta produce a lower HW. 15% of the mares even showed HW (height at withers) below 160 cm after three years, which is at the lower limit of breeding goals for this Czech warmblood. This progeny is called "later."

Sixta (2006), emphasizes the quality of the stallion 2626 Sahiba Kubišta and alleges that this stallion needs quality mothers from proven families.

Figure 5 Comparison of HWs of mares by 1028 Manillona Rouge and 2626 Sahib Kubišta



CONCLUSION

The results of the first database (A) unequivocally confirm the importance of keeping the families in the breeding. In the long term, the bred families demonstrably confirm the impact on the evaluation of exterior indicators, such as HW (height at withers), CCH (circuit chest), CCB (circuit cannon bone), which are directly related to the health and performance of the evaluated horses. It should be noted that in the breeding stable of Selle Français great emphasis is put on the family, just as at ŠCHK – Kubišta s.r.o. by the founder - Josef Kubišta. Therefore, I believe that when preparing mating plans, we should not forget about the high-quality and proven families.

The second and third databases (B and C) have given us positive results, which confirm the correctness of breeding work at ŠCHK – Kubišta s.r.o. and of the decision to choose stallion 1028 Manillona Rouge, which lays a good foundation for the breeding, as is confirmed by the results in Database A - eight parent families, as well as relates to the stallions who were used for the breeding in the past, for example, 366 Taarlo, 2626 Sahib Kubišta and 206 Frühesch. With these and other excellent results, one can conclude that the stallion 1028 Manillona Rouge was an improver of the breed.

I would also like to note that 2626 Sahib Kubišta has its own mare lines, which are successors of some of the above families and later mated with stallion 1028 Manillon Rouge. Progeny of this combination has been proved to be very solid and promising, not only in sports but also in farming. It follows that a great position in the case of 2626 Sahiba Kubista is the position of the grandfather, and in the case of 1028 Manillon Rouge – the position of the mothers' father. The concept of the breeding employed by ŠCHK – KUBIŠTA s.r.o., according to these results, is on track for the production of high quality horses.

Figures 6 and 7 Breed stallion 1028 Manillon Rouge. Source: ŠCHK – KUBIŠTA s.r.o.

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I would like thank my thesis supervisor Assoc. prof. Ing. Iva Jiskrová, Ph.D.

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THE EFFECT OF PERIOD FROM CATCHING OF TURKEYS TO SLAUGHTERING ON BREAST MEAT PSE INCIDENCE

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Abstract: The experiment involved 24 flocks of turkeys. Ten flocks were slaughtered within one hour after catching while fourteen flocks were transported to the slaughter for 80–100 km and the period between catching and slaughtering was 3–4 hours. The determination of PSE was done by staff visually and by palpation and they decisions were confirmed by water losses. The incidence of PSE meat was higher ($P<0.05$) for turkeys slaughtered within 1h after catching (5.7% vs. 1.4%). The loss of water for PSE free meat ranged from 10.1 to 13.1%, whereas PSE meat reached values from 14.2 to 15.6%, the differences were significant ($P<0.05$). It was confirmed that experienced staff at slaughter can very precisely and quickly recognize PSE meat visually and by palpation.

Key Words: turkeys, growth, PSE meat, turkey meat production

INTRODUCTION

The consumption of turkey meat in the Czech Republic is annually around 2 kg per person but especially breast meat belongs to quality dietary meat. Given that heavy turkey hybrids with high carcass yield are currently used for fattening it is not necessary to fatten the high number of animals to cover the demand for this meat. Therefore it is important to greatly reduce stress and its adverse effect on the animals which can cause lower carcass yield of breast meat and possibly lead to the occurrence of defects meat PSE (pale, soft, exudative) and the subsequent confiscation was the most valuable parts of the turkey´s carcass.

The muscle showing pale color, fine texture and exudative character was firstly described in pork. The occurrence of these symptoms is mainly in white meat with predominance of white myofibrils. Based on these findings the abbreviation PSE (pale, soft, exudative) was accepted for such meat defects even for poultry meat. However the term PSE is still debated because the causes causing PSE differ for pigs and poultry mainly in genetic background, therefore for poultry expression PSE-like is also used. The halogen screening method, which have been used for the detection of sensitive pigs to stress and consequently prone to PSE, does not work in turkeys or broilers (Fujii et al. 1991, Ingr 2003).

PSE meat defect arises primarily as a result of stress. During fattening there is no significant stressful situations, however the situation is changed at the end of the fattening period when the animals are caught to the cages and transported to the slaughterhouse and here they are handled again (Ingr 2003). This stress starts a very rapid progress of degradation of ATP and glycogen to lactic acid and inosine and the pH drops in one hour postmortem to 5.80 or lowers (Ingr 2003).

Fast glycogenolysis releases a lot of energy and it increases muscle temperature up to +43 °C (Ingr 2003, Chae et al. 2007). Increased acidity and temperature of the muscle causes partial denaturation of the muscle proteins, which results in deterioration of water holding capacity of the meat. All meat quality characteristics show great variability and in this spirit PSE defects are manifested in intensity from barely noticeable to very significant (Ingr 2003).

Owens et al. (2009) reported that the incidence of PSE meat on slaughters in the USA is in the range from 5 to 40%. Breeders in USA are also the largest producers of turkeys and turkey meat and therefore this defect meat causes considerable financial losses, which are estimated at up to 200 million dollars annually. In the EU the incidence of this defect is quite high, nearly 40% (Barbut et al. 2005). Petracci et al. (2009) sum the main possible causes of poultry PSE-including genetics, seasons, stress factors and conditions before slaughtering.

Breast muscle exhibiting PSE defect is not suitable for direct consumption, because of water level, softness and paleness. PSE meat can be used for processing, canning or in sausage production but with less water holding capacity it is necessary to mix the meat with the meat of standard quality (Skřivan et al. 2000). Carcass quality and breast meat yield is also affected by transport and particularly by transport cages size and its design. There is risk of breast meat of other body turkey parts damage due to the lack of space among the animals. Other factors which include hanging, stunning, undercutting, bleeding, scalding, plucking and storing meat can also affect carcass yield and breast meat quality (Skřivan et al. 2000, Smith and Northcutt 2009).

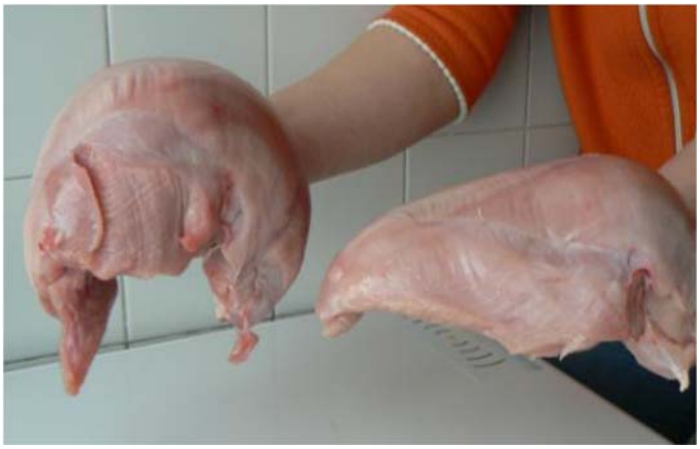
The aim of the experiment was to determine the effect of period from catching to slaughtering including transport on occurrence of PSE breast meat in turkey.

MATERIAL AND METHODS

Twenty-four flocks were included in the evaluation of PSE incidence in turkey, both sex hens and toms. Ten flocks (2 460 turkeys) were fattened in the halls near the slaughterhouse. These animals were slaughtered within one hour after catching in the hall. The remaining 14 flocks (3 784 turkeys) were transported from farms 80–100 km distant when the period from catching to slaughtering ranged between 3–4 hours. The effect of both periods from catching to slaughtering and sex on the PSE incidence in breast meat was evaluated. The live weight of turkeys, carcass weight, PSE meat weight and breast muscle weight were measured too.

The determination of PSE meat was done by two methods. First, visually and by palpation was done by staff in the slaughter that has long time experience with PSE. Correctness of the staff assessment was judged by laboratory control by water holding capacity (see Figure 1).

Figure 1 Evaluation of PSE by staff (PSE free right side and PSE left side)



Legend: Photographer Iveta Vdolčeková

Water holding capacity (WHC) was determined in samples of the breast meat which were visually assessed as PSE by staff. The samples were taken from the caudal part of the deep breast muscle weighting 50 g, 24 hours post mortem. PSE free meat was assessed too. The samples were homogenized, 2 g of the meat were inserted between two papers Watman No. 2 and between two glass plates and loaded weight 500 g for 5 minutes. The samples were weighted before and after the treatment and percentage of water loss was calculated (Grau and Hamm 1953). There were done two observations, in the first 20 samples of PSE free and 16 samples of PSE were analyzed for WHC and in the second one 15 PSE free and 30 samples of PSE were analyzed. Water loss was expressed by mean and standard error of mean. Variability of the measurement was characterized by the coefficient of variation. For the evaluation of the effect of sex end transport on the observed characteristics Kruskal-Wallis one-way analysis of variation and the Mann-Whitney U test were used. Statistical analysis was performed using the UNISTAT 5.1 (Unistat Ltd, England).

RESULTS AND DISCUSSION

The period from turkey catching to slaughtering (4 hours vs. 1 hour) had significant effect on PSE incidence ($P<0.05$). Table 1 shows the higher incidence of PSE meat defects in turkeys slaughtered

within 1 hour from catching compared turkeys that were transported from a distance 80–100 km. PSE meat incidence ranged in turkeys slaughtered in one hour at a level of 5.7% while in turkeys slaughtered in 3–4 hours the level was 1.4%. Figure 2 shows the incidence of PSE in each flocks and there is a noticeable higher incidence of PSE meat in all flocks slaughtered within one hour after removal from halls. The sex of turkeys had no statistically significant effect (P>0.05) on the incidence of PSE meat which shows Table 2.

The results of water loss in both observations are shown in Table 3. The staff right decision about meat (PSE or PSE free) was confirmed by water losses according to Grau and Hamm (1953). The PSE free samples had statistically significantly lower water loss (P<0.05) compared to PSE samples in both cases. This observations demonstrated that employees thanks connection with acquired practice are able precisely recognize the PSE meat just by quick palpation (P<0.05).

Table 1 Influence of period from catching to slaughtering on the incidence of PSE meat defects in turkeys [%]

PSE breast meat		
Period	Average ± SE*	v_x
Till 1 h	5.7 ± 0.42[a]	0.23
Till 4 h	1.4 ± 0.15[b]	0.40

Legend:
SE- standard error*
a, b - means of the same order designated by different letters are significantly different (P<0.05)
v_x- coefficient of variance (%)

Table 2 Effect of turkey sex on PSE meat incidence [%]

PSE breast meat		
Group	Average ± SE*	v_x
Turkey cockerels	2.8 ± 0.63[a]	0.63
Turkey hens	3.4 ± 0.66[a]	0.78

Legend:
SE- standard error*
a, b - means of the same order designated by different letters are significantly different (P<0.05)
v_x- coefficient of variance (%)

Figure 2 The incidence of PSE meat in all observed flocks [%]

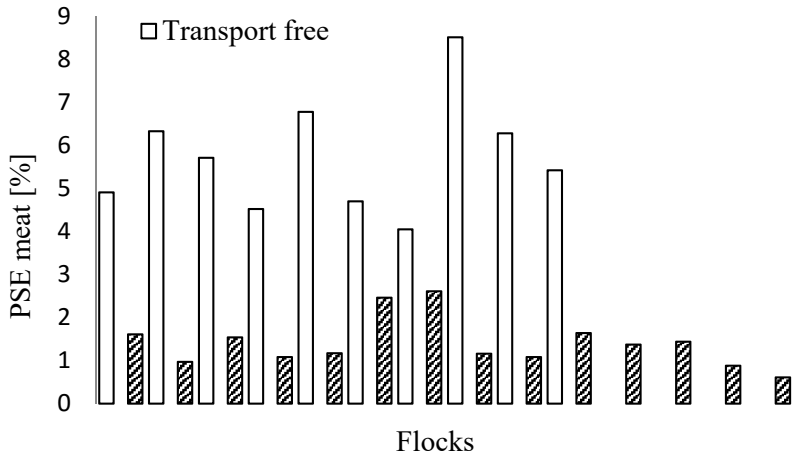


Figure 3 show sum of live weight, carcass weight and breast meat weight in all flocks according to the period from catching to slaughtering. It is clear, that more animals were transported from distance 80–100 km. On the other hand, figure 4 shows the absolute weight of PSE meat for both periods. Notwithstanding the live weight of turkeys slaughtered within one hour was lower about 16.5t (Figure 3), the weight of PSE was much higher for these birds (337 kg vs. 109 kg, Figure 4).

Table 3 Water losses of PSE and PSE free as determined by staff [%]

		Water losses	
Observation	Breast meat	Average ± SE*	v_x
1.	PSE free	13.1 ± 0.47[a]	0.16
	PSE	15.6 ± 0.72[b]	0.18
2.	PSE free	10.1 ± 0.86[a]	0.33
	PSE	14.2 ± 0.43[b]	0.16

Legend:
SE- standard error*
a, b - means of the same order designated by different letters are significantly different (P<0.05)
v_x- coefficient of variance (%)

The effect of time from catching to slaughtering on the incidence of PSE also discussed Owens et al. (2000). They used for PSE meat determination pH and breast meat temperature as other possible factors characterizing PSE meat and they showed that breast meat of transported turkeys had significantly higher pH in 2 hours and 24 hours after slaughter. These authors however did not notice the difference in the loss of water. In contrast the authors Marques et al. (2016) point out the significant influence of transport lasting two hours, the time from catching to slaughtering, on turkey stress, expressed by gene expression in the liver, which corresponds with our results.

Hoof (1979) showed that with increasing length of turkey transport decreased the levels of glycogen and ATP, which can be connected with lower incidence of PSE in turkey after longer transport. They measured the glycogen and ATP levels after 4 hours of transport (260 km).

Figure 3 The monitored carcass parameters depending on the period from catching to slaughtering [kg]

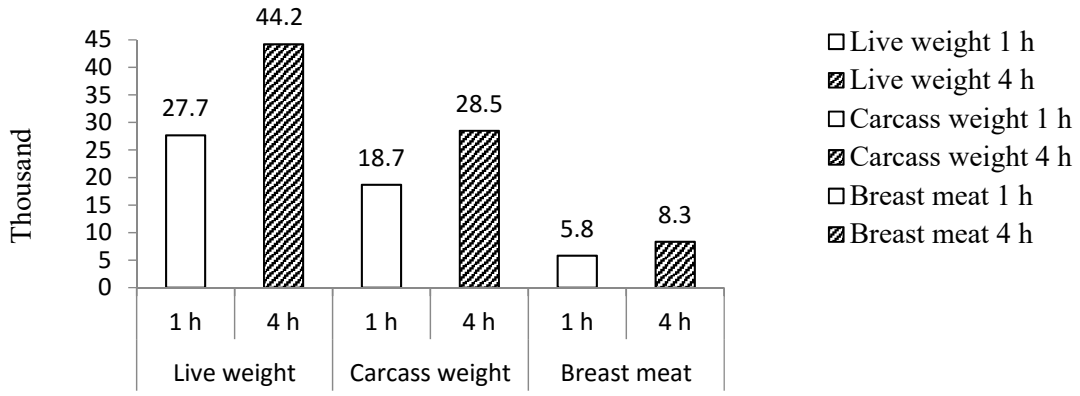
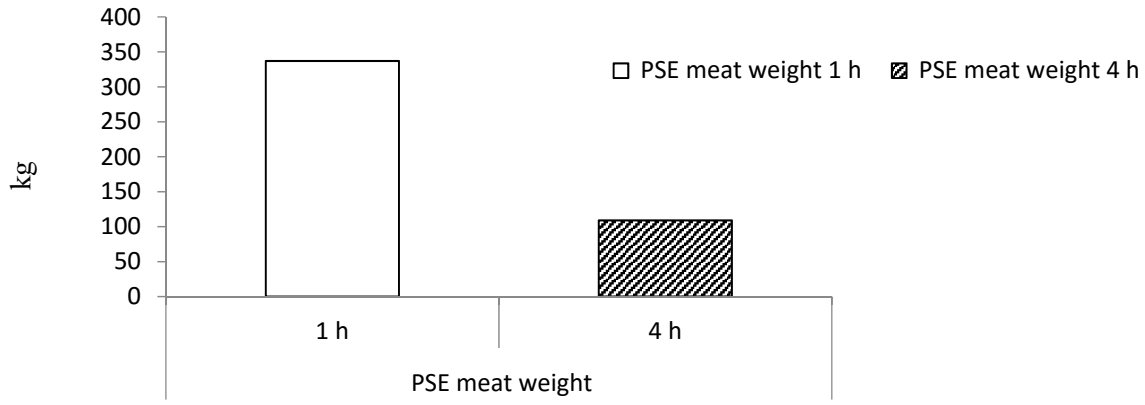


Figure 4 PSE breast muscle weight to about 1 hour to 4 hours to defeat of the total weight of the breast muscle turkey hens and turkey cockerels in the period [kg]



Owens et al. (2009) reported that the incidence of PSE meat on slaughters in the USA is in the range from 5 to 40%. Breeders in USA are also the largest producers of turkeys and turkey meat and

therefore this meat defect causes considerable financial losses, which are estimated at up to 200 million dollars annually. In the EU this defect is found in meat quite often nearly 40% (Barbut et al. 2005).

CONCLUSION

The period from catching to slaughtering had statistically significant effect ($P<0.05$) on incidence of PSE breast meat in turkey. The incidence of PSE meat was higher for turkeys slaughtered within 1h after catching, namely 5.7%. In contrast the incidence of PSE meat in turkey slaughtered within 3–4 hours was 1.4%. The influence of sex was no statistically significant ($P>0.05$). The loss of water for PSE free meat ranged from 10.1 to 13.1%, whereas PSE meat reached values from 14.2 to 15.6%, the differences were significant ($P<0.05$). It was confirmed that experienced staff at slaughter can very precisely and quickly recognize PSE meat visually and by palpation.

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THE INFLUENCE OF GENOTYPE ON THE YIELD, QUALITY AND TECHNOLOGICAL PROPERTIES OF MILK OF COWS KEPT UNDER IDENTICAL CONDITIONS

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Abstract: The objective of this paper was to assess the influence of the genotype on yield, quality and technological properties of cow´s milk. In this study we observed 18 dairy cows kept under indentical conditions of one dairy farm. There were three genotypes evaluated: Czech Fleckvieh (C), Holstein (H), and Ayrshire (A), 6 cows of each breed. In total 120 milk samples, taken weekly, were analysed in milk-laboratory. Following charakteristics were analysed: milk yield per standardized lactation (305 days); fat, protein and dry matter content, non-fat solids content, density, active and titrable acidity, rennet coagulation time (RCT) and curd class quality. The results were also compared with the milk recording in CZ in years 2014/15. The highest yield was recorded for Holstein cows (8802 kg) followed by Czech Fleckvieh (7591 kg) and Ayshire breed (7097 kg). The best technological properties was shown by the milk of the Czech Fleckvieh (RCT 220 s, titratable acidity 7.29 SH). The technological properties of Holstein (RCT 248, titratable acidity 6.96 SH) and Ayrshire (RCT 246, titratable acidity 6.72 SH) milk were significantly ($p<0.01$) worse than those of Czech Fleckvieh. Concerning to the milk composition, the significantly ($p<0.01$) higher contents was recorded in the milk of the Czech Fleckvieh. In the Holstein milk , compared with Ayrshire, we recorded higher protein content (3.21%), but Ayrshire had higher fat content (4.23%). Findings suggest that Czech Fleckvieh cows at the farm yielded 451 kg of milk more, and Ayrshire cows 15 kg more, than the general average for mentioned breeds in Czech Republic (CZ). The Holstein cows yielded 744 kg of milk less than the general average for this breed in CZ. Fat content (at farm) was higher than general average in CZ for all three breeds. In the contrary, protein content was for all breeds lower.

Key Words: Technological properties, milk content, Ayrshire, Holstein, Czech Fleckvieh, identical conditions

INTRODUCTION

Czech Fleckvieh is national and native cattle breed of Czech Republic (CZ). It is a part of Fleckvieh family, which widely used in central and western Europe for its great milk and meat yield. The ratio between meat and milk production was generally set to 40:60. Czech Fleckvieh cows produce in average 7140 kg of milk per lactation with average fat and protein content of 3.98% and 3.52% Weight of cows is between 650 and 750 kg. The average weight of Czech Fleckvieh bull is over 1200 kg (SCHČSS 2015).

Holstein cattle breed is the most used dairy breed in the world (producing 9546 kg of milk in average in Czech Republic) (SCHHS 2015). Breeding is focused on only milk production. According to Sambraus (2006) cows can weigh 650–750 kg, bulls over 1000 kg. Heifers of Holstein cattle reaches breeding maturity very soon. They can be impregnated in 14–15 months of age which leads to first calving in age of 24 months. Concerning to the milk technological properties, the fat resp. protein content is in averae 3.78% resp. 3.43% (SCHHS 2015).

Ayrshire cattle breed is, as well as Holstein, used only for a dairy production. The average yield is 6982 kg of milk with fat content of 4.12% and protein content of 3.47% (SCHHS 2015). Ayshire cows can weigh 550–620 kg, bulls can reach 950 kg (Sambraus 2006).

Contents of fat, protein and lactose are the main parts of breeders interest besides the yield, and they are also pointers of milk quality. Each breed has different milk content and can be used for different field of milk production (cheese making, consumption of liquid milk, yogurts etc.). All the nutrients come to milk from feed through blood vesels. For one liter of milk there has to flow aproximatelly 500 liters of blood through udder. Also, milk synthesis is not in same intensity throughout whole day. The most intenstive synthesis occurs 3 hours after milking (Jelínek et al. 2003).

Technological properies such as non-fat solids content (SNF), titratable acidity and rennet coagulation time (RCT) are also very important for the further processing of milk in dairy factory. These qualities are determined by breed, genotype or individuality of dairy cows (Bayram et al. 2009, Matějíček et al. 2008). Enviromental influence like temperature also has large influence (Summer et al. 2003). For example Falta et al. (2014) report differences in titrable acidity of milk with changing temperature.

MATERIAL AND METHODS

Experiment for our paper took place in ZD Okrouhlička, between Jihlava and Havlíčkův Brod (CZ). The observed period was since March 2015 till September 2015 (6 months). In our experiment there were 18 cows of all three breeds (Holstein, Czech Fleckvieh, Ayrshire) with genotypes H 100, C 100 and A 100.

There were six milk samples of each breed analysed. We acquired the milk samples with aparature for the yield control in a day of monthly milk recording scheme. All samples were then freezed and analyzed aproximately two weeks after sampling in laboratory of applied lactology, Department of Animal Breeding of Mendel university in Brno.

Analysis itself started with the defrosting in water base at temperature 20 °C. After complete defrosting of sample we analysed titratable (SH) and active (pH) acidity, rennet coagulation time (RCT). Milk composition (fat and protein content, non-fat solids %), density (cm^3) and curd quality (on 1-5 scale). Total yield was analyzed by results of milk recording control scheme.

Titrable and active acidity were analyzed by instrument HI902C1–02 by Hanna Instruments company. This instrument can determine acidity by using ion selective electrodes.

RCT was determined by turbidimetric detector. This device can detect milk coagulation by light permeability of sample (Přibyla and Čejna 2006, Chládek et al. 2011).

Milk composition was analyzed by MilkoScope Julie C5. This instrument can analyze content of fat, protein, non-fat solids and density. Dry matter was calculated from fat and non-fat solids.

Curd quality was evaluated by method according to Gajdušek (1997), that uses 5-point scale (1 = best, 5 = worst) for determination of curd quality. This evaluation is used after rennet is added into the milk, when curd and whey fully appear.

RESULTS AND DISCUSSION

In Table 1 we can see effect of breed on milk composition and yield. Is obvious, that the highest overall amount of milk was provided by Holstein cows. Concenring the milk composition, highest fat and protein content was recorded in milk of Czech Fleckvieh cows. Nevertheless, we have to mention, that neither Holstein and Czech Fleckvieh cows on the observed dairy farm met the requirements of their breeding goals when it comes to protein content, that is 3.30% for Holstein and 4.0% for Czech Fleckvieh (SCHHS 2005; SCHČSS 2012). This also applies for cows of Ayrshire breed. Average protein content in CZ in 2015 was 3.47% (SCHHS 2015). Insufficiency in protein content of all three breeds can indicate lacks in feed, housing, or care factors. The fact these insufficiencies appears with all three breeds shows, that they should not affect experiment results whatsoever.

Table 1 Milk composition and yield characteristics (n =120)

	Genotype		
Indicator	Ayrshire	Holstein	Czech Fleckvieh
Fat (%)	4.23 (±0.62)^{a}	3.99 (±0.48)^{a}	4.4 (±0.76)^{b}
Protein (%)	3.12 (±0.07)^{Ab}	3.21 (±0.06)^{a}	3.25 (±0.06)^{B}
Dry matter (%)	12.68 (±0.93)^{A}	12.63 (±0.9)^{A}	13.24 (±1)^{B}
Non-fat solids (%)	8.5 (±0.19)^{Ab}	8.75 (±0.16)^{a}	8.84 (±0.17)^{B}
Yield (kg)	7097 (±769.09)^{A}	8802 (±932.68)^{B}	7591 (±1109.56)^{C}

Values in the same line marked with different symbols (a to d, or A to D, respectively) are different (P < 0.05 or P < 0.01, respectively), ± marks standard deviation.

Table 2 Technological properties of analysed milk samples (n = 120)

	Genotype		
Indicator	Ayrshire	Holstein	Czech Fleckvieh
Active acidity (pH)	7.37 (±0.24)	7.34 (±0.20)	7.34 (±0.22)$^{n.s.}$
Titrable acidity (SH)	6.72 (±0.69)^{a}	6.96 (±0.52)^{a}	7.29 (±0.49)^{b}
Density (g.cm^{-3})	1.028 (±0.001)^{Ab}	1.029 (±0)^{a}	1.029 (±0.001)^{B}
RCT (s)	246 (±11)	248 (±30)	220 (±22)$^{n.s.}$
Curd quality (class)	1.97 (± 0.7)	1.69(±0.6)	1.64$^{n.s.}$(±0.7)

Values in the same line marked with different symbols (a to d, or A to D, respectively) are different (P < 0.05 or P < 0.01, respectively), ± marks standard deviation. The n.s. stands for non-significant result.

Table 2 has shown, that the best technological properties had milk of the Czech Fleckvieh cows. It needed the shorter time to coagulate, and it also showed the best curd quality. This could be caused by higher amount of B-casein allele in herd of this breed. This allele causes better technilogical properties of milk (Hanuš et al. 1995). This statement also supports Samknová (2012) who claims, that genetic variant BB of K-casein is responsible for shorter RCT and higher milk yield. Concerning the other two breeds, we can say that Ayrshire milk had shorter RCT, but worse curd quality, than milk of Holstein cows.

CONCLUSION

The majority of Czech dairy farmers keep Holstein cows. This is mainly because of the amount of milk that compensates lower milk content. According to our work we can see, that both higher milk content and better technological properties was shown by Czech Fleckvieh. This breed was created exactly for our climate and enviromental conditions. It could be the main reason for better milk quality indicators as showed above. Reason for the lower yield is that Czech Fleckvieh is breed with dual purpose (combined) performance in contrast to the Holstein cattle, which belongs to the breeds with purely dairy performance.

Concerning the Ayrshire breed, it showed higher milk content in some aspects than Holstein, but as well as Holstein, in milk quality it cannot compete to Czech Fleckvieh.

Results of our work can be usefull for farmers decision what kind of breed to use for their particular farm depending on the local conditions. This is important for achieving competitiveness and self-sufficiency of milk prodiction of the Czech Republic.

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THE EFFECT OF SEASON AND LIGHTING ON DUROC BOARS EJACULATES QUALITY

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Abstract: The aim of the study was to evaluate abiotic factor such as microclimatic conditions of boar's stable in the artificial insemination center. The intensity of daily light and light permeation to the naturally-lit stable and lights indicators in the stable with artificial lighting were evaluated. In the each stable was evaluated the influence of season on the boar´s ejaculate quality. The measurements were always carried out on a level with the head in animal zone environment. Obtained results of quantitative ejaculate indicators, sperm volume (ml), motility (%), sperm concentration (thousand/mm^3) and occurrence of pathological sperm (%) were statistically processed. Our results suggest the impact of longer photoperiod length increases a percentage of pathological changes in the end of summer month. The volume of ejaculate and the semen concentration is increased, furthermore the sperm motility is decreased.

Key Words: Stable facility, boar, light intensity, season, manifest of organism

INTRODUCTION

The daily light is an integral part of human and animal life. The necessary level of physiological lighting should be ensured by the technical construction for new and refurbished stables in the case of livestock breeding animals. If the daily light is not sufficient, it must be supplemented by appropriate artificial lightening. The appropriate stable lighting has a proven positive effect on feed consumption, state of health and related yield of livestock productivity (Doležal and Černá 2006). According to common technical requirements for stable construction, the stable intended for the livestock must be constructed to ensure the balanced internal environment microclimate and protect the housing and animal husbandry (Pogran et al. 2011). Due to this reason it is necessary to monitor microclimatic aspects of environment such as lightening, which have a direct influence on livestock in animal premises and stables (Karandušovská et al. 2012). The minimum recommended standard of 40 lx was kept for 8 hours.

European wild boar has highly seasonal character of reproduction. The mating season of wild boars start with declining intensity of daylight (usually in the half of November) and consequently parturition fall on the spring season during a light intensity prolongation (Hansen-Catta 2008). Although this seasonal character is suppressed by domestication and selection, it could persist as an increased susceptibility to seasonal factors (Čanderle 2002).

The process of spermatogenesis (evolution process from spermatogonia to mature sperm) is very long and complex process lasting around 40 days (Hrudka et al. 1962). Up to 10−14 days is necessary for resistance formation and sperm capacitation which takes place in epididymis. A whole cycle of spermiogenesis takes about 50 days. Afterwards, the sperm accumulate in the ligament of the epididymis (Říha et al. 2003). Therefore, if this process is influenced by light regime the ejaculate quality should be evaluated after this period.

MATERIAL AND METHODS

The aim of the study was to evaluate abiotic factors of microclimate, including photoperiod and also, confirm the hypothesis that these factors affect the quality of the boars semen in the two experimental stables (natural and artificial lighting). The experimental measurements of selected

parameters was carried out in the artificial insemination center in the district of Žďár nad Sázavou. The company is located in the Vysočina region characterized by typical inland climate, with average annual rainfall of 594 mm and a mean annual temperature 7.2 °C. The experiment lasted for one year. 39 Duroc boars breeding stocks were included in the experiment (*Sus scrofa domestica*) and they were in the same age. Totally were processed 3100 data records from 602 samplings. Control group (stable A, 15 specimens) and experimental group (stable B, 20 specimens under the artificial light intensity 200 lx) was stabled in the same technologic conditions as well as the same feeding, with regular sampling of ejaculate - 3 times per week and evaluation. Obtained results of quantitative ejaculate indicators, sperm volume (ml), motility (%), sperm concentration (thousand/mm^3) and occurrence of pathological sperm (%) were employed according to the methodology for the evaluation of sperm parameters which is valid for semen collection center boars. The data of the sperm parameters were statistically processed using the software STATISTICA 8.0 (StatSoft, 2007).

RESULTS AND DISCUSSION

The light conditions in the stable are expressed by summer months (with increasing photoperiod lenght) and winter months (the decreasing photoperiod lenght). The duration of the photoperiod influenced the individual parameters of evaluated ejaculate. It was determined the mean intensity of daylight C = 54.9 lx with standard deviation 16.99. Was observed using the ambulatory measurement during the time of the experiment in the control stable with combined light. The differences were evaluated on the basis of minimum (50.9 lx) and maximum values (59.4 lx). The experimental stables with combined lights showed the mean intensity of daylight E = 217.2 lx with a standard deviation of 52.34, the minimum value of light intensity 163.7 lx and a maximum value 232.1 lux. Some variation in ejaculate quality in dependence on increasing or decreasing photoperiod could be observed by fluctuations as a result of seasonality. Zasiadczyk et al. (2015) reported that the seasonality disappeared under the domestication; others have been inclined to the opinion the reproduction functions are stimulated or repressed with increasing photoperiod. Seasonal divergences in the ejaculate quality are explained in the mentioned publication. There is clarified that the daylight and the production of corticosteroids are regulating the level of testosterone. Finally, the seasonal divergences are affected by heat stress during the summer months. Moreover, the negative influence is described on the boar reproductive physiology during spring and summer season. Furthermore, increasing photoperiod length and higher level of environment temperature could effects seasonal infertility of boars (Peña et al. 2016). Fraser et al. (2016) described the largest seasonal effect is connected with boars sperm motility and the highest resistance of sperm was observed in pigs in the age of 19−30 months and in the autumn and winter period. During the sampling, sperms were more susceptible to lipid peroxidation and regardless of the age group in the spring and summer months. It may be in accordance with the declaration of the fact, during the time of the experiment sperm motility from 72.55 % to 70.81 % decreased, but without statistically significant difference. Similar trends were observed in the stable with experimental light intensity of 200 lx, demonstrated by reduced sperm motility from 76.88 % in the spring to 70.87 % in the winter months ($P<0.05$).

Further results confirmed the hypothesis of the influence of the season which shows an important influence on the reproductive indicator of boars semen volume and sperm concentration. With the increasing photoperiod lenght in summer months, the changes are lately appeared on the beginning of winter months. In the spring and summer months boars ejaculate was collected at average volume of 272.13 ml and 272.41 ml in the control group. In the autumn and winter months were collected the volume of 287.76 ml and 263.13 ml. Among individual sampling there were no statistically significant differences. In the stable B where the artificial lightening was applied, the similar changes were confirmed in the winter months as a consequence of summer photoperiod. The artificial lightening showed the significant influence on increasing of ejaculate volume, which was found the lowest value in the spring of 243.96 ml and autumn period value of 356.82 ml. These indicators were statistically significant ($p<0.001$). Similar results were published by Kunavongkrit et al. (2005). They evaluated the collected boar semen in different seasons in Thailand. Their results were explained by the high temperature, which may be the main cause of the hypothesis. It was confirmed, the heat stress induces excess production of corticosteroids, with the highest incidence of stress of sensitive specimens.

Savić and Petrovic (2015) declared that the influence of the season could be evinced on the boars ejaculate quantity and a higher libido in longer period than 12 hours of daily light, whereas volume of ejaculate and the intensity of ejaculation is the highest in daily light shorten than 12 hours but only in the case of decreasing photoperiod length.

By the analysis of the results, which characterized the sperm concentration, was observed the mean negative correlation R = -0.4484 between semen volume and sperm concentration, with the highest concentration observed in the control group in the winter months (500.93 thousand/mm^3) in the comparison with the experimental group in the spring (442.08 thousand/mm^3). Smital (2002b) presented another changes affected by season of the year such as lower level of testosterone in the blood and semen plasma, lower secretion of *Glandulae vesiculosae* and *Glandulae bulbourethralis* and lower *Libido sexualis* in the spring season. Next study by the Murase et al. (2007) confirmed the level of testosterone reach the maximum during the October and November. Another studies supported the higher levels of testosterone in younger boars in comparison with older boars.

The evaluated values of pathological changes were statistically significant from the high difference ($P<0.01$). In the summer month the determined values were 7.15% in the control group and 6.74% in the experimental group. The highest percentage of abnormalities was found in both groups during the winter months (C = 12.16 %, E = 10.82 %). The statistically highly significant increases in abnormalities were observed in the winter months. The similar results were reported by Montserrat et al. (2005). However, their evaluated statistically significant increase of the number of abnormalities is not caused by photoperiod, but by differences in microclimate parameters which are differ (mainly temperature and relative humidity) in various seasons. This effect is attributed mainly relative humidity. Moreover, Aust a Bazala (2006) observed the increased sperm tolerance against dilution in the spring season and moreover they observed the deteriorated sperm survival in the stored samples of diluted ejaculate.

As an ideal season the autumn was proved, although the increased sperm motility was estimated. However, the highest ejaculate volume with high sperm concentration and lower values of pathological changes was found.

Table 1 Evaluation parameters of ejaculate in relation to the season in the control stable

Evaluated parameter of ejaculate	Stable A							
	Spring		Summer		Autumn		Winter	
	x	sd	x	sd	x	sd	x	sd
Volume (ml)	272.13	78.775	272.41	81.746	287.76	87.941	263.13	101.996
Motility (%)	71.79	4.771	72.55	7.230	70.81	6.033	71.20	7.063
Concentration (ths/mm^3)	408.45	133.692	398.33	137.860	425.32	154.150	500.93	171.606
Abnormality (%)	8.54	5.987	7.15	5.243	7.73	5.685	12.16	8.563

Table 2 Evaluation parameters of ejaculate in relation to the season in the experimental stable

Evaluated parameter of ejaculate	Stable B							
	Spring		Summer		Autumn		Winter	
	x	sd	x	sd	x	sd	x	sd
Volume (ml)	243.96	95.183	279.8	109.332	356.82	121.441	302.57	110.406
Motility (%)	76.88	4.854	74.2	5.018	71.82	5.430	70.87	7.463
Concentration (ths/mm^3)	442.08	172.595	415	157.734	428.32	134.255	421.55	139.926
Abnormality (%)	7.6	5.332	6.74	2.705	7.45	4.533	10.82	8.423

CONCLUSION

The evaluation of daily light and artificial light of stable was observed the significant differences of studied parameters of ejaculate evaluation, mainly the volume, concentration, pathological changes of ejaculate and sperm motility. From the perspective of necessity of the highest possible quality of sperm it must be ensured the zoo-sanitary measures including temperature, air circulation, hazardous of gases as well as lightening which have considerable effect on the production of sperm, hence the yield of livestock productivity. The season influenced the quantitative and qualitative parameters of ejaculate. From the obtained results could be concluded the effect of seasonal fluctuations in the sperm production in domesticated pigs.

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THE EFFECT OF HIGH TEMPERATURE ON SELECTED PARAMETERS OF SEMEN QUALITY AND ANTIOXIDANT ACTIVITY OF SUPEROXIDE DISMUTASE AND COPPER IN DUROC BOARS EJACULATE

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Abstract: The aim of the study was to investigate the effect of high temperature on selected parameters of semen quality of duroc boars at the insemination station in Velke Mezirici (N 49°23.46667', E 15°52.70135') in summer season from May to September. In the stable the temperature (°C) and relative humidity (%) were monitored at hourly intervals for whole period of this study. For purpose of the experiment were chosen 20 boars of the Duroc breed, divided into two groups. Group A – the control group (n = 10) has average quality of ejaculate a group B – the experimantal group (n = 10) showed below-average long-term quality of ejaculate. Analysed parametres were volume of ejaculate, concentration of sperm, motility and rate of abnormal sperm. We also monitored concentration of superoxiddismutase (SOD) and copper in ejaculate.

The results of the experiment shows that the volume of ejaculate from both monitored groups increased at the same rate ($P > 0.05$). Concentration of sperm of group A decreased, whereas concentration of sperm of group B was at the same level during the experiment. The motility of sperm of group A at the end of the experiment increased and motility of sperm of group B has intensively decreased ($P < 0.05$). In the both groups there was an increase of amount of the abnormal sperm in an ejaculate. The concentration of SOD and copper in 2nd and 3rd period intensively increased ($P < 0.05$). In our experiment the effect of the season had no significant influence on boars with the average quality of ejaculate (group A), but there was found a tendency to deterioration of motility of sperm and amount of the abnormal sperm in group of boars (group B), whose quality of ejaculate has below-average values before the start of the experiment. But the heat stress had significant influence on concentration of SOD and copper. In periods 2 and 3 there were temperatures above the thermoneutral zone (25 °C) and the concentration of copper in both groups significantly decreased and concentration fo SOD increased.

Key Words: temperature, quality ejaculate, antioxidant, boar

INTRODUCTION

Characteristic sign of boar semen is high volume of ejaculate and low concentration of sperm, however the total rate of sperm is high (Hájek et al. 1992). Boar ejaculate is composed of 3–7% spermatozoa and 93–97% seminal plasma. Specific sign is a lenght of ejaculation which can take 5–7 minutes. It also changes the composition of semen during ejaculation. As indicated in Louda et al. (2001), we distinguish three fractions of ejaculate: prespermatic (pH adjusting and emulsifying sows sheath), spermatic (the main sperm-rich part of the ejaculate) and postspermatic (characterized by high viscosity, which ensures maintenance of sperm fraction in sows genital tract). The average volume of the semen in an adult boar is between 200 to 300 cm^3, concentration between 250 to 400 000 in 1 mm^3 and activity (motility) should range between 60 to 90% (Louda et al. 2001).

The increase in body temperature by several degrees than the normal is called thermal shock and can be fatal, as it directly affects the cell function. Disrupts the membrane permeability, protein structure and appears loss of fluid and electrolytes (Hansen, 2009). Testes, since they are located outside the abdominal cavity, have usually a 2.5 °C lower temperature, than the body core. This temperature must

be maintained for optimum fertility of boars (Gadd 2011). If the testicular tissue is exposed to a higher temperature, it reduces sperm production, their motility and increases the occurence of morphologically abnormal sperm (Hansen 2009). Heat stress in boars begins during prolonged exposure to a temperature above 25 °C (Hajek et al. 1992). In an experiment, where was daily increased ambient temperature by 1 °C for 20 days (within the temperature range 20–40 °C), it was noted that the critical limit of temperature is 30 °C, when the motility of sperm has significantly decreased (Smital 2001). Motility in healthy boar in satisfactory conditions ranging up to about 95%, but when exposed to temperatures above 40 °C motility rapidly decreases to 5% (Gadd 2011). Because a high temperature environment occurs mainly in the summer months, this temporary reduction in fertility is called "seasonal fertility disorder" (Hajek et al. 1992). The thermoneutral zone in boars in the range from 12 to 20 °C (Pulkrábek 2005).

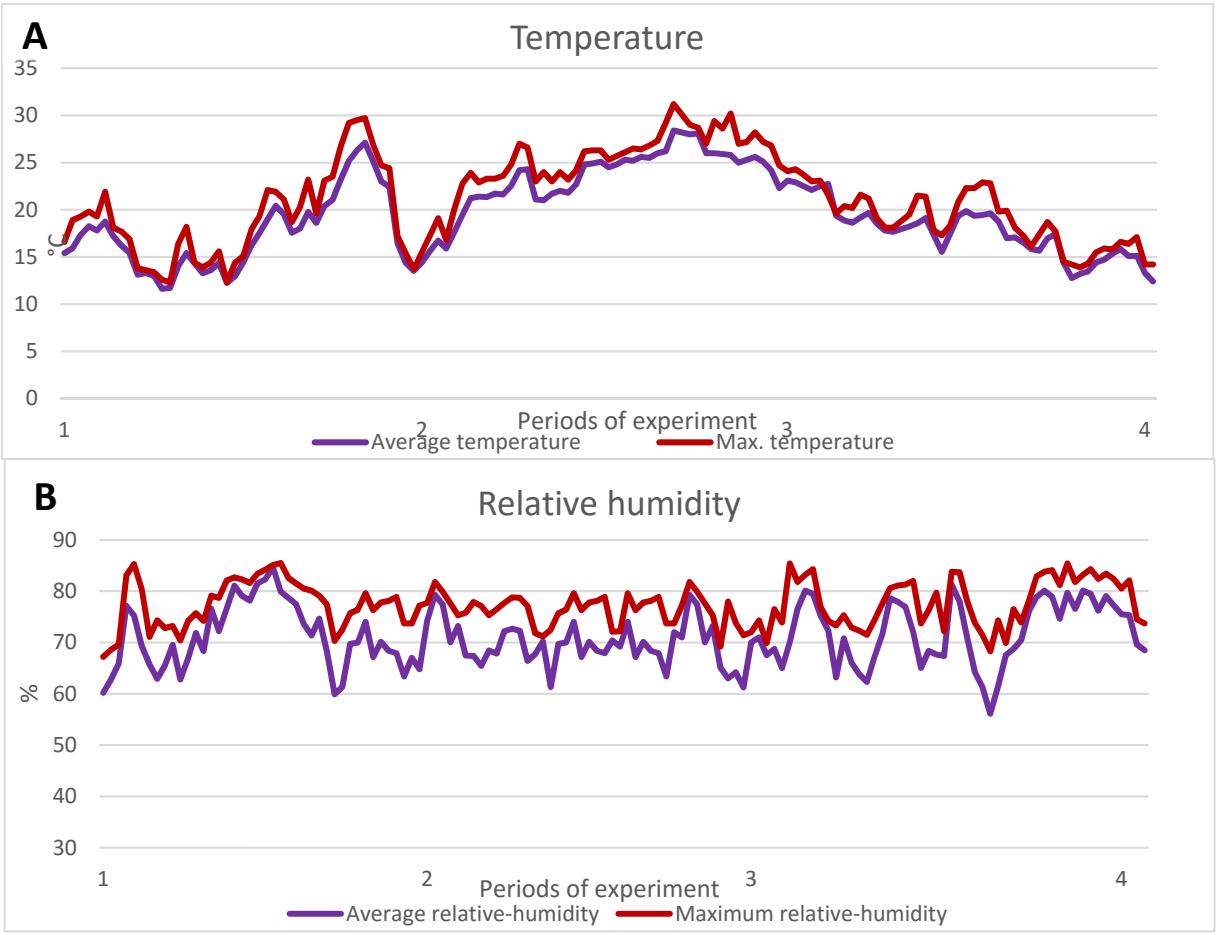
Selenium belongs in the nutrition of farm animals into the group essential trace elements. It is an integral part of the antioxidant capacity of the organism, where plays a major role in the protection against damage of tissues by free radicals as an integral part of enzyme glutathione peroxidase (Horký et al. 2013). Copper is a part of the enzyme superoxide dismutase, which together with glutathione peroxidase represents main defense mechanism against free radicals (Štípek et al. 2000). They are created in the body during normal physiological processes and also under stress situations as heat stress, childbirth, and excessive physical burden. Excessive production of free oxygen radicals can be caused by an excessive intake of mycotoxins, ions of zinc and cadmium, or lead. When an imbalance between the production of free radicals and antioxidants available in the organism creates oxidative stress, oxidative damage of tissues, proteins, lipids, or DNA occur (Horký et al. 2013).

MATERIAL AND METHODS

The experiment was performed at the station of insemination in Velké Meziříčí (N 49°23.46667', E 15°52.70135'). For purpose of the experiment were chosen 20 boars of the Duroc breed (*Sus scrofa domestica*). The average age of the boars was 2 ± 0.3 years and the average weight of the boars was 255 ± 20 kg. The length of the experiment was set at 135 days (May – September). Experimental animals were housed individually (2.5 × 2.5 m) and had ad libitum access to water. All animals were fed by 3.3 kg of basic feed. ME_P content was 12.6 MJ/kg feed. Boars were divided into two groups. The first group of boars (group A, n = 10) had a value of ejaculate without pathological changes (72 % sperm motility, sperm concentration was 499 000/mm^3; volume of ejaculate 203 mm^3, percentage of pathological sperm 6 %). The second group (group B, n = 10) included animals that had problems with the quality of the produced semen just before start of the experiment (low motility – 67% and the concentration of sperm 430 000/mm^3). Throughout the experimental observation was at hourly intervals determined ambient temperature and relative humidity using a datalogger device (Voltcraft DL-121TH, Germany), which was placed in a living animal zone (1 m above the ground). From these determined values were calculated the average temperature and relative humidity in each day. The resulting values of air temperature and relative humidity are shown in Figure 1 (Section A - average and maximum temperature measured during monitoring; Section B - average and maximum relative humidity during monitoring).

Ejaculate was taken from boars once a week. For the biochemical analyses was used the ejaculate that was removed at the beginning of the experiment (day 0), the 45th, 90th and 135th day of the experiment. Boar semen was sourced by a jump to the phantom. Volume of ejaculate, motility, sperm concentration and the percentage of abnormal spermatozoa were collected before the start of the experiment from each group of 30 samples (1st period - the control period). In the 2nd period (day 1 to 45) were analyzed 64 samples from group A and 61 samples of semen from the group B. In the 3rd period (day 46 to 90) were analysed 60 samples from group A and 63 samples from group B. In the last, 4th period (day 91 to 135) were analysed 62 samples from group A and 60 samples from group B. Samples of semen were evaluated and analyzed according to criteria CSN 467116.

Figure 1 The resulting values of air temperature and relative humidity



Determination of the volume of ejaculate

The volume of ejaculate was measured using a measuring cylinder with an accuracy of ± 0.1 ml.

Determination of the concentration of sperm in the ejaculate

The concentration of sperm in the ejaculate is expressed as the number of sperms in 1 mm^3. The concentration is determined photometrically using Spekol 11. Measurement was performed at a wavelength in the range 340–850 nm. By a dispenser for small amount of fluid was picked up in thin walled tubes 9 ml of 1M HCl, using varipipety was added 0.25 ml of the sample of mixed native semen and subsequently mix. The tube is inserted into the adapter Spekol 11 and deducting the measured value. According to the calibration table was determined the concentration of sperm.

Determining of the percentage of pathological sperm

Percentage of pathological sperm was determined from the first collection in the month. To prepare the smear: drop of semen was coated with a glass rod on a glass slide; at an angle of 45° was spread out over the edge by a grounded smears glass. Morphological assessment (evaluation of abnormal sperm in five ocular fields - all individually numbered with abnormal sperm), staining and evaluation of sperm carried the district veterinarian.

Determination of motility

Determination of motility was performed within 15 minutes after collection of boar, microscopically of gently kneaded semen. Semen was taken up with a glass rod, a drop of semen was coated on a preheated microscope slide (about 42 °C) and overlaid with a coverslip. Slides were preheated on preheating table, microscope had also preheating plate. Microscopically determined by a subjective estimate of the percentage of sperm with rectilinear motion forward for a head at body temperature and sampled at 1:40. The mobility rate is determined in five ocular fields.

Preparation of samples for biochemical analyses

Firstly, 0.5 ml volume of native thawed ejaculate was pipetted with subsequent addition of 2 ml of liquid nitrogen and 0.5 ml of phosphate buffer. Subsequently, the sample was homogenized in an ULTRA-TURRAX T8 homogenizer (IKA, Konigswinter, Germany) at 3000 rpm for 2 minutes. After homogenization, 1 ml of phosphate buffer was added. Sample modified like this was homogenized in a vortex (Vortex-2 Genie Scientific Industries, New York, NY, USA) at 2000 rpm for 15 minutes. Subsequently, the sample was centrifuged in a Universal 32 R centrifuge (Hettich-Zentrifugen GmbH, Tuttlingen, Germany) at 16000 rpm at 4 °C for 20 minutes. Finally, supernatant was removed and used for analyses (1.5 ml).

Determination of SOD

Kit 19160 SOD (Sigma Aldrich, USA) was used for assay of superoxide dismutase (SOD, EC 1.15.1.1.). A 200 µL volume of reagent R1 (WTS solution diluted 20 times with buffer) was pipetted into a plastic cuvette and agent was incubated at 37 °C for 108 s. Afterwards, a 20 µL volume of sample was pipetted and in 378 s, the reaction was started by adding a 20 µL volume of reagent R2 (enzyme solution 167 times diluted with buffer). It was incubated for 72 s and then absorbance was measured at $\lambda = 450$ nm. Kinetic reaction was measured for 180 s and absorbance was read every 9 s.

Preparation of samples for electrochemical determination of copper – microwave digestion

To 10 µl ejaculate 500 µl of digestion mixture (350 µl HNO_3 + 150 µl H_2O_2) was added. Samples were digested in MW Anton Paar, rotor MG-65. The program (SUP 6) begins and ends with the same ten-minute-long-step, beginning with the power of 50 W and ending with the power of 0 W. Microwave power was 100 W in the main part of the program (30 min.).

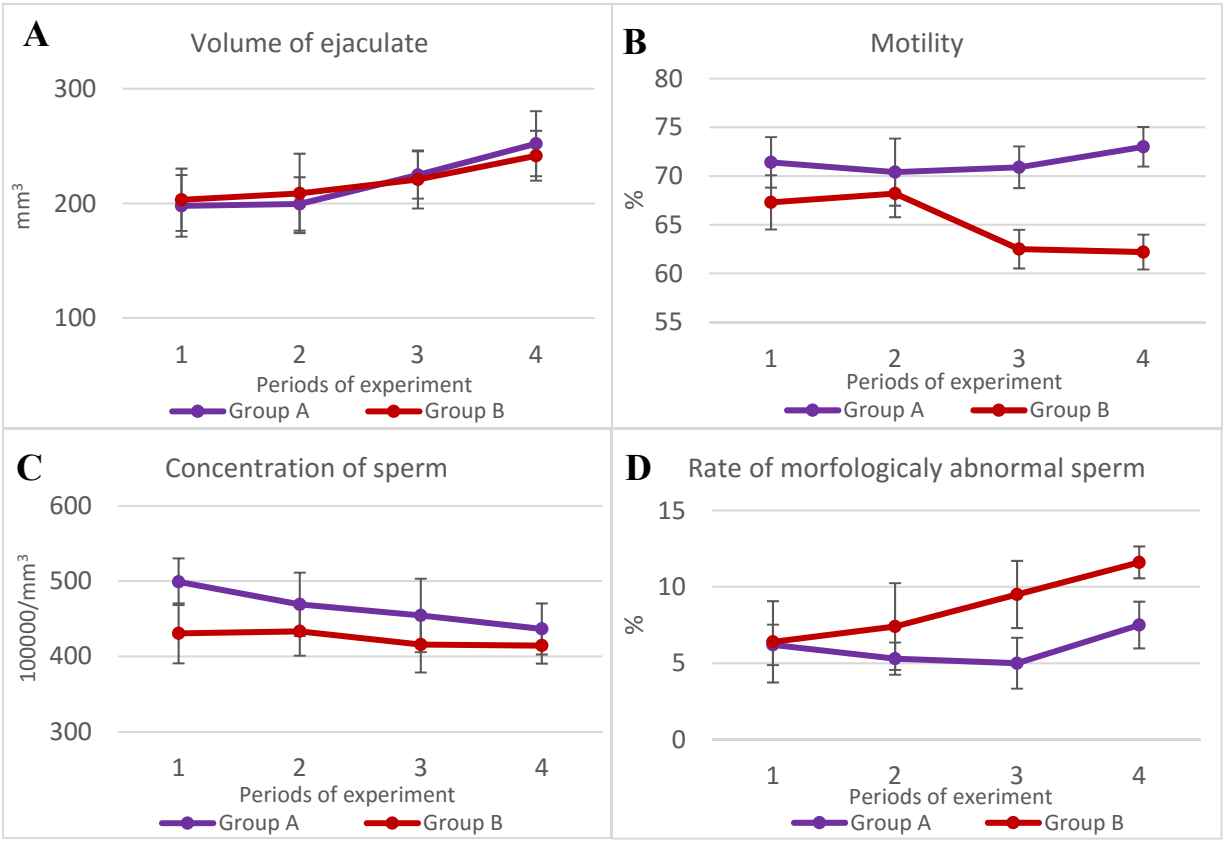
RESULTS AND DISCUSION

The effect of heat stress on the quality of ejaculate produced by breeding boars is shown in Figure 2 (Section A - ejaculate volume, Section B - sperm motility, Section C - sperm concentration; Section D - abnormal sperm).

The results of the experiment shows that the volume of ejaculate from both monitored groups increased at the same rate ($P > 0.05$) and in group A from 198 mm^3 to 252 mm^3; in group B from 203 mm^3 to 241 mm^3. Concentration of sperm of group A decreased (from 499 000/mm^3 to 436 000/mm^3), whereas concentration of sperm of group B was at the same level during the experiment. The motility of sperm of group A at the end of the experiment increased (from 71.4 % to 74.0 %) and motility of sperm of group B has intensively decreased (from 67.3 % to 62.2 %) ($P < 0.05$). In the both groups there was an increase of amount of the abnormal sperm in an ejaculate and in group A from 6.2 % to 7.5 % ($P > 0.05$); in group B from 6.4 % to 11.6 % ($P < 0.05$). In our experiment the effect of the season had no significant influence on boars with the average quality of ejaculate (group A), but there was found a tendency to deterioration of motility of sperm and amount of the abnormal sperm in group of boars (group B), whose quality of ejaculate has below-average values before the start of the experiment.

The aim of this study was to determine how the boar body reacts on heat stress and how it will affect the quality of semen. During monitoring the quality of semen produced at the insemination center, it was observed that the highest quality is achieved in the winter months. Conversely, in summer and autumn, volume of ejaculate increased and sperm concentrationto decreased. Seasons had no significant effect on motility of sperm (Knecht et al. 2014). In the case of our experiment was observed similar effect of the summer (heat stress), when the maximum daily highs reached even over 30 °C. Given that our experiment included two groups of boars (one with low and second with high quality of semen), we can say, that according to our results, the heat stress raises problems in pigs, which suffered long-term low sperm quality (low motility, low concentration). As Knecht et al. (2014), we observed a reducing sperm concentration, increasing of volume of ejaculate and we also confirmed, that summer season has no significant effect on sperm motility of boars with high quality of ejaculate. In contrast, in another study was observed, that in the summer is significantly reduced sperm motility compared with the spring, autumn and winter (Barranco et al. 2013).

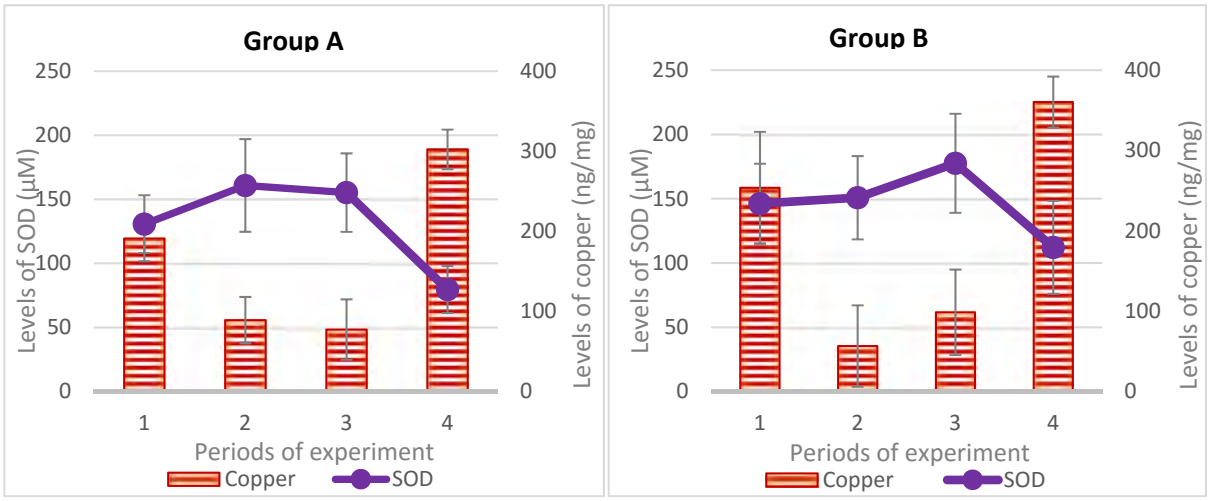
Figure 2 The effect of heat stress on quality of produced ejaculate of both experimental groups



The concentration of SOD was at the 1st period 130,6 ng/mg in group A and 146.2 ng/mg in group B. In 2nd and 3rd periods there was a increase of concentration of SOD on average 25 ng/mg in both groups. In the 4th period there was decrease of concentration of SOD in group A at 79.6 ng/mg and in group B at 112,1 ng/mg. The concentration of copper was at the 1st period 191.1 µM in group A and 253.6 µM in group B. In 2nd and 3rd periods there was a significant decrease (more than 50% form initial value) of concentration of copper in both groups. In the 4th period the concentration of copper significantly increased at 302.3 µM in group A and 360.3 µM in group B.

The heat stress had significant influence on concentration of SOD and copper. From the 2nd to 3rd period there were temperatures above the thermoneutral zone (25 °C) and the concentration of copper in both groups significantly decreased and concentration fo SOD increased. It is the result of antioxidant aktivity, when is a higher increase of free radicals in organism.

Figure 3 The effect of heat stress on concentration SOD and copper in ejaculate of both experimental groups



CONCLUSION

The results of observations shows, that the volume of ejaculate in both groups is increasing at the same rate, while sperm motility in the experimental group compared to the control group sharply decreased, while increasing the amount of abnormal sperm. Effect season in our case had no significant effect on boar semen with average values (control group), but was found a tendency to significant deterioration of the individual parameters in the group of ejaculate boars whose ejaculate exhibited below average values before the start of monitoring (experimental group). Temperatures above 25 °C had significant influence on concentraton of superoxide dismutase and copper. This means that there are antioxidant processes in the organism due to a higher increase of free radicals, caused by heat stress.

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ANALYSIS OF THE NOISE EXPOSURE OF MILKING PARLOUR OPERATORS DURING WORKING SHIFT AT DIFFERENT TECHNOLOGICAL SOLUTIONS

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Abstract: Generally, working environment means all tangible and intangible factors that act directly on the employee and his work. Employees working in conditions of farms are exposed to different unnatural influences. These factors may include also noise. Noise always arises with a certain energy conversion. In cattle farms, the sources of noise are represented by various mechanical equipment and machines that are used for enabling the operations of the farm. The aim of the paper was to analyze the exposure of operators in milking parlours, during their day routine. The measurement took place at three different farms with different technological solution of milking system. In this article, automatic milking system, herringbone milking parlour and rotary milking parlour was evaluated. Values were processed statistically, showed in graphs and compared with values under the Directive of the European Parliament and the Council Nr. 2003/10/EC, which gives the exposure limit values $L_{AEX, 8h}$ (noise exposure with weighting filter "A") and upper and lower action value of exposure $L_{AEX, 8h,}$ but also the values of L_{CPk}.

Key Words: working environment, milker, cattle

INTRODUCTION

Currently, high demands are given on environmental protection. The envi-ronmental protection must be part of person's life in twenty-first century (Gálik et al. 2014). Each production process is characterized by certain conditions in which it is performed (Opáth and Kažimírová 2013). Into production process of milk is entering various means of mechanization.

Cattle bred in farm buildings are exposed to noise, which can come either from outside or from inside of the building. Several published studies demonstrate different sounds that can occur inside the building for animal husbandry (Castelhano-Carlos and Baumans 2009). Noise sources on farms can be, in addition to ordinary activities (opening and closing doors, washing, speech of employees, dispensing feed, etc.), also machinery, basal levels of noise caused by mechanical ventilation, animal activity (climbing to barriers, chewing on barriers) and their own vocalization (Mihina et al. 2012). Other sources of noise can also be mechanization used on farms, because of the noise either of the engine or hydraulic systems (Janoško et al. 2010). Besides noise from technical and mechanized equipment, in animal

production there are also noise emissions caused by biological noise of animals. This noise is by dairy cows in the range of 73.7 dB to 83.8 dB (Šístková et al. 2010).

A great deal of research has been done on the effects of noise on performance (Kjellberg and Landström 1994). Algers et al. (1978) detected noise levels in the milking parlours and states values from 75 to 90 dB. According to Kauke (2007) is the noise intensity in most cases unacceptable for dairy cows and also for operator (milker).

Therefore, careful planning should be made before construction of animal buildings, in order to avoid stressful environmental sounds both for the animal and personnel (Brouček 2014).

MATERIAL AND METHODS

Research place

The experiment was conducted in three cattle farms in the Czech Republic.

First measurements were performed in the farm with 840 production dairy cows, with rotary milking parlour (year of manufacture 2007) with 36 parlour places. This parlour is equipped with oil-vane vacuum pump Fullwood Ambassador. Dairy cows are milked 2 times per day and they and the milking lasted 407 minutes. This parlour is served by three workers (milkers).

Second measurements were performed in the farm with capacity for 205 dairy cows, milked with three identical automatic milking systems (AMS) (year of manufacture 2006). Every AMS unit is equipped with claw vacuum pump Mink MM 1104 A VM. Cows are milked according to their needs, in some cases up to 3 times a day. This system of milking is served and controlled by one worker (zootechnician). The working shift lasted 640 minutes.

Third measurements were performed in the farm with 536 production dairy cows, with herringbone milking parlour 2 x 12 with quick exit (year of manufacture 2009). This parlour is equipped with oil-vane vacuum pump Fullwood Q4. Dairy cows are milked 2 times per day and the milking lasted 348 minutes. Parlour is served by two workers (milkers).

Measuring device

Personal Noise Dosimeter 3M eg4 Edge Dosimeter was used for measuring of noise exposure. For calibration, before the measurement was used Acoustic Calibrator 3M AC–300. Conditions during measurement were recorded by digital meteorological station WS–1600.

Data acquisition

During measurement, the operators of milking parlours were exposed to normal, everyday conditions, when the milking system was running. The noise pressure levels were measured in area, where the employees are located during the working shift and where their work tasks are performed. The measuring device was located on workers right shoulder, so that his work tasks were not obstructed by wearing this device (Figure 1). Duration of each measurement was equal to the duration of the work shift in a given milking parlour.

Figure 1 Location of noise dosimeter

Data analysis

Directive of the European Parliament and the Council Nr. 2003/10/EC indicates the exposure limit values $L_{AEX,\ 8h}$ (noise exposure during weighting filter "A" for 8 hour shift) and upper and lower exposure action value $L_{AEX,\ 8h}$. Determining the daily noise exposure at working place with a shorter or longer time of duration T_e to the nominal duration of the working day 8 hours T_0 can be with normalizing of equivalent sound pressure level A at nominal time of working day according to relationship (1).

$$L_{AEX,8h} = L_{Aeq} + 10\lg(T_e/T_o),\ dB \qquad (1)$$

where:
$L_{Aeq,Te}$ - equivalent sound pressure level during the period of T_e;
T_0 - nominal duration of working day – 8 hours

Software package SAS ver. 9.2 (SAS, 2009) was used to carry out given statistical procedures.

RESULTS AND DISCUSSION

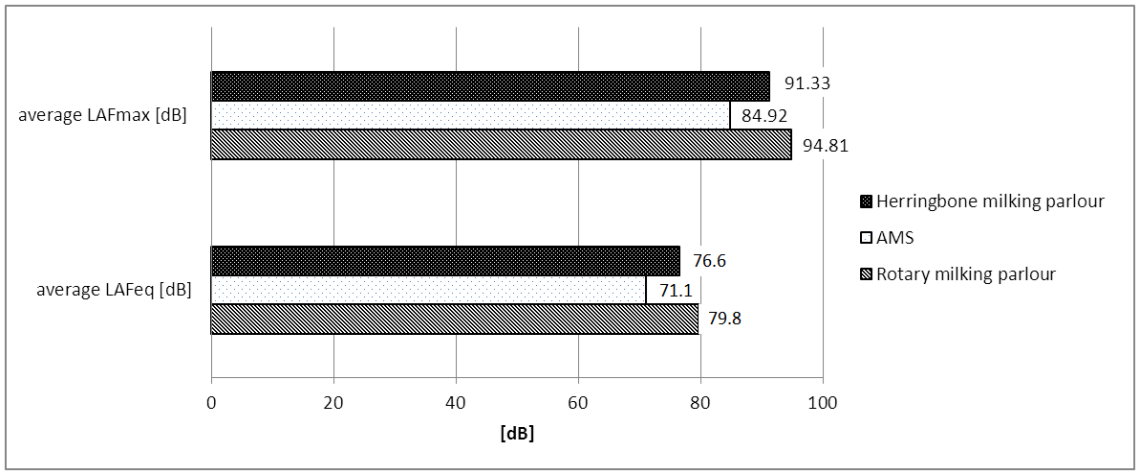
The measurements were conducted under the conditions specified in Table 1.

Table 1 Climatic conditions during measurement

Location of measurement	Air temperature [°C]	Relative humidity of air [%]	Atmospheric pressure [hPa]
Rotary milking parlour	19.1	64	943
AMS	18.1	42	959
Herringbone milking parlour	20.1	54	991

When analyzing the summary statistics of basic noise measures, we found out, that the highest L_{AFeq} was found in rotary milking parlour (79.75±5.04 dB) while the lowest value was measured in AMS (71.11±9.13 dB). The same situation was observed in L_{AFmax} values. The highest peak value (L_{CPk}) was found in AMS (114.16±5.20 dB). Graphical overview of mean L_{AFmax} and L_{AFeq} in various milking parlour types is presented in graph (Figure 2).

Figure 2 Average values of noise in milking parlours



As can be seen in the graph (Figure 2), the average maximum sound pressure levels are quite high. This is due to the fact, that during milking are occurring different noises, caused by various noise sources, such as hitting metal parts to each other(metal barriers, namely chains, locking mechanisms of barriers and so on), or other adverse sounds. In reality, these values were in the range of 59 dB to 121 dB.

Equivalent levels were in rotary milking parlour in range of 62.4 dB to 100.2 dB, in AMS in range of 59.2 dB to 96.9 dB, and in herringbone milking parlour in range of 59.8 dB to 97.2 dB. Arithmetic averages of measuered values showed in graph (Figure 2) indicate, that in rotary milking parlour and in herringbone milking parlour the values were higher than in AMS, where the levels were around 71 dB.

The differences within mean L_{AFeq} values between various milking parlours types were highly statistically significant ($P<0.001^{***}$). The same result was observed in case of L_{AFmax} and L_{CPk} values as well.

The differences of correlation coefficients within the individual types of milking parlours were not important, therefore correlations were calculated from common database of all measured values in all types of milking parlours toghether. As expected, the most important correlation was found between L_{AFeq} and L_{AFmax} ($r = 0.9115^{***}$). Moderate relations were found between L_{AFmax} and L_{CPk} (r = 0.39161***)

and L_{AFeq} and L_{CPk} (r = 0.29874***). Measured values of L_{CPk} include especially sudden noises and these values did not significantly affect L_{AFeq} and L_{AFmax}.

Table 2 Calculated $L_{AEX, 8h}$, and maximum values of L_{CPk}

Type of milking parlour	Average LAFeq	Total milking time (duration of the work shift)	LAEX, 8hours	Maximum Value of LCPk
	[dB]	[min]	[dB]	[dB]
Rotary milking parlour	79.75	407	79.03	132.8
AMS	71.11	640	71.11	109.6
Herringbone milking parlour	76.57	348	75.17	109.5

In the table (Table 2) are shown calculated noise exposure values of operators $L_{AEX,8hod}$ weighted with weighting filter "A" (dB) and maximum values of L_{CPk}.

According to Directive of the European Parliament and the Council Nr. 2003/10/EC, exposure limit value $L_{AEX, 8h}$ has a value L = 87 dB (resp. L_{CPk} = 140 dB by single impulses) and with this value, the worker can´t be exposed under any circumstances, therefore after use of methods for reducing noise. The upper exposure action value $L_{AEX, 8h}$ has a value a = 85 dB (resp. L_{CPk} = 137 dB by single impulses), and the lower exposure action value $L_{AEX, 8h}$ has a value a = 80 dB (resp. L_{CPk} = 135 dB by single impulses). These action values are noise values in working place, beyond which is the employer obliged to carry out actions (shares) to reduce noise.

As can be seen from the values in the table (Table 2), the noise level did not exceed even the value of the lower exposure action value of 80 dB in any of parlours.

CONCLUSION

Noise generated during the milking process depends not only on technological equipment of parlours and their age. Important factors are also the number of animals milked at the same time, and thus the number of parlour places.

The noise exposure directly depends on the way of working of operators, especially on the speed of work (more noise is produced in haste), precision and accuracy of teat cups application (if improper application, unpleasant noise can occur), the volume of mutual communication of operators and by chasing the dairy cows into the parlour, or other activities (flushing water during milking).

In our experiment, noise exposure levels didn´t exceed the values given in The Directive of the European Parliament and the Council Nr. 2003/10/EC. In terms of noise, most favorable working environment was in case of automatic milking system.

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EC Directive, (2003), Directive of the European Parliament and the Council Nr. 2003/10/EC from 6 February 2003 on the minimum health and safety requirements regarding the exposure of workers to the risks arising from physical agents (noise) (Seventeenth individual Directive within the meaning of Article 16(1) of Directive 89/391/EEC)

THE EFFECT OF DIVERGENT SELECTION FOR SHAPE OF GROWTH CURVE IN JAPANESE QUAIL ON EGG QUALITY

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Abstract: The HG (high growth intensity from 11 to 28 d) and LG (low growth intensity form 11 to 28 d) lines of meat-type Japanese quail, divergently selected for relative body weight gain from 11 to 28 d but constant 49 d weight were used in the experiment. Eggs were analyzed six times during laying period, form 11 to 33 weeks of age. In total 488 eggs of HG and 497 eggs of LG were analyzed for weight of eggs, weight and proportion of yolk, eggshell and albumen, and for yolk colour. Except yolk colour the line had significant effect on all observed characteristics ($P<0.05$). Age of quails also significantly affected all parameters ($P<0.05$). Egg weight was higher in LG (13.4 vs. 12.6 g) compared with HG ($P<0.05$) and yolk weigh was 3.9 g for HG and 4.0 g for LG ($P<0.05$). Except egg and yolk weight and yolk proportion significant interaction between age and line was calculated ($P<0.05$).

Key Words: egg weight, yolk, albumen, eggshell

INTRODUCTION

Japanese quail is today the smallest poultry species kept for eggs and meat on some farms. It is also accepted as a laboratory animal widely used for biological and genetic studies because of its small body size, resistance to diseases, rapid growth, easily handling, and possibility of using a large number of animals in a limited space (Narayan et al. 1998, Minvielle et al. 2007, Tarhyel et al. 2012, Hyánková et al. 2008, Hyánková and Knížetová 2009, Hyánková and Starosta 2012). The evaluation of the production and quality of meat quail eggs is of fundamental importance for the breeding; genetic differences between the strains may change the egg´s internal and external quality, as well as their production. Meat type quails are also suitable laboratory animal for broiler breeders. The quality of hatching eggs is imperative because eggs provide both physical protection and nutrition for the growing embryo. Therefore the aim of the study was to evaluate the egg quality of two lines of meat type Japanese quails divergently selected for shape of growth curve. The HG and LG lines of meat-type Japanese quail, divergently selected for relative body weight gain from 11 to 28 d but constant 49 d weight, provide a suitable model for study of the relationship between the shape of the growth curve, the efficiency of meat production and reproductive traits. Selection in these lines for high (HG) and low (LG) relative body weight (BW) gain between 11 and 28 d of age ($RG_{11–28}$ = gain from 11 to 28 d of age/BW at 28 d of age), modified the slope of the linear part of the growth curve. Subsequent selection for constant BW at 49 d of age (90 to 95% of adult BW), kept adult BW at a similar level for both lines. Details concerning the selection process of the lines have been reported previously (Hyánková et al. 2001).

MATERIAL AND METHODS

The HG and LG quail used for the experiments were the progeny of generation 51obtained from a single hatch. The husbandry was essentially similar to that provided during selection. The quail were fed ad libitum on a breeder diet (195 g/kg CP and 11.1 MJ/kg ME). Temperature was maintained at approximately 22 °C. The birds received 14 h of light followed by 10 h of darkness per day. In ± 90 quails per line (2 to 3 females/cage), egg quality (weight of egg, yolk, eggshell and albumen, yolk colour) were recorded in age 11; 17; 21; 26; 30 and 33 weeks. In total 488 eggs of HG and 497 eggs of LG were analyzed. Eggs were weighed and broken open, and wet yolk weights were recorded. The color of the yolk was measured using the DSM Yolk Color Fan. Shell weight was determined after washing and

drying of shells. The albumen weight was calculated by the following equation: egg weight- (shell weight + yolk weight). Weight proportion of yolk, albumen and eggshell were calculated and expressed in percentage. The data were analyzed using a general linear model procedure of the UNISTAT 5.1 (Unistat Ltd, England). The effects of line, age and their interactions on egg quality were evaluated.

RESULTS AND DISCUSSION

The results of egg quality are shown in Table 1. Egg weight, yolk, albumen and eggshell weight were significantly higher (P<0.05) in LG in comparison with HG. The line LG has higher live body weight at 11days of age and lower growth intensity form days 11 to 28 but consequently grow with higher intensity and final body weight should be the same as in HG, anyway can be slightly lower than in HG. Hyánková and Starosta (2012) published in these lines in 39 generation in 5 month of age egg weight 12.2 g for HG and 13.1g for LG, this means that the difference between the lines remained almost the same for next 12 generations. It is necessary to pay attention to hatching egg quality, because lower yolk weight in HG can cause lower weight of offspring in 11 days of age, as it was described by Ulmer-Franco et al. (2010) in broilers.

Table 1 The effect of lines HG and LG on egg quality

		HG		LG		P values		
		mean	v_x	mean	v_x	line	age	line x age
Egg weight	g	12.6^a	0.10	13.4^b	0.09	<0.05	<0.05	ns
Yolk weight	g	3.9^a	0.13	4.0^b	0.13	<0.05	<0.05	ns
Albumen weight	g	7.6^a	0.11	8.3^b	0.09	<0.05	<0.05	<0.05
Eggshell weight	g	1.0^a	0.11	1.1^b	0.12	<0.05	<0.05	<0.05
Yolk colour	−	4.9^a	0.14	5.0^a	0.14	ns	<0.05	<0.05
Yolk proportion	%	31.0^b	0.08	30.0^a	0.07	<0.05	<0.05	ns
Albumen proportion	%	60.7^a	0.04	62.1^b	0.03	<0.05	<0.05	<0.05
Eggshell proportion	%	8.3^b	0.09	8.0^a	0.09	<0.05	<0.05	<0.05

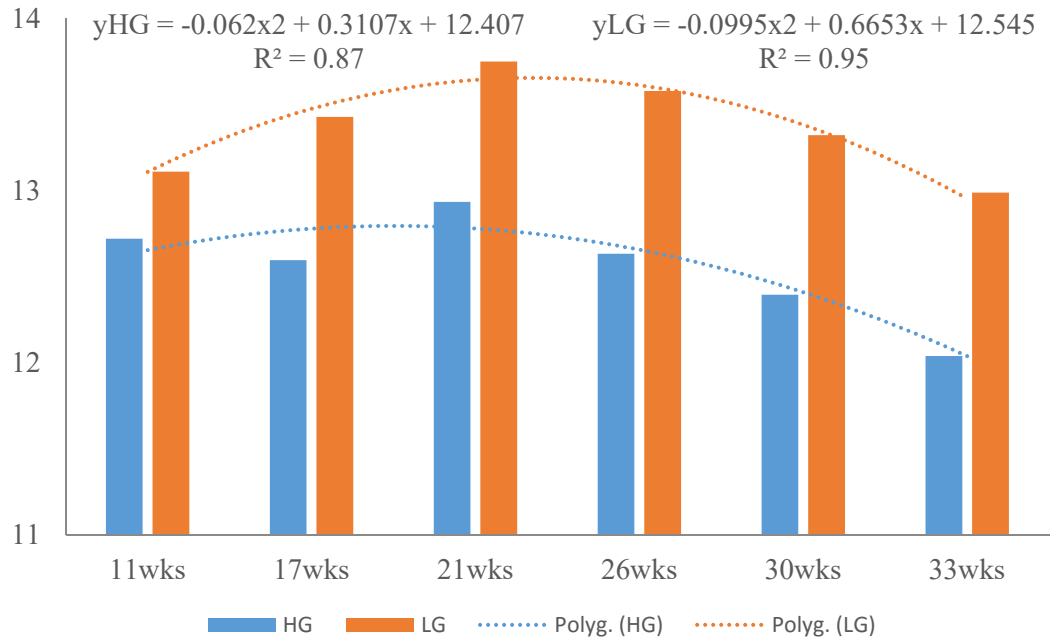
Legend:
a, b - means of the same order designated by different letters are significantly different (P<0.05)
v_x- coefficient of variance

Drumond et al. (2014) evaluated egg quality of four different meat quail genotypes from 7 to 20 weeks of age and they published average egg weight from 13.1 to 14.3 g, which is slightly higher range than in our study. Hakan et al. (2015) also observed egg quality of quails with different colour of feathers. Their quails had lower live weight, than HG and LG and consequently the egg weight was also lower and ranged from 11.7 to 13.2 g. Silva et al. (2013) published positive genetic correlations between body weights and average egg weight, depending on the age, from 0.37 to 0.57 g, which can explain lower egg weight in quails with lower body weight. The live body weight in HG and LG is about 255g at 49 days.

In the study of Hakan et al. (2015) the feather colour had significant effect on all observed parameters, except eggshell thickness. However despite to lower egg weight in their study the weight of yolk was almost the same or even higher than in our study (3.97–4.5 g). It seems that higher egg weight means mainly higher weight of albumen as it was found in chickens (Stanishevskaya and Toritisna 2007). Similar observation reported Anderle et al. (2014) who evaluated egg quality of Czech primitive breed and they found relatively high weight of yolk. There was no significant effect of line on yolk colour as both of them fed the same diets.

Figure 1 shows the effect of age on egg weight in both lines. The egg weight increased with age and after 21st week of age decrease again. In contrast in broiler breeders the egg weight increase with age (Tona et al. 2001).

Figure 1The effect of age on egg weight [g]



CONCLUSION

Statistically significant effect of divergent selection of Japanese quails for shape of growth curve on eggs quality was found. LG line, with higher weight at 11 d and lower growth intensity from 11 to 28 d, had significantly higher egg weight, yolk, albumen and eggshell weight, than HG line ($P<0.05$). This observation is important from hatching eggs point of view and offspring quality, as both lines have the same final live body weight. Age of quails had significant effect on all observed characteristics ($P<0.05$).

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RELATION BETWEEN THE AMOUNT OF SOMATIC CELLS AND THE LACTOSE IN THE COW MILK OF ORGANIC FARMING ORIGIN

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Abstract: The aim of this study was to evaluate the relation between the number of somatic cells and the amount of lactose at a dairy herd of cattle managing in the organic farming mode. Individual samples of milk were monitored for a period of one year, when the samples were taken once per month and underwent analyses in the Brno – Tuřany laboratory. Apart from the somatic cells count (SCC) (thousands/ml) and lactose (L) in milk, we monitored the milk yield (kg), the contents of fat (%), proteins (%), urea (mg/100ml) and free fatty accids (FFA) (mmol/100g of fat). After the evaluation it is clear that the relation between the number of somatic cells and the amount of lactose is in mutual negative correlation. The results can be used in a zootechnical practice when filtering off the results of non-infectious mastitis.

Key Words: organic farming, milk, somatic cell, lactose, mastitis

INTRODUCTION

Currently in a dairy technology the most widely used type of milk is the cow milk which is a product of a mammary gland of domestic cattle (*Bos primigenius f.taurus*) (Samková 2012). It is a complex food, when most of the nutrients contained in milk do not act in isolation but in cooperation with a variety of other components. Further on the domestic cattle milk contains many inorganic substrates and biocatalysts (Vorlová et al. 2015). A complex system, such as raw cow milk, contains apart from basic components, such as water, milk fat, proteins, milk sugar and mineral substances, also a variety of other compounds of organic and inorganic origin. Raw cow milk being an important material for milk technologies contains more than a hundred substances with different chemical and physical features (Semjan 1987). The essential, and indeed the only component of carbohydrates in cow milk is lactose ($C_{12}H_{22}O_{11}$). Carbohydrates, such as glucose, galactose or oligosaccharides, are present in raw cow milk in negligible quantities (Samková 2015). Lactose is present in milk in the range of 4.4–4.7%. It is a disaccharid with low sweetnes and good digestibility for a human being (Navrátilová et al. 2012). Other nonseparable components of cow milk, besides others, are also somatic cells. Their number is an important value of hygienic quality. The number of somatic cells in milk (SCC) indicates the physiological balance and state of health of the dairy cow and its mammary gland. SCC is one of the basic criteria for national and international regulation of milk quality. According to the Regulation of European Parliament and Council No. 853/2004 the upper hygienic limit for purchase of raw cow milk was established for SCC at the number of 400 000 in 1 ml of a pool sample of milk (Samková 2015).

MATERIAL AND METHODS

Characteristics of the Farm and the Herd Reared in Organic Farming

Branná, the organic farm, has been kept under the organic farming mode since 2000. The main focus of the farm is a livestock production and only partially a crop production. The organic farm produces bulk feed itself, that is hay, haylage and silage, to provide feed supplies for animals. In

livestock production the farm breeds cows with market production of milk and cows without market production of milk (Hereford a Galloway breeds). The dairy herd consists of Czech Pied cattle breed. Summer ration of dairy cows (from 15/5/2015 to 20/10/2015) is provided by the animals grazing on surrounding lands. The walking distance for the dairy cows to the pasture is up to one kilometer. After milking the dairy cows are served with energetic feed in a form of a mixed ration of meal and silage. At the farm the night feast is also applied. In winter months the animals are fed on conserved forage, grass silage and hay. From organic mill operation the farm purchases mill by-product, grits and bran to fill the energy compound of the feed ration of the animals bred.

The farm operates a newly reconstructed parlor, a half-size version of herringbone type 1 x 11 places for dairy cows; it is a semi-automatic milking technology. The milked milk is then pumped off through a mechanical filter into a tank, where being sprayed on cooled walls of the tank it is cooled onto 4.8–5.2 °C. Milking takes place twice per day, that is always in the morning at 3:30 a.m. and in the afternoon at 15:30.

Sets of Milk Samples

For the analyses, individual samples of raw milk of dairy cows were used. At the monitored farm the milk of dairy cows was led from the milking unit to milk metres behind which drip collecting vessels were temporarily installed, being able to prepare a milk sample from the entire time of milking one animal. In total, on average 105 individual milk samples were collected per month. Milk samples were collected once per month. Collections were conducted at regular intervals in the range of 14th – 20th day of a month. Further on the milk was cooled and transferred to subsequent analyses.

Milk of the dairy cows which milk was anyhow nonstandard (increased number of somatic cells, medically treated dairy cows, dairy cows after calving, milk with blood), was collected with a clean ladle from milking cans allotted for milking nonstandard milk.

Analytical Methods

Analyses of milk were carried out in an accredited laboratory (according to CNS EN ISO/IEC 17025:2005) for milk analysis in Brno – Tuřany (number of the test laboratory 1312.3, number of the certificate 750/2015) according to methods:

- Determination of milk composition (fat, protein, lactose), urea and freezing point by infrared spectroscopy SOP 01, CNS 570536:1999.
- Determination of number of somatic cells by fluoroopto-electronic method SOP 02, CNS EN ISO 13366-2:2007

Lactose: this is a quality parameter of milk. The amount of lactose in analyzed milk of the dairy cows is determined by infrared absorption analyzer which measures the amount of light that is absorbed by hydroxil groups. It is an indirect method of measurement (determination of lactose monohydrate). The amount of lactose (% of monohydrate) is reported in g/100g (www.cmsch.cz).

Somatic cells: the amount of somatic cells in milk is a quantitative indicator. The number of somatic cells in raw cow milk is determined by fluoro-opto-electronic method. At this procedure the somatic cells are particals that have a minimum intensity of fluorescence due to staining with fluorescent dye. In a flow cytometer the dyed somatic cells create an electrical impulse and it is registered. The number of somatic cells is reported in thousands in 1 ml of milk. At samples of milk with high number of SCC, a bacterial examination of the presence of mastitis pathogenes was performed. A control measurement of SCC was performed also directly at the organic farm. For the control measurement the MT05 device of Slovak producer PISOFT was used. This device is used for immediate detection of somatic cells count in fresh milk. It is used when monitoring the decline of somatic cells count at dairy cows after calving (the end of a colostric period), when daily controlling the pool samples of milk etc.

To the measured milk an agent is added, which affects somatic cells in milk and this way it changes the milk viscosity so that the rate of the viscosity change is directly proportional to the somatic cells count in the milk of the dairy cow. For this accurate viscosity measurement a special ball in the device is used. The ball moves in an inclined glass tube by an angle of 25° according to the milk viscosity. The device analyzes a sucked milk sample in a way that in the glass tube with the sample a metal ball moves downwards for specific time, after that the tube automatically offsets horizontally

and on the scale the values of somatic cells count can be read. The measurement time of one sample is 30 seconds.

Statistical Evaluation

To analyze the parametres the statistical software R was used. Basic statistical parametres such as geometric mean and standard deviation were calculated. To describe the relations between the number of somatic cells and lactose, the method of constructing a biplot was used. These relations were described for all the analyzed compounds of milk (milk yield (kg), fat (%), protein (%), lactose (%), somatic cells (SCC), (tis./ml), urea (mg/100ml), free fatty acids (FFA) (mmole/100g fat).

RESULTS AND DISCUSSION

The number of somatic cells in milk was evaluated in orginal values. The amount of lactose was evaluated in a logaritmically transformed form. Selective standard deviations (SSD) (observation deviations from selective average) were counted from logaritmical data.

Achieved values of milk yield are shown in the Table 1 and the Figure 1. The average yield is relatively stable, that is through all the monitored period. Low yield is caused by feeding in an organic farming system, location of the farm in a mountain region, and the breed bred (Czech Pied cattle).

The slight increase was observed in the months of June and October. This can be attributed to the pasture maturity of overgrowing areas for pasture in spring and after summer mowing.

Table 1 Basic statistic characteristics of milk yield and milk compounds

Month	Milk yield (kg)	Milk yield SSD	SCC (tis./ml)	SSD of SCC	Lactose (%)	Lactose SSD
I.	8.796	0.413	283.445	1.529	4.558	0.176
II	11.287	0.381	335.947	1.426	4.490	0.271
III	11.814	0.359	407.256	0	4.633	0.162
IV	not collected					
V	11.319	0.431	586.533	1.327	4.512	0.137
VI	12.391	0.487	779.613	1.159	4.382	0.263
VII	9.577	0.529	1030.655	1.334	3.912	0.507
VIII	10.009	0.437	575.277	1.517	4.326	0.281
IX	10.433	0.392	394.822	1.379	4.451	0.173
X	11.808	0.360	220.005	1.221	4.747	0.116
XI	9.805	0.387	216.536	1.399	4.571	0.249
XII	9.557	0.378	258.472	1.380	4.612	0.256

Figure 1 Development of milk yield in average of the reference year

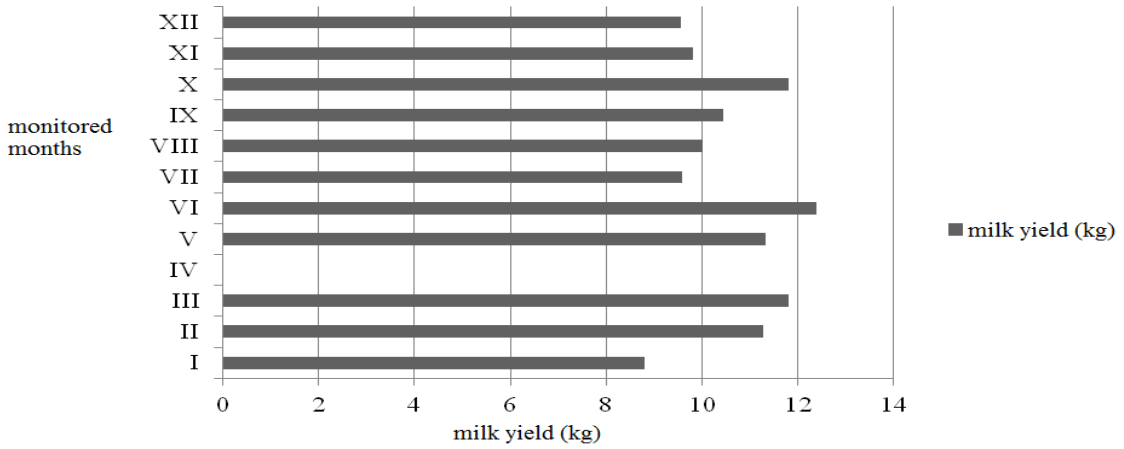
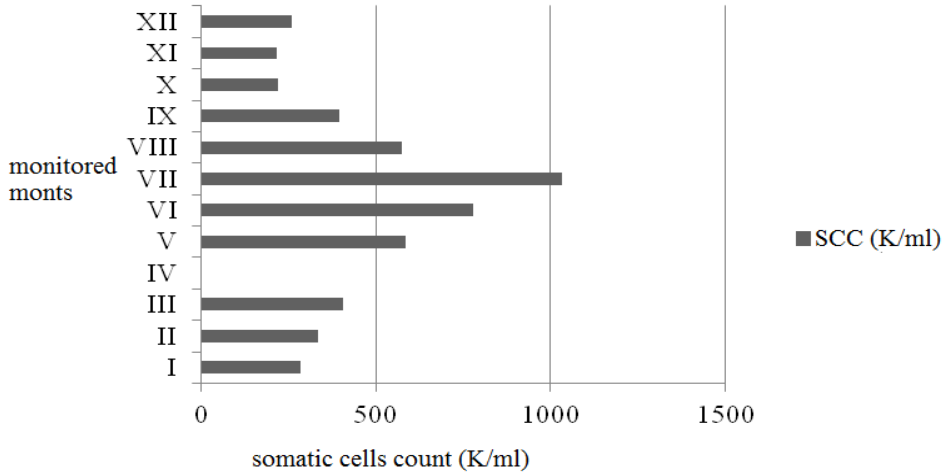


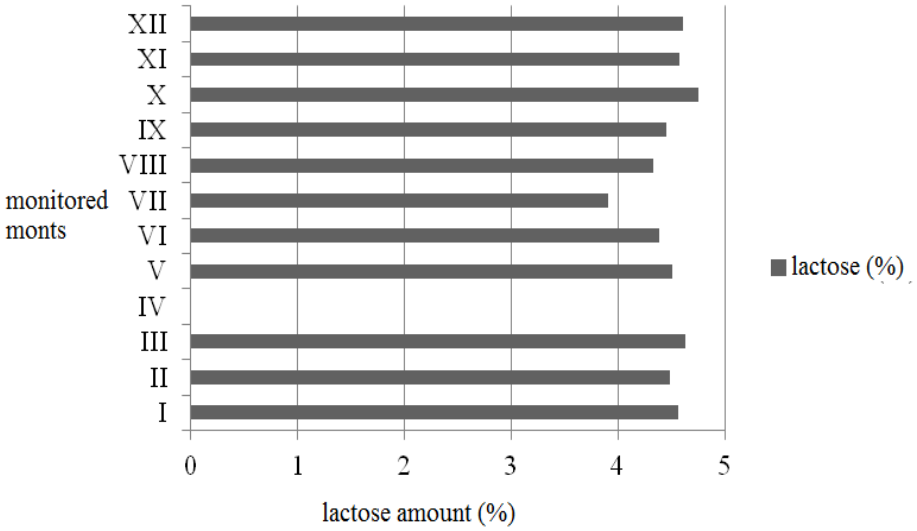
Figure 2 Values of somatic cells count (SCC) (thousand/ml)



Achieved values of SCC are shown in the Table 1. and the Figure 2. The analysis of the somatic cells count in the organic farm milk proved an increase over the permitted norm (400 thosand/ml) in the months of March and September. High increase of somatic cells count in milk indicates inflammations of mammary glands, so called mastitis at dairy cows bred.

At the microbiological analysis of the milk the most spread disease agents were found as *Streptococcus uberis* a *Streptococcus agalactiae.* At selected dairy cows antibiotic treatment was started. After an examination more serious cases of mastitis were selected off the herd for slaughter.

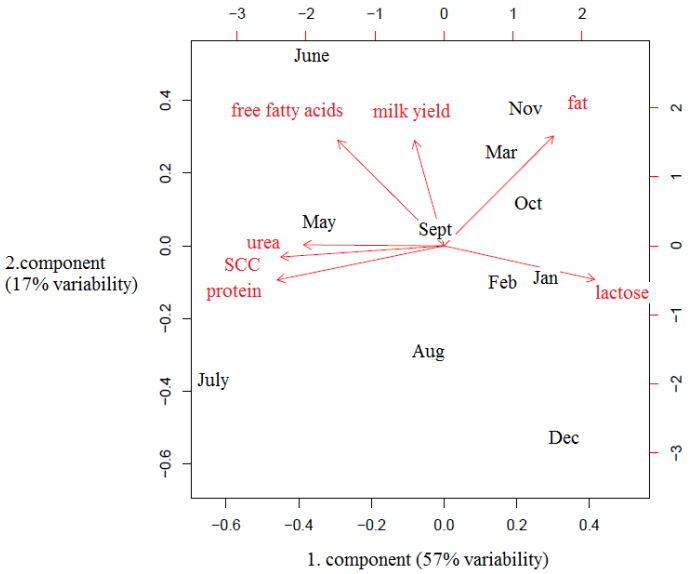
Figure 3 Values of lactose amounts (%)



Achieved values of lactose are shown in the Table 1 and the Figure 3. In the analyzed milk a significant decrease of lactose in milk occurs from the month of April to July. This is caused by an increase of mastitis in dairy farming.

At healthy dairy cows the lactose amount is stable in the terms of milk composition and it moves on the level of 4.5–5%.

Figure 4 Biplot – display of variables and observations for analyzed parametres



To build a biplot for the farm analysis values scaling was used to suppress the high scattering of the first two variables, because scaled variables have dispersion equal to 1. Since the centering was carried out, they are based in point [0,0] (zero mean values of variables).

Evaluation of biplot :

- The maximum amount of fat is present in months of Oct, Nov, Mar (October, November, March)
- The minimum amount of fat is present in July
- Lactose is high in winter months, lower in summer months
- SCC and lactose are jsou negativly correlated variables
- Urea, SCC and proteins are positively correlated variables. They have smaller values of variables generally in winter months, in summer months generally average and above average.

The results on the farm and milk analyses through the monitored year proved negative correlation between SCC and lactose amount, which corresponds with results of more works (Renner 1972, Bergmann 1978, Hanuš and Suchánek 1991, Hanuš et al. 1995, 2009). In these studies the same negative correlation between somatic cells count and lactose amount was proved. This result can be led close to a possible mastitis in dairy cows and development of mastitis. The process of the disease causes increasing pathogenic activity which was proved in milk samples. This process reduces the secretory epithelium and formation of lactose in the mammary gland. It is therefore an intensification of immune reaction of the organism when increasing somatic cells count. Lactation stage can be also an agent of decreasing the amount of lactose and increasing somatic cells count along lactation progression, mainly towards its end (Hanuš et al. 2010).

CONCLUSION

The aim was to determine the relation between the number of somatic cells and the amount of lactose at a dairy herd of cattle managing in an organic farming mode. Monitored were individual milk samples through a period of one year. Based on the results it can be stated that the relation between somatic cells count and the amount of lactose is in a mutual negative correlation. The results can be used in zootechnical practice not only when filtering the results of non-infectious mastitis, but also when analyzing in rutine monitoring systems, screening a herd or when managing a prevention of occurance of product disorders of dairy herd.

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INFECTIOUS AGENTS OF CALF MORTALITY IN NEONATAL PERIOD

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Abstract: The aim of this study was detection of the percentage of infectious agents in mortality of calves in the neonatal period. The study was conducted on 37 carcasses of Holstein-Friesian calves, aged between 2 and 14 days from 10 farms located in south-western Poland. Standard set of organs (liver, spleen, lung and intestine) were examined to presence of bacteria (general microbiology, isolation of *Salmonella*, *Campylobacter*) and *Candida*. Isolated strains of *E. coli* were evaluated for virulence genes. The results obtained from research indicate that the main cause of mortality of examined calves was diarrhea (64.9%). In 13.5% there was found sepsis, and in case of 21.6% there could not be determined the cause of death. Among the pathogenic strains of *E. coli* had most of the F5 (K99). In testing material there was no found *Salmonella* sp.

Key Words: calves, mortality, diarrhea, etiology

INTRODUCTION

The health of replacement calves is an important component of cattle producers worldwide. The productivity of the herd can be negatively affected by high mortality rate of calves, in fact it increases veterinary costs, limits genetic selection and increases the need for acquisition of replacement dairy animals. The highest morbidity and mortality rates generally occur in neonatal calves prior to weaning (Cho and Yoon 2014). High mortality rate of calves can be related to the larger number of calves in a herd, employee performance, severe weather, and the neonatal period covering the first 4 weeks of life (Uetake 2013).

Diarrhea of calves is a commonly reported disease and is attributed to both infectious and non-infectious factors (Foster and Smith 2009, Bednarski et al. 2015, Azimpour and Pourtaghi 2016). While the diarrhea of neonatal calves is usually associated with bacterial background (*E. coli*) or rotaviruses and coronaviruses infections, *Cryptosporidium* spp. seems to be the most common cause of diarrhea of older, 3-week-old animals (Foster and Smith 2009, Ok et al. 2009). Salmonellosis in calves occurs mostly at 2–6 weeks of age (Barrow et al. 2010). Dehydration and septicemia are the major contributors of mortality and morbidity among calves. In addition, early-life enteric disease among calves might have influence on calf raisers' ability to detect respiratory disease later in life (Hulbert and Moisá 2016). Mortality in the cattle industry is not only relevant with regard to animal health and welfare but also to economic losses (Uetake 2013).

This study is aimed at evaluation of the infectious agents in the neonatal dairy calf mortality in south-western Poland farm.

MATERIAL AND METHODS

The study included 37 carcasses of Holstein-Friesian calves, aged between 2 and 14 days from 10 farms located in south-western Poland in 2015. A full necropsy of all animals was performed. Samples of organs were taken for the microbiological examination. Standard set of organs (liver, spleen, lung and intestine) were examined to presence of bacteria. Each sample was investigated by standard microbiological mediums (Columbia blood agar with 5% defibrinated sheep blood, McConkey/XLD

agar, Sabouraud medium, brein-heart-infusion, Oxoid, GB). Additionally isolation of *Salmonella* and *Campylobacter* were performed according protocol described above (Bednarski et al. 2011, Jawor et al. 2012). Isolated strains of bacteria were kept at - 80°C in Microbank Storage Boxes (Pro-Lab Diagnostic, Canada). All *E. coli* strains were evaluated by multiplex PCR for identification of 8 virulence genes (Table 1) typical for entorotoxigenic strains (ETEC) (Casey and Bosworth 2009). The conditions for DNA template amplification were described before by Bednarski et al. (2015). The *Campylobacter* species were identified using methods described above (Wojciech et al. 2005). Identification of isolated strains was performed using Api20E (BioMerieux, France). Apart from the microbiological tests there were also carried out epidemiological inquiries. All data were evaluated by standard statistic methods.

Table 1 Sequence of primers for toxins and fimbrias of E. coli used in this study (Casey and Bosworth 2009)

Virulence factor	Name of target gene	Primers sequence (5'-3')	Length of product (bp)
STb	*estB*	TGCCTATGCATCTACACAAT CTCCAGCAGTACCATCTCTA	113
STa	*estA*	ACTGAATCACTTGACTCTT TTAATAACATCCAGCACAGG	158
F5 (K99)	*fanA*	AATACTTGTTCAGGGAGAAA AACTTTGTGGTTAACTTCCT	230
LTb	*eltB*	GGCGTTACTATCCTCTCTAT TGGTCTCGGTCAGATATGT	272
F18	*fedA*	TGGTAACGTATCAGCAACTA ACTTACAGTGCTATTCGACG	313
987P (F6)	*fasA*	AAGTTACTGCCAGTCTATC GTAACTCCACCGTTTGTATC	409
F4 (K88)	*faeG*	GTTGGTACAGGTCTTAATGG GAATCTGTCCGACJAATATCA	505
F41	*fedA*	AGTATCTGGTTCAGTGATGG CCACFATAAGAGGTTGAAGC	612

RESULTS AND DISCUSSION

Studies have shown that the most important cause death of calves were diarrhea (64.9% of calves), then septicemia (13.5%). In 21.6% the cause of death were unknown (Table 2).

In cases of diarrhea, ETEC strains were found in 12 cases (32.4%), mixed infection of ETEC and *C. jejuni* was observed in 10.8%. The most important causes of diarrhea in newborn calves are infections with enterotoxigenic strains of *E. coli*, rotavirus, coronavirus and *Cryptosporydium* sp. (Bednarski et al. 2015, Foster and Smith 2009). Minor role in this group age plays *Salmonella* or other pathogens like *Campylobacter* sp. (Bednarski et al. 2011). Strains of ETEC characterizes by the presence fimbriae and ability for the production of enterotoxins. Fimbriae allows colonization of the intestine by adhesion to enterocytes. In our study we found the dominance of fimbriae F5 (K99) (Table 3). There are also known strains isolated from clinical cases, which do not include fimbriae K99, but only F41 or F17. Strains of ETEC including F4 (K88) fimbriae are able to elicit both diarrhea and sepsis calves (Bednarski et al. 2015, Kolenda et al. 2015). In cases of illness caused by the ETEC strains in calves usually ca. 15–30% of the animals may show symptoms of the disease and requires rehydration and replenish of electrolytes. The clinical status of an animal may further deteriorate due to acidosis and in chronic cases lactic acidosis, which has been reported in diarrheic calves (Bednarski and Kupczyński 2016). Morbidity may reach the 50% in the case of meat breeds and even 75% in the case of dairy breeds. Process and the number of drops depends on many factors such as the virulence of the strain, exposure of animals to infection, the presence of other pathogens and resistance of calves and their appropriate care. The mortality of diarrheic calves ranges from 5 to even 50% (Kolenda et al. 2015, Uetake 2013).

Table 2 Reason of death and etiological factor

Reason of death	No. of cases (%)	Etiology	No. of cases (%)
Diarrhea	24 (64.9%)	ETEC	12 (32.4%)
		ETEC + *C. jejuni*	4 (10.8%)
		C. jejuni	1 (2.7%)
		Candida sp.	2 (5.4%)
		Unknown	5 (13.5%)
Septicemia	5 (13.5%)	*E. coli*	4 (10.8%)
		Pseudomonas aeruginosa	1 (2.7%)
Unknown	8 (21.6%)	-	

Table 3 Characteristic of E. coli strains (n=16) isolated from case of diarrhea from intestine

Virulence factor	No. of isolated	%
F5 (K99) + STa	9	56.3%
F41 + STa	4	25.0%
F18	2	12.5%
F41	1	6.3%

Campylobacter sp. was isolated in only three calves. This bacteria is or can be an part of normal gastrointestinal microflora in adult cattle (Busato et al. 1999, Wesley et al. 2000). The agents can be isolated from healthy calves and as well as calves with diarrhea. The role of *C. jejuni* and *C. coli* as primary enteropathogen in calves is uncertain. However in experimental infection this pathogen will cause mucoid diarrhea. The isolation of *Campylobacter* is as well of some importance because of its zoonotic impact (Busato et al. 1999, Wesley et al. 2000, Bednarski et al. 2011).

In our study, there was no occurrence of *Salmonella* sp.. Currently, infection of this pathogen in cattle in Poland is sporadic (Jawor et al. 2013). Research has indicated occurrence of *Candida* infection in 2 instances (5.4% of the causes of death). Development of candidiasis usually occurs as result of the antibiotic therapy. Infection of *Candida* in calves is considered as an opportunistic infection as result of dysbacteriosis (Wada et al. 1994).

Septicemia occurs commonly in calves and can be a significant cause of economic loss in cattle farm (Fecteau et al. 2009). Our research has shown that septicemia is responsible for the 13.5% of the calves death with predominance of septicemic colibacillosis. *E. coli* strains were isolated from cases of septicemia calves are a relatively small number of serogroups O 8, 9, 15, 26, 35, 45, 78, 86, 101, 115, 117, 137. In Europe the most dominating serotype of E. coli is O78: K80, whereas the most common isolated serotype in North America is O137: K79. However, there are reports which indicate that other serotypes could be most common cause of colisepticemia of calves. There are *E. coli* strains capable of inducing sepsis characterized by a specified virulence traits. Among them, the most important are: the ability of invasive, presence of genes which allows their penetration and multiplication in the blood and internal organs (Fecteau et al. 2009, Kolenda et al. 2015).

We can prevent the occurrence of diseases neonatal period, by proper treatment of the calf after birth (caring for calves), colostrum-drinking calves, taking care of the hygiene of the environment in which calves are born and reside, disinfection of premises and mulch as well as specific active immunization of pregnant cows.

CONCLUSION

In the presented study, as the most important cause of death in ten calves there was found diarrhea. ETEC strains were found in 32.4% of the group. In the case of *E. coli* strains dominated fimbria F5 type. Also, an important cause of death calves were mixed ETEC infection and *C. jejuni*. In the case studies, there was no occurrence of *Salmonella* sp. recognized. However, occurrence of the identified pathogens in dairy cattle herds can be prevented through proper herd management, prevention, bioassurance and vaccination.

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THE INFLUENCE OF FEEDING WHEAT WITH PURPLE GRAIN TO PERFORMANCE AND BIOCHEMICAL PARAMETERS OF BROILER CHICKENS

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Abstract: Purple wheat contains a higher amount of anthocyanins than common wheat cultivars. The aim of this study was to evaluate the influence of feeding wheat with purple grain to metabolism and liver function and some performance parameters of broilers. A total of 50 Ross 308 hybrid cockerels were fattened on conventional deep litter system to 39 days of age. Cockerels were allocated randomly to 2 dietary treatments using a randomized complete block design with five replicates per treatment. There were 5 chickens per replicate pen. The experimental group (n = 25) received feed mixtures containing 60% of purple wheat RU 687-12 (experimental group) with content of anthocyanins 36.66 mg/kg dry matter. The other control group (n = 25) received feed mixture with 60% of common wheat with content of anthocyanins 4.88 mg/kg dry matter. The blood samples were collected at the end of the experiment. The blood biochemical parameters related to hepatocyte damage or metabolism of liver were analysed (aspartate aminotransferase, gamma-glutamyl transferase, alkaline phosphatase, alanine aminotransferase, lactate dehydrogenase, albumin, total protein, cholesterol, triglycerides, urea). The highest carcass yield was found in the experimental group but differences between groups were not significant ($P > 0.05$). During the trial were not observed significant differences between both experimental groups in monitored basic biochemical parameters of blood.

Key Words: colour wheat, anthocyanins, poultry nutrition, metabolism, liver

INTRODUCTION

The colour grain of wheat cultivars contains a higher amount of anthocyanins than common wheat cultivars grain. Many previous analyses confirm this statement (Mareš et al. 2015, Karásek et al. 2016, Šťastník et al. 2016). Anthocyanin pigmented wheats have been discovered in the 19th century in Ethiopia. Many cultivars of purple, red, blue and black grained wheat have been developed (Zeven 1991). In last years, the interest towards functional properties of foods has increased progressively. Increasing interest of anthocyanins rich food are caused by their health benefits. Anthocyanins are antioxidant flavonoid compounds occur in fruits and vegetables, as well as in the pigmented varieties of many cereals (Mazza and Miniati 1993). The composition of the anthocyanin fraction has been found to be characterized by cyanidin 3-O-glucoside and peonidin 3-O-glucoside in purple grained wheats, while delphinidin 3-O- rutinoside and delphinidin 3-O-glucoside prevail in blue wheats (Abdel-Aal and Hucl 2003, Abdel-Aal et al. 2006, Ficco et al. 2014, Liu et al. 2010). Anthocyanins demonstrate anti-inflammatory (Subarnas and Wagner 2000) and antioxidative activity (Subarnas and Wagner 2000, Wolniak 2002, Wang 2000), and they are able to chelate metal ions (Wawer 2001). Anthocyanins increase the resistance of hepatocytes to oxidation, activate liver enzymes (aspartate aminotransferase, alanine aminotransferase and lactate dehydrogenase) and lower the reduced glutathione concentration in the liver (Kowalczyk et al. 2003).

Whether a higher compounds contained in purple wheat it may have positive potential to liver health and it may improve antioxidant potential, consequently, may positively influence growth. So, the aim of this study was to evaluate the influence of feeding wheat with purple grain to liver health and performance of broilers.

MATERIAL AND METHODS

A total of 50 sexed Ross 308 hybrid cockerels were fattened on conventional deep litter system. Wood shavings were used as bedding material. The trial was conducted from day 12 to day 39 of chicken's age. Room temperature and humidity were controlled. Lighting system was 16 hours light and 8 hours dark. Cockerels were allocated randomly to 2 dietary treatments using a randomized complete block design with five replicates per treatment. There were 5 chickens per replicate pen. The experimental group received feed mixtures containing 60% of purple wheat RU 687-12 cultivar (EXP group) with content of anthocyanins 36.66 mg/kg dry matter (DM). The control group (C group, n = 25) received feed mixture with 60% of common wheat with content of anthocyanins 4.88 mg/kg dry matter. The wheat-based diet was designed to ensure the highest dose of anthocyanins to chickens. Table 1 shows the compositions of experimental rations. The feed mixtures were calculated according to the Nutrition Specifications (Aviagen Group, 2014). Chemical compositions of experimental feed rations show Table 2. The chickens were fed *ad-libitum.* Health status was evaluated daily and live weight measured every week during the trial.

*Table 1 Composition of feed mixture (*g/kg*)*

Component	C	EXP
Wheat - purple	0	600
Wheat - common	600	0
Soybean meal	235	235
Maize	33.5	44.5
Rapeseed oil	60	60
Wheat gluten	31	20
Premix*	30	30
Monocalciumphosphate	7.5	7.5
Limestone milled	3	3

** Premix contains (per kg): lysine 60 g; methionine 75 g; threonine 34 g; calcium 200 g; phosphorus 65 g; sodium 42 g; copper 500 mg; iron 2500 mg; zinc 3400 mg; manganese 4000 mg; cobalt 7 mg; iodine 30 mg; selenium 6 mg; tocopherol 450000 mg; calciferol 166700 IU; phylochinon 350 mg; thiamine 140 mg; B2 230 mg; B6 200 mg; cobalamine 1000 mg; biotin 7 mg; niaciamid 1200 mg; folic acid 57 mg, calcium pantothenate 450 mg; choline chloride 6000 mg; salinomycin sodium 2333 mg.*

Table 2 Chemical composition of ration – as fed (per kg of diet)

	C	EXP
Dry matter (g)	880	880
AME (MJ)	12.87	12.87
Crude Protein (g)	201.1	202.6
Ether extract (g)	72.8	71.7
Crude fibre (g)	28.8	24.5
Crude ash (g)	53.3	54.4
Cyanidin-3-glucoside (mg)	4.88	36.66

At the end of experiment 6 birds were selected randomly from each group, weighed and slaughtered. Blood was collected into the heparinized tubes and centrifuged for 15 minutes at 3,000 rpm. The separated blood plasma was frozen (-20 °C) until biochemical examination. Feathers were removed and chickens were eviscerated. Carcass yield was calculated as a percentage of live weight. Breast muscle and leg muscle were deboned and weighed in these selected chickens. These values were calculated as a percentage of live weight.

The biochemical profile of blood plasma was analysed with the use of Ellipse (AMS Spa, Italy) analyser. The blood parameters related to hepatocyte damage were selected, it was showed functional activity of liver, respectively. The individual parameters were analysed using individual tests produced by Erba Lachema (Brno, CZ): albumin (Alb 500); total protein (TP 500); AST - aspartate

aminotransferase (AST/GOT 500); GGT - gamma-glutamyl transferase (GGT 250); ALP - alkaline phosphatase (ALP AMP 500); ALT - alanine aminotransferase (ALT/GPT 500); LD - lactate dehydrogenase (LDH-L 100); cholesterol (CHOL 250); TG - triglycerides (TG 250), and by Randox, UK: Urea (Urea, cat. No. UR 107) and Alb/Glob ratio was calculated.

Data has been processed by Microsoft Excel (USA) and Statistica version 12.0 (CZ). We used one-way analysis (ANOVA). To ensure evidential differences Scheffe´s test was applied and $P < 0.05$ was regarded as statistically significant difference.

RESULTS AND DISCUSSION

The mean bodyweight of chickens during the experiment were presented in Table 3. During the trial were not observed significant differences between both experimental groups ($P > 0.05$). In accordance with the performance targets for ROSS 308, the average body weight of cockerels would be 2,705 g at 39 days of age (Aviagen Group 2014). It was achieved live weight 2,636 g in EXP group in our experiment at the day of slaughter (39th day of age).

Table 3 Mean bodyweight per trial (g)

Day of age		12	19	26	33	39
Group	n	Mean ± standard error				
C	25	315 ± 3.7	682 ± 9.6	1,210 ± 22.8	1,872 ± 37.5	2,613 ± 73.3
Exp	25	304 ± 5.0	675 ± 13.7	1,170 ± 27.8	1,764 ± 41.5	2,636 ± 55.2

Differences between groups are not statistically significant (P > 0.05)

Table 4 Body composition (%)

Group	n	Carcass	Breast meat	Leg meat	Liver tissue
		Mean ± standard error			
C	6	67.8 ± 0.37	19.0 ± 0.23	15.7 ± 0.29	2.3 ± 0.13
Exp	6	69.3 ± 0.66	19.6 ± 0.66	15.6 ± 0.16	2.1 ± 0.03

Differences between groups are not statistically significant (P > 0.05)

The highest carcass yield (Table 4) was found in the experimental group (69.3 ± 0.66%) but differences between groups were not significant ($P > 0.05$). Carcass yield stated in the technological procedure for ROSS 308 (Aviagen Group 2014) is the 72.77% for 2,600 g of live weight. Percentages of breast meat and leg meat of body weight are presented in Table 4. In the manual of hybrid Ross 308 (Aviagen Group 2014) is stated 22.03% of breast muscle at 2,600 g of live weight. The same manual (Aviagen Group 2014) indicates a yield of leg meat 16.08% for 2,600 g live weight. The differences among groups in slaughtering yields were not statistically significant ($P > 0.05$). In our previous experiments (Šťastník et al. 2014, Karásek et al. 2016) were found no statistically significant effect on the weight of broilers performance and carcass parameters when fed with colour (purple and blue) wheat. On the contrary, in study Karásek et al. (2014) was found increased antioxidant capacity of the liver in rats fed with purple wheat.

Results of the biochemical analysis are presented in Table 5. The AST, GGT, ALT, ALP and LD activities indicate liver tissue status. The TG and cholesterol concentrations characterized fat metabolism. The urea, TP, and albumin indicate nitrogen metabolism of organism. All these parameters interfere with metabolism of liver and have influence to it. This argument is also confirmed by Dufour et al. (2000), which state the concentration of AST and ALT in the blood reflects the extent of the tissue damage. Our expectation was that the content of anthocyanins in feed mixture may affect liver enzymes. This effect was not discovered in our experiment. This phenomenon may be due to relatively short life of broiler chickens. In accordance with our findings, Abadi et al. (2014) conducted an experiment with wheat based feed mixture, which monitor blood parameters cholesterol and TG. In their experiment was not found significant differences in biochemical parameters, as well. Similar results are discussed by Bhaswant et al. (2015), who carried out an experiment with added cyanidin-3-glucoside into rats feed,

there was not found differences in cholesterol and TG between control and experimental group. On the other side, significant lower plasma cholesterol concentration was found in our experiment (Šťastník et al. 2016) with blue wheat feeding with content of cyanidin-3-glucoside 47.63 mg/kg in comparison to the control group. This results also suggests a positive effect of coloured wheat feeding.

Table 5 Biochemical blood parameters of broilers

Parameter	C			Exp		
	n	6		6		
	Mean ± standard error					
ALT (µkat/l)	0.47	±	0.031	0.45	±	0.017
AST (µkat/l)	4.34	±	0.190	4.80	±	0.389
GGT (µkat/l)	0.44	±	0.037	0.43	±	0.067
ALP (µkat/l)	145.27	±	12.216	147.22	±	15.640
LD (µkat/l)	31.40	±	8.632	28.45	±	2.039
TP (g/l)	32.08	±	0.844	32.33	±	0.964
Alb (g/l)	13.93	±	0.301	14.60	±	0.254
Glob (g/l)	18.15	±	0.613	17.73	±	0.809
Alb/Glob	0.77	±	0.020	0.83	±	0.033
Chol (mmol/l)	3.04	±	0.126	3.10	±	0.130
TG (mmol/l)	0.93	±	0.066	0.93	±	0.107
Urea (mmol/l)	644.50	±	28.365	649.17	±	39.917

Differences between groups are not statistically significant (P > 0.05)

CONCLUSION

In conclusion, based on biochemical characteristics and performance the purple wheat feeding at dose 60% with content of cyanidin-3-glucoside 36.66 mg/kg does not show positive or negative effect in our experiment.

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THE EFFECT OF HEMP BY-PRODUCTS FEEDING ON GUT MICROBIOTA AND GROWTH OF BROILER CHICKENS

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Abstract: The *Cannabis sativa* L. is an annual plant and well known as an important source of fiber, food, feed, dietary oil and medicine for thousands of years in many countries. The aim of the experiment was to study whether hemp by-products may affect the microbial colonization of the gut, because it would affect the absorption and utilization of nutrients also. This could have effect to performance of chickens. A total of 60 sexed Ross 308 hybrid cockerels were fattened on conventional deep litter system. The trial was conducted from day 10 to day 37 of chicken's age. Cockerels were divided into three equal groups with 2 replicates per treatment. There were 10 chickens per replicate pen. Cockerels were allocated randomly. The two experimental groups received feed mixtures containing 2.5% of hempseed expellers or 1% of pellets from technical hemp plant tops. The pellets from hemp plant tops are flowers and seeds with a bit of shives. It was crumbled before mixing into feed mixture. The third group was without hemp addition. On behalf of each bacterial species were not detected statistically significant differences ($P > 0.05$) between groups at 37th day of age. Based on our results we can conclude that the content of cannabidiol 0.03% and 0.15% (hempseed expellers and hemp plant tops, respectively) not affect monitored microbiological parameters of intestinal contents. It was achieved non-significant ($P > 0.05$) differences in average body weight in our study. The same trend was found in carcass yield.

Key Words: hempseed expellers, hemp plant tops, carcass yield, *Lactobacillus*, *E. coli*

INTRODUCTION

The *Cannabis sativa* L. is an annual plant and well known as an important source of fiber, food, feed, dietary oil and medicine for thousands of years in many western and eastern countries in Old World (Callaway 2004, de Padua et al. 1999). The two main proteins in hempseed are edestin and albumin. Both of these high-quality proteins are easily digested and contain nutritionally important amounts of all essential amino acids. In addition, hempseed has very high level of the amino acid arginine (Callaway 2004). Industrial hempseeds have a low content (~0.3%) of tetrahydrocannabinol (THC) which stimulates appetite (Konca et al. 2014, Hampson et al. 2000, Koch 2001). Cannabinol (CBN) is a metabolite of THC, with potential immunosuppressive and anti-inflammatory activities (Pubchem 2015). Another metabolite of THC is cannabidiol (CBD). It is reported that hempseed's cannabidiols have antimicrobial, immunomodulatory, antioxidative, antihypertensive and mineral binding activities (Korhonen and Pihlanto 2003). The nutrient composition of hemp by-products can be quite different. This statement confirms our previous study where the content of CBD in hempseed expellers was 0.017% and crude protein content was 27% (Stastnik et al. 2015).

With the increasing production of hemp (*Cannabis sativa* L.) it´s by-products are more often. These products can be used in animal nutrition. This study was aimed to investigate the possibility of use hemp by-products in animal nutrition. We decided to examined hempseed expellers and hemp plant tops, in our study. Thus, the aim of this experiment was to study whether hemp by-products may affect

the microbial colonization of the gut, because it would affect the absorption and utilization of nutrients also. This could have effect to performance of chickens. The addition of hempseed expellers at dose 15% negatively affected the growth of chickens in our previous experiment (Stastnik et al. 2015). So, we decide to include a lower dose of expellers to feed mixture now.

MATERIAL AND METHODS

The experiment was performed with 60 sexed Ross 308 hybrid cockerels which were fattened on conventional deep litter system. Wood shavings were used as bedding material. The trial was conducted from day 10 to day 37 of chicken's age. Room temperature and humidity were controlled. Lighting system was 18 hours light and 6 hours dark. Cockerels were divided into three equal groups with 2 replicates per treatment. There were 10 chickens per replicate pen. Cockerels were allocated randomly. The two experimental groups received feed mixtures containing 2.5% of hempseed expellers or 1% of pellets from technical hemp plant tops (groups HSE and HP, respectively). The pellets from hemp plant tops are flowers and seeds with a bit of shives. It was crumbled before mixing into feed mixture. The third group was without hemp addition (C).

Table 1 shows chemical composition of used hemp by-products. The used hempseed expellers contained 0.03% of cannabidiol (CBD) and pellets from hemp plant tops contained 0.15% of CBD. The content of tetrahydrocannabinol (THC) and cannabinol (CBN) are non-detectable in feed either in feces. The compositions of experimental rations are presented in Table 2. The rations were calculated according to the Recommended nutrient content in poultry diets and nutritive value of feeds for poultry (Zelenka et al. 2007). The chickens were fed *ad-libitum*. The live weight was measured every week during the trial.

Table 1 Chemical composition of used hemp by-products

	HSE	***HP***
Dry matter (g/kg)	100	100
Gross energy (MJ/kg)	20.54	17.52
Crude protein (g/kg)	306.4	157.7
Ether extract (g/kg)	96.2	68.9
Crude fibre (g/kg)	367.1	288.7
Crude ash (g/kg)	74.2	163.8
CBD* (%)	0.03	0.15

**CBD - cannabidiol*

At the end of experiment 6 birds were selected randomly from each group, weighed and slaughtered. Microbial analyses were carried out at the 37th days of broilers age. Fresh digesta were obtained from the lower ileum. The digesta were diluted into a fluid state with saline solution. Weighed digesta (3 g) was homogenised in centrifuged tube. Into Petri dishes 1 ml of sample was inoculated. Three groups of microorganisms were determined. The *Escherichia coli* (*E. coli*) on Rapid Ecoli 2 agar (Biorad, USA) for 24 hrs at 37 °C. *Enterococci* spp. on Slanetz-Bartley agar (Merck, Germany) for 72 hrs at 37 °C. *Lactobacilli* spp. cultivated anaerobically on MRS agar (Biokar Diagnostics, France) for 48 hrs at 37 °C. After cultivation, counts of microorganisms were expressed as log CFU/g (Colony Forming Unit). Feathers were removed and chickens were eviscerated. Carcass yield was calculated. Breast muscle and leg muscle were deboned and weighted in these selected chickens. These values were calculated by the percentage of live weight.

Table 2 Composition of feed mixtures (g/kg)

Component	C	HSE	HP
Wheat	400	400	400
Soybean meal	260	260	260.5
Maize	205	182.3	192
Rapeseed oil	40.5	45	44
Premix*	30	30	30
Hempseed expellers	0	25	0
Hemp plant tops	0	0	10
Wheat gluten	31	24.4	29.5
Maize starch	20	20	20
Monocalciumphosphate	7.5	7	7.5
$CaCO_3$	3	2.8	3
L-Lysine	1.5	2	2
DL-Methionine	1.5	1.5	1.5
Chemical composition - as fed (per kg of diet)			
Dry matter (g)	880	880	880
AME (MJ)	12.61	12.60	12.58
Crude Protein (g)	200.7	198.4	195.7
Ether extract (g)	60.4	61	62
Crude fibre (g)	45.8	55.2	48.6
Crude ash (g)	58.6	57.1	55.5

** Premix contains (per kg): lysine 60 g; methionine 75 g; threonine 34 g; calcium 200 g; phosphorus 65 g; sodium 42 g; copper 500 mg; iron 2500 mg; zinc 3400 mg; manganese 4000 mg; cobalt 7 mg; iodine 30 mg; selenium 6 mg; tocopherol 450000 mg; calciferol 166700 IU; tocoferol 1500 mg; vit K 350 mg; thiamine 140 mg; B2 230 mg; B6 200 mg; B12 1000 mg; biotin 7 mg; niaciamid 1200 mg; folic acid 57 mg, calcium pantothenate 450 mg; choline chloride 6000 mg; salinomycin sodium 2333 mg.*

Data has been processed by Microsoft Excel (USA) and Statistica version 12.0 (CZ). One-way analysis (ANOVA) was used. To ensure evidential differences Scheffe´s test was applied and $P < 0.05$ was regarded as statistically significant difference.

RESULTS AND DISCUSSION

The mean bodyweight of chickens during the experiment is presented in Table 3. In accordance with the performance targets for ROSS 308, the average body weight of cockerels would be 2,493 g at 37 days of age (Aviagen Group 2014). Whereas non-significant ($P > 0.05$) the highest average body weight 2,216 g was achieved in control group in our study at the day of slaughter. In accordance with this, Afzali et al. (2015) found inclusion of extruded hempseed into feed mixture for broilers had no significant effect on performance. In another study Barani et al. (2015) observed that addition of hempseed at dose 10% significantly decreased feed intake and body weight of broilers. On the other hand, Khan et al. (2009) found that mean body weight gain and dressing percentage of chickens was significantly higher ($P < 0.05$) in group with 20% cannabis addition against to the control group at the end of the experiment. Nevertheless, our results of earlier trial (Stastnik et al. 2015) suggested that addition of hempseed expellers at dose 15% negatively affected the growth of chickens, because the final body weight (at 37 days of age) with part of hempseed cakes in feed mixture was significantly lower ($P < 0.05$). A higher proportion (15%) also worsened feed conversion ratio. Data of carcass yield were not affected ($P > 0.05$) by higher inclusion of hempseed cakes.

Table 3 Mean live weight of broilers per trial (g)

Day of age	n	10	17	24	31	37
		Mean ± standard error				
C	20	221 ± 5.57	535 ± 10.45	1,023 ± 20.97	1,623 ± 32.41	2,216 ± 47.30
HSE	20	230 ± 5.21	530 ± 11.75	915 ± 31.42	1,521 ± 46.44	2,141 ± 62.58
HP	20	239 ± 4.98	534 ± 12.86	928 ± 31.44	1,471 ± 42.10	1,998 ± 52.36

Differences between groups are not statistically significant (P > 0.05)

Table 4 shows the mean content of *E. coli, Lactobacillus* and *Enterococcus* in gut of broiler chickens. On behalf of each bacterial species were not detected statistically significant differences (P > 0.05) between groups at 37th day of age. Based on our results we can conclude that the higher content of cannabidiol not affect monitored microbiological parameters of intestinal contents. Jakubcová et al. (2014) in experiment with supplementation of chamomile extract found that lactic acid bacteria were higher in the group without chamomile extract. On the other hand, Lichovnikova et al. (2014) observed in their trial with added red grape pomace (*Vitis Vinifera* L.) that this had a positive effect on the content of *Lactobacillus*.

Table 4 The mean content of microorganism's log CFU per grams of digesta of the broilers

Group	n	*Escherichia coli*	*Lactobacillus* spp.	*Enterococcus* spp.
		Mean ± standard error		
C	6	6.28 ± 0.491	9.10 ± 0.187	6.47 ± 0.117
HSE	6	6.65 ± 0.402	9.36 ± 0.380	6.55 ± 0.072
HP	6	6.56 ± 0.218	8.79 ± 0.116	6.66 ± 0.064

Differences between groups are not statistically significant (P > 0.05)

The highest carcass yield was found in the control group (Table 5) but differences between groups were not significant (P > 0.05). The lowest value was observed in experimental groups. Carcass yield stated in the technological procedure for ROSS 308 (Aviagen Group 2014) is the 72.08% for 2,200 g of live weight. Percentages of breast muscle of body weight (Table 5) were non-significant (P > 0.05) highest for control group (21.30 ± 0.982%), while the lowest values were observed in experimental groups again (18.0 and 18.13%, respectively). In the manual of hybrid Ross 308 (Aviagen Group 2014) is stated similar percentage of breast muscle of body weight to our results. Percentages of thigh muscle of body weight was attempted the highest for HSE group (Table 5). The manual for the hybrid Ross 308 (Aviagen Group 2014) indicates a yield of leg meat 16.03% for 2,200 g live weight. The differences among groups in slaughtering yields were not statistically significant (P > 0.05). Similar results achieved Eriksson and Wall (2012) who conducted an experiment with organic broilers with supplementation of hempseed cakes at dose 100 and 200 g/kg in feed mixture (grower and finisher, respectively). They observed non-significant differences in carcass yield among experimental and control groups.

Table 5 Carcass yield (%)

Group	n	Carcass weight	Breast meat	Leg meat
		Mean ± standard error		
C	6	68.77 ± 0.712	21.30 ± 0.982	14.24 ± 0.310
HSE	6	66.84 ± 1.082	18.00 ± 0.987	14.40 ± 0.438
HP	6	66.03 ± 1.387	18.13 ± 1.364	15.14 ± 0.625

Differences between groups are not statistically significant (P > 0.05)

CONCLUSION

The addition of hempseed expellers and hemp plant tops had no positive or negative effect to growth of chickens. On behalf of each bacterial species were not detected differences between control and experimental groups at 37th day of chickens age. So, we can conclude that the content of cannabidiol in amount 0.03% and 0.15% (hempseed expellers and hemp plant tops, respectively) not affect monitored

microbiological parameters of intestinal contents, based on our results. Consequently, it was achieved non-significant differences in average body weight in our study. The same trend was found in carcass yield.

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THE EFFECT OF DIETARY ZINC AND CALCIUM CONTENT ON THE FEMUR BONE STRENGTH OF BROILERS

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Abstract: In this experiment, two organic zinc (Zn) sources, zinc chelate of proteinate (Zn-proteinate) and zinc chelate of glycine (Zn-glycin) and three calcium (Ca) levels (4 or 9 or 14 g/kg) were evaluated for their effects on the strength of broiler femur bone. Total of 48 male broiler chicks Ross 308 were divided into six groups according to 2x3 factorial arrangement (2 zinc sources and 3 supplemented calcium levels). The basal diet contained 33 mg/kg Zn and 3.2 g/kg Ca and experimental premix was modified by adding 100 mg/kg Zn either as Zn-proteinate or Zn-glycin and by calcium levels to achieve total calcium content 4 or 9 or 14 g/kg in the diet. Calcium was supplied as $CaCO_3$. The feed mixture and water were offered ad libitum. The experiment started at 11 days of broiler age and chicks were fattened up to 35 days of age. At the end of the trial, birds were slaughtered and femur bone from right leg was dissected. The bone breaking strength as force in the moment of bone breaking was measured. The calcium level had significant effect ($P<0.05$) on the strength of broiler femur bone between group with Zn-glycin + 4 g/kg Ca and group Zn-glycin + 9 g/kg Ca, however zinc source had no significant effect.

Key Words: zinc, calcium, broiler, femur bone

INTRODUCTION

Breeding chickens for superior meat production is associated with higher body weight which loads chicken skeleton. Orthopaedic problems may cause reduced feed intake, weight gain, low productivity, mortality and economic loss. Weak bones can cause bone fractures during catching, transporting and processing of chicken meat (Rath et al. 2000).

In poultry management, nutrition is central to the maintenance of skeletal health. The extent of bone mineralization affects bone strength, and poor mineralization has been associated with increased risk of fractures. The strength of bones is affected by age, gender, nutrition, physical activity, infection and disease, hormones, toxins and antinutrients (Rath et al. 2000).

Calcium (Ca) together with phosphorus are primary inorganic nutrients in the bone, because they form 95% of the mineral matrices. Calcium deficiency can lead to skeletal deformation, rickets and bone fracture. Calcium deficiency does not seem to be a problem, but the interaction among calcium and other elements can impair Ca absorption (Rath et al. 2000). It is well established that zinc can permeate a variety of calcium channels and on the other side very high levels of calcium may inhibit zinc absorption (EFSA 2014). Zinc (Zn) is an essential trace mineral necessary for growth, bone development, feathering, reproduction, immune system, disease resistance, maintaining insulin levels, it is part of the list of zinc metaloenzymes. Deprivation of zinc is characterized by loss of appetite, growth depression, abnormalities of the skin or outgrowths (hair, wool, feathers, hoof, horn) and reproductive disorders (Suttle 2010). Deficiency of zinc in chicks can cause decreased growth, frizzled feathers, shortened and thickened legs or enlarged hocks (Nielsen 2012). Feed materials for poultry are usually either too low in zinc or show reduced availability of zinc to cover the animal requirements, so animal feeds are routinely supplemented with zinc (EFSA 2014). Zinc is added to broiler diets in inorganic sources (usually zinc oxide, zinc sulphate, zinc chloride) or in organic forms complexed to amino acids, proteins, or carbohydrates.

The National Research Council (1994) estimated the dietary requirement for broilers as 40 mg Zn/kg and 9 g Ca/kg. Recommended values are usually higher than NRC requirements for ensuring adequate performance. According to Ross Broiler Management Handbook (2014) recommended added zinc content is 110 mg/kg of the diet. Recommended calcium content is 8.7 g/kg complete diet from 11 to 24 days of age and 7.9 g/kg from 25 days of age.

The aim of this study was to investigate the influence of different zinc sources and calcium levels in the diet on strength of broiler femur bone.

MATERIAL AND METHODS

The experiment was conducted with 48 male chicks of hybrid Ross 308. Birds were marked by wing tags and housed in the balance cages in a room that had a temperature set according to Ross Broiler Management Handbook (2014). The chicks were divided into 6 groups and they had free access to feed and water throughout feeding trial. The lighting system was set on 18 hours light and 6 hours dark. Temperature and relative humidity was recorded every day. The experiment started at 11 days of broiler age and chicks were fattened up to 35 days of age.

Birds were fed by the diet (composition of the diet is shown in Table 1) formulated to meet or exceed NRC (1994) nutritional requirements except zinc and calcium. It was used a modified vitamin-mineral premix with minimum amount of zinc and calcium. The basic diet contained 33 mg/kg Zn and 3.2 g/kg Ca, originated from feedstuffs and the zinc-calcium-low premix. The experimental premix was modified by adding 100 mg Zn/kg either as Zn-proteinate or Zn-glycin and by calcium levels to achieve total calcium content 4, 9 or 14 g/kg in the diet. This was achieved by adding different amounts of $CaCO_3$. Dietary treatments are shown in Table 2.

Table 1 Composition of the diet

Ingredient	g/kg
Maize	260
Wheat	342
Soybean meal	305
Rapeseed oil	40
Vitamin-mineral premix [1]	20
Experimental premix[2]	30
Chromium oxide	3

[1]Supplied per kilogram of premix: lysine 101.65 g, methionine 135.63 g, threonine 51.22 g, calcium 200 g, phosphorus 98.19 g, natrium 62.89 g, sulphur 0.39 g, chlorine 119.69 g, copper 752.5 mg, iron 3768.6 mg, zinc 44.73 mg, manganese 6046.07 mg, cobalt 11 mg, iodine 47.95 mg, selenium 8.96 mg, vitamin A 680000 IU, vitamin D 250000 IU, vitamin E 2250 mg, K_3 74.8 mg, B_1 206.44 mg, B_2 344 mg, B_6 300.44 mg, B_{12} 1999.2 mg, biotin 11 mg, niacinamid 1793.4 mg, calcium pantothenate 676.2 mg, folic acid 82.8 mg, cholinechlorid 9000 mg

[2] Content different sources of zinc and levels of calcium according to the dietary treatments

Table 2 Added zinc and calcium content in the dietary treatments

Group	Zinc source	Zn (mg/kg)	Ca (g/kg)
P4	*Zn-proteinate*	100	4
P9	*Zn-proteinate*	100	9
P14	*Zn-proteinate*	100	14
G4	*Zn-glycin*	100	4
G9	*Zn-glycin*	100	9
G14	*Zn-glycin*	100	14

At the end of the feeding trial (35 days of broiler age), birds from each group were slaughtered and femur bone from right leg was dissected from fresh carcass and stripped of soft tissues. They were stored individually in plastic bags in a freezer (-20 °C) for 3 weeks and then they were thawed at room temperature the day before tests of strength. The tests of strength of bones were carried out on a universal testing machine TIRATEST 27025 using a three point bending test. The bones strength were measured

as the force in the moment of bone breaking. Breaking force was determined automatically by the device. The loading rate was 100 mm/min up to moment of bone breaking.

Data has been processed by Statistica version 12.0 (CZ). We used two-way analysis (ANOVA). Sheffe´s test was applied to defined statistical differences and differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

In this experiment, it was added 100 mg/kg Zn diet from two different sources: Zn-proteinate and Zn-glycin. The strength of femur bone was not significantly affected by these zinc sources. The breaking force was significantly affected by calcium level ($P<0.05$). Significant differences ($P<0.05$) were recorded between groups G9 (307.7 N) and G4 (175.2 N) with diet contain 9 g Ca/kg and 4 g/kg Ca diet, both supplemented by Zn-glycine. The effects of supplemented zinc sources and calcium levels in this study on the strength of broilers femur bones are presented in Table 3.

Table 3 The effects of dietary zinc and calcium on the strength of broilers femur bones

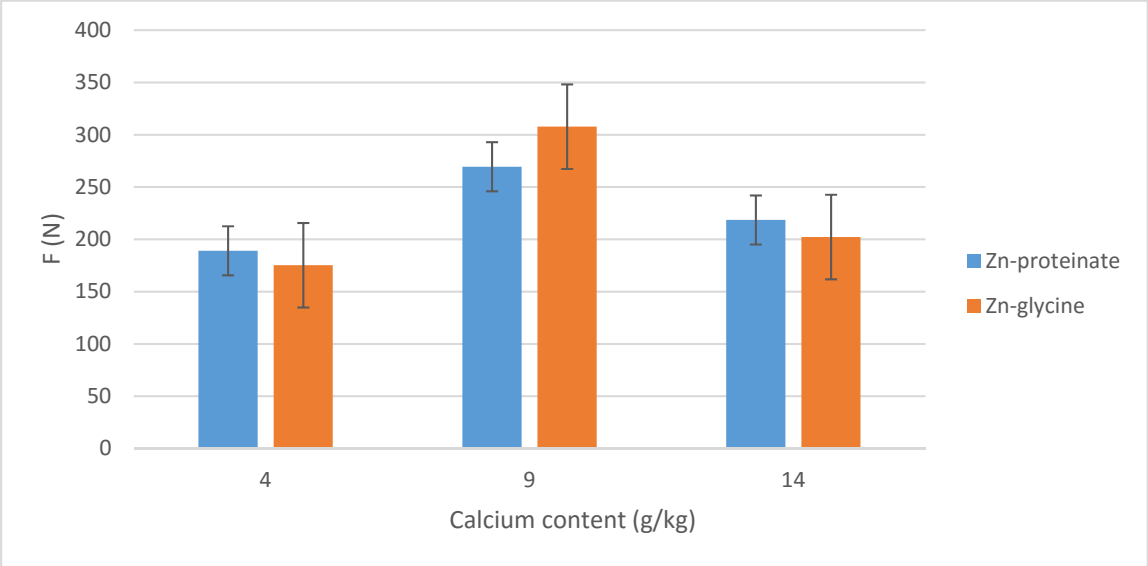
Group	Zinc source	Calcium level (g/kg)	Breaking force (N) ± SD
P14	Zn-proteinate	14	218.5 ± 61.2
P9	Zn-proteinate	9	269.4 ± 12.0
P4	Zn-proteinate	4	189.0 ± 38.7
G14	Zn-glycine	14	202.2 ± 52.2
G9	Zn-glycine	9	307.7 [a] ± 40.2
G4	Zn-glycine	4	175.2 [b] ± 19.0
Zn source			NS
Ca level			*
Zn source x Ca level			NS

*NS: not significant; * P<0.05; Different letters [a,b] in the columns indicate significant differences at a level of P<0.05*

The bone strength is affected by the extent of bone mineralization. Many studies were realized to determine the influence of dietary zinc on concentration of zinc in tibia bones. Scrimgeour et al. (2007) claimed that the tibiotarsal bone compared to the femoral bone is more sensitive to different Zn levels. Underwood and Suttle (1999) found a decrease in Zn concentration in bones caused by lower zinc content in the feed and Mohanna and Nys (1999) recorded that tibia zinc content increases with dietary zinc level. Štofaníková et al. (2011) confirm the differences in bone biomechanical competence in broilers fed diet with different zinc levels. The significant reduction in tibial strength was observed in broilers fed 50 mg Zn/kg in comparison with 100 mg/kg Zn. Rama Rao et al. (2006) recorded tibia breaking strength significantly higher by increasing the dietary Ca level from 6 to 7 g/kg Ca diet. Lander et al. 2014 dealt with the effect of freezing (at −20 °C for 21 days) on the microstructure of bone, and they found microcracks and cracks on the surface of the frozen bone samples, but no significant differences were observed. Similarly Tersigni (2007) also reported no significant microstructural changes on frozen bone samples and control samples.

In our study, maximum breaking force occurred at a calcium content of 9 g/kg diet and then showed declining trend with increase in dietary Ca level (14 g/kg). The lowest values was found in groups G4 and P4 with calcium content of 4 g/kg feed mixture independently of zinc source (Figure 1).

Figure 1 The effects of dietary zinc and calcium on the femur bone strength of broilers



F = force in the moment of bone breaking

The strength of femur bone was not improved when calcium was increased from 9 to 14 g/kg diet. There is some other contraindicative factors except zinc which can interfere with Ca absorption, such as high levels of phytate and cellulose fibers in the diet. (Rath et al. 2000).

CONCLUSION

In this experiment, different zinc sources and calcium levels were evaluated for their effects on the breaking strength of femur bone of broiler at the age of 35 days. This parameter increased independently of zinc source from calcium content of 4 g/kg and 14 g/kg Ca and the best results were achieved at level of 9 g/kg Ca. In case of zinc sources, zinc bounded to proteinate suggested better results with 4 g Ca and 14 g/kg, however the best strength of femur bone was noticed in group with zinc bounded to glycin and 9 g/kg a. Zinc source had no significant effect on breaking force. The increase of calcium content from 4 to 9 g/kg in Zn-glycine groups significantly ($P<0.05$) increased force required for bone breaking.

ACKNOWLEDGEMENT

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EFFECT OF AIR TEMPERATURE ON RUMINATION ACTIVITY AND MILK PRODUCTION OF HOLSTEIN COWS

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Abstract: The aim of this study was to evaluate the effect of air temperature on rumination activity and milk production of Holstein cows. Monitoring took place at the University farm in Žabčice within 4 weeks with a lower (L.) – 11.3 °C and 4 weeks with a higher (H.) – 25.5 °C average daily temperature, in 2016. Two groups of cows – 75 animals each – were included into experiment. From both groups there were always randomly selected 10 lying and chewing cows. For these cows the length of rumination cycle (s), the number of masticatory movements (n), the intensity of rumination (n/s) and the daily milk yield (kg) were evaluated and recorded. We found a statistically significant difference in the length of ruminal cycle (L. 58.1; H.53.0), the number of ruminal movements (L. 65.9; H. 57.0) and the intensity of rumination (L. 0.88; H. 0.93). Daily milk yield values were not statistically significant (L. 37.4; H. 36.8).

Key Words: Holstein, chewing movements, intensity of rumination, daily milk yield

INTRODUCTION

Rumination, or chewing, is a process in which the feed gets from the rumen back to the oral cavity (Reece 2011). It is an ability, which allows feed intake in relatively short time and its mincing can be dealt with later, more precisely, to complete its perfect processing (Drevjany et al. 2004, Lee et al. 2009). Studies have demonstrated that although the period of rumination is primarily determined by the amount and the quality of the diet, chewing behaviour is innate need of cattle, regardless of the quantity of ingested feed (Lindström and Redbo 2000). It is a reflective act, although this process may be interrupted or stopped voluntarily (Lee et al. 2009). Dairy cows spend 8–10 hours a day ruminating (Welch 1982), or 4–9 hours a day, respectively (Vomočilová and Voslářová 2014). However the time devoted to this activity varies depending on the diet (Reece 2011). Ruminal cycle includes regurgitation of chyme (flowing of gastric contents into the oesophagus and oral cavity), then the rumination which is associated with the repeated salivation (mixing with the saliva, which contains bicarbonate and phosphate buffers) and the final phase-once more swallowing chewed feed (Drevjany et al. 2004, Hulsen and Aerden 2014, Skřivánek 2001). Re-swallowing of the bite ends the ruminal cycle and the new one begins approximately 5 seconds later (Reece 2011). At the moment the bite gets into oral cavity it is deprived of fluid by compression and while mixing with saliva it is being chewed (Hulsen and Aerden 2014, Vomočilová and Voslářová 2014). For chewing one mouthful (100–200 g) cattle perform 20 to 90 chewing movements, per minute it is 55 movements (Vomočilová and Voslářová 2014), or 50–75 respectively, according to the moisture of mouthful (Hulsen and Aerden 2014), or 66–69, depending on selected diet (Vaculíková and Chládek 2016). For the correct course of the entire ruminal cycle it is important for the daily ration to contain sufficient amount of effective fibre (contained in roughages with a length exceeding 2.5 cm), because the rumen needs roughage for good digestion and stabilization of rumen pH (Heinrichs 2011, Kulovaná 2001). Rumination is one of many different signals that can show us that rumen is working properly (Dussert 2012).

MATERIAL AND METHODS

For the purpose of this study, the behavioural monitoring of high producing dairy cows of Holstein breed was conducted at the University farm in Žabčice in March (average daily temperature of

11.3 °C) and in June and July 2016 (average daily temperature of 25.5 °C). The experiment included two groups of dairy cows (75 animals each). From both groups, 10 lying and chewing animals were always randomly selected. For selected dairy cows (160 animals) these parameters were evaluated and recorded: length of rumination cycle (s) – time required for chewing one mouthful, number of masticatory movements required for chewing one mouthful (n) intensity of rumination (n/s) and daily milk yield (kg). Throughout the entire study cows were fed total mixed ration (TMR), but the TMR was not identical the whole time. Temperature was always measured on the day of observation, specifically at 10.00, in the middle of the stable for dairy cows. Obtained results of ethologic monitoring were processed by conventional mathematical-statistical and graphical methods.

RESULTS AND DISCUSSION

Table 1 Effect of air temperature on rumination activity and milk production Holstein dairy cows

Monitored parameters	$\bar{x}$	Ø temperature
The time of rumination cycle (s)	58.1^{A}	L
	53.0^{B}	H
Number of masticatory movements (n)	65.9^{A}	L
	57.0^{B}	H
Intensity of rumination (n/s)	0.88^{A}	L
	0.93^{B}	H
Daily milk yield (kg)	37.4^{a}	L
	36.8^{a}	H

Statistical significance of the difference: a = P > 0.05; A, B = P < 0.01; L.: low temperature; H.: high temperature

In Table 1 we can see the average values of monitored parameters of selected dairy cows, namely the length of rumination cycle (s) - time required for chewing one mouthful, number of masticatory movements (n) required for chewing one mouthful, intensity of rumination (n/s) and daily milk yield (kg). All parameters are given at lower and higher temperatures. These values imply that the length of rumination cycle under this observation was greater at lower temperature (58.1 s) than at higher temperature (53.0 s). We can concur with Doležal et al. (2010), who claim that higher temperature shortens rumination time up to 10%. Number of masticatory movements reached at lower average daily temperature the value of 65.9 (n) per one rumination cycle. The number of masticatory movements at higher average daily temperature was lower – 57.0 (n). Both of these values agree with the statement of Hulsen and Aerden (2014), who indicate the number of 50–75 masticatory movements per one minute. Also the number of masticatory movements (approximately 55) given by Vomočilová and Voslářová (2014) corresponds with our obtained results. In contrast, the intensity of rumination achieved at lower temperature was lower (0.88 n/s), than at higher temperature (0.93 n/s). The observed values of rumination at lower temperature agree with the data from Vomočilová and Voslářová (2014), who indicate approximately 55 masticatory movements per one minute, which means intensity of rumination of 0.92 n/s. In all above mentioned values high statistical significance was found ($P < 0.01$). The intensity of rumination of 0.93 n/s could be also caused by the change of daily ration during the monitoring. In this case, we can agree with the statement of Vaculíková and Chládek (2016), who claim that addition of fibre to the TMR of dairy cows tends to increase the intensity of rumination. The average daily milk yield in selected cows during monitoring was at lower temperature only slightly higher (37.4 kg), than at higher temperature (36.8 kg). These values have not been proven statistically significant ($P > 0.05$).

CONCLUSION

From the results of this experiment it can be said, that if we judged our results only under the exposition to lower (11.3 °C) or higher (25.5 °C) average daily temperature, we could say that the temperature clearly acts on the rumination activity of high producing dairy cows. Namely to the length of rumination cycle, when at lower temperature this cycle is longer ($P < 0.01$) and also to the number of masticatory movements, when the low temperature clearly works in favour of higher values. That

means that dairy cows, when exposed to lower temperatures, perform during one rumination cycle more masticatory movements ($P < 0.01$). It has also impact on intensity of rumination, when in case of our results greater intensity of rumination was found when exposed to higher temperature ($P < 0.01$). In daily milk yield there were not found statistically significant differences while exposed to lower or higher average daily temperatures. As part of this monitoring, one cannot determine with certainty whether the aforementioned statistically significant differences were caused by temperature or by changes in daily ration during the monitoring.

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Section – Fisheries and Hydrobiology

SEASONAL DYNAMICS OF ERGASILOSIS IN RESERVOIR FISH

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Abstract: We evaluated ergasilosis seasonal dynamics at two reservoirs (Hubenov, Koryčany) in the Morava River Basin (Czech Republic). Samples of fish were obtained at monthly intervals between April and October 2014. In total, 189 fish of 11 species were caught using electrofishing and seine nets. Highest overall values for *E. sieboldi* infection intensity and abundance were recorded at Hubenov in June and September. Prevalence ranged between 80 and 100%. Two highest peaks of infection intensity and abundance were recorded at Koryčany in May and the second around September and October, prevalence ranging between 33 and 100%. As the nauplius and copepod stages of arthropods form part of the zooplankton assemblage, they will be found at highest numbers in reservoirs with low predatory pressure, i.e. where predatory fish suppress zooplanktonophagic fish species.

Key Words: parasite, *Ergasilus sieboldi*, arthropods, predatory pressure, biomanipulation

INTRODUCTION

If predatory fish suppress zooplanktonophagic fish sufficiently, for example, the quantity of zooplankton increases, positively affecting the quality of raw drinking water (Jurajda et al. 2016). On the other hand, such a reduction in cyprinids may increase the occurrence of parasites whose development is bound to that of zooplankton as the free-living nauplius and copepod stages of parasites such as *Ergasilus sieboldi*, for example, form part of the zooplankton assemblage. Adult *E. sieboldi* females then attach themselves to the gills of many fish species, feeding on the gill epithelium and blood, eventually impairing the respiratory function of the gills. When parasitic invasion is particularly high, or oxygen levels particularly low, the affected fish may suffocate and die (Abdelhalim et al. 1991, Hoole et al. 2001).

The seasonal development of ergasilosis usually starts in April and lasts until November. In Central Europe, the first spring generation reaches sexual maturity in mid-June, with the second generation appearing from mid-August to mid-September. Occasionally, a third generation may also develop at the end of the season (Lester and Hayward 2006). During the season, parasite abundance will change, mainly in relation to zooplankton development. A second important factor affecting parasite abundance is water temperature as on which egg development in the egg sacs is dependent. In general, the higher the temperature the faster eggs develop (Piasecki et al. 2004).

Long-term ichthyological monitoring by the Morava River Basin State Enterprise, in cooperation with the University of Veterinary and Pharmaceutical Sciences Brno, indicates that ergasilosis is one of the most frequent fish diseases detected in reservoirs of the Morava river basin (Czech Republic). Based on these results, it was decided to undertake a more detailed survey of ergasilosis at two reservoirs differing in known intensity of ergasilosis and predatory fish stock. Here, we test the hypothesis that an

increasing proportion of predatory fish in the fish assemblage will threaten fish health status due to increased preponderance of *E. sieboldi* (Navrátil et al. 2015).

MATERIAL AND METHODS

Reservoir characteristics

Two contrasting water-supply reservoirs under the management of the Morava River Basin State Enterprise were chosen for this study, Hubenov and Koryčany. Experience has shown that intensity of infection and abundance of *E. sieboldii* tends to be higher in Hubenov than Koryčany. The two reservoirs also differ in typology and typography, i.e. altitude, area, depth (Matějíček and Rotschein 2006), trophic status (Geriš and Jahodová 2014) and fish stock composition. While predatory fish are stocked in both reservoirs to improve the quality of raw drinking water by suppressing zooplanktonophagic fish (unpublished data; personal communication), Hubenov supports higher numbers.

The 55 ha Hubenov reservoir (49°23'41.7"N, 15°29'05.2"E; 524 m a.s.l.) is located in the Vysočina Region, 7.5 km from the town of Jihlava, and has served as a drinking water storage reservoir for the Jihlava region since entering into operation in 1972. It has a storage capacity of 2.4 million m³ and a total volume of 3.4 million m³ (Matějíček and Rotschein 2006).

The 35 ha Koryčany reservoir (49°06'49.2"N, 17°12'04.3"E; 325 m a.s.l), located about 1 km east of Koryčany in the Zlín region, first entered service in 1963. Koryčany fulfils a similar role in the landscape to Hubenov, though its main role is the supply of drinking water to the Kyjov region. It has a storage capacity of 2.1 million m³ and a total volume of 2.6 million m³ (Matějíček and Rotschein 2006).

Water temperature at both reservoirs is measured every day at 7:00 a.m.by representatives of the Morava River Basin State Enterprise using a manually calibrated thermometer.

Fish sampling and parasitological examination

We undertook seven monthly fish surveys at each reservoir between April and October 2014. All fish were caught using electrofishing apparatus (EFKO FEG 13000, Honda 13 kW, ca. 300 V, 60 A) and seine nets (30 m long, 4,5 m deep, with mesh sizes ranging from 70 to 135 mm), based on standard methodologies for fishing in still waters (Kubečka and Prchalová 2006).

Fish were transferred alive to the laboratory, where they were humanely stunned and immediately killed before preparation for parasitological examination. Immediately prior to examination, each fish was determined to species, measured (total length [TL], nearest mm) and weighed (nearest mg) and the fish's nutritional status assessed. Finally, scales were sampled for age determination and the gills examined for *E. sieboldi* parasites. The number of *Ergasilus* sp. was assessed macroscopically on all four pairs of gill arches and the species determined by microscopic examination (microscope Olympus, magnification 40 x) (Ergens and Lom 1970, Schäperclaus 1979). The results are presented as infection intensity, abundance and percentage prevalence (Bush et al. 1997).

Statistical analysis

Analysis of variance (ANOVA) was used to evaluate changes in water temperatures over the year in and between the two reservoirs. The statistical analysis was undertaken using the UNISTAT statistical software package.

RESULTS AND DISCUSSION

During the seven fish surveys undertaken, we caught a total of 189 fish (Hubenov - 98, Koryčany - 91) of 11 species: common bream *Abramis brama*, northern pike *Esox lucius*, perch *Perca fluviatilis*, asp *Leuciscus aspius*, roach *Rutilus rutilus*, chub *Squalius cephalus*, rudd *Scardinius erythrophthalmus*, zander *Sander lucioperca*, bleak *Alburnus alburnus*, nase *Chondrostoma nasus* and an *A. brama* and *R. rutilus* hybrid (see Table 1).

Table 1 Number, total length (TL, min.–max.) and age of fish caught in the Hubenov (Hub.) and Koryčany (Kor.) reservoirs over 2014

Species	Number Hub./Kor.	TL [mm] Hub./Kor.	Age Hub./Kor.
Abramis brama	26/19	318–475/228–375	3–9+ / 2–6+
Esox lucius	15/18	434–530/309–600	2–6+ / 1–6+
Perca fluviatilis	16/14	205–346/149–329	2–6+ / 2–5+
Leuciscus aspius	17/12	413–568/340–585	4–7+ / 4–8+
Rutilus rutilus	14/8	140–310/144–288	2–8+ / 2–4+
Squalius cephalus	6/6	330–393/204–410	3–5+ / 2–5+
Scardinius erythrophthalmus	1/9	169/150–289	3+ / 3–5+
Sander lucioperca	1/4	356/466–534	3+ / 3–5+
Alburnus alburnus	0/1	0 / 164	0 / 2+
Chondrostoma nasus	1/0	383 / 0	4+ / 0
Hybrid *A. brama x R. rutilus*	1/0	310 / 0	6+ / 0

Highest infection intensities were recorded in *L. aspius* and *E. lucius*. Highest overall values for *E. sieboldi* infection intensity and abundance were recorded at Hubenov in June and September. Prevalence ranged between 80 and 100% (see Figure 1). Two peaks in mean infection intensity and abundance were also recorded at Koryčany, the first in May (earlier than at Hubenov) and the second around September and October. Prevalence ranged between 33 and 100% (Figure 2).

Figure 1 Mean intensity (ind.), abundance (ind.) and prevalence (%) of Ergasilus sieboldi in the Hubenov reservoir from April (IV) to October (X) 2014

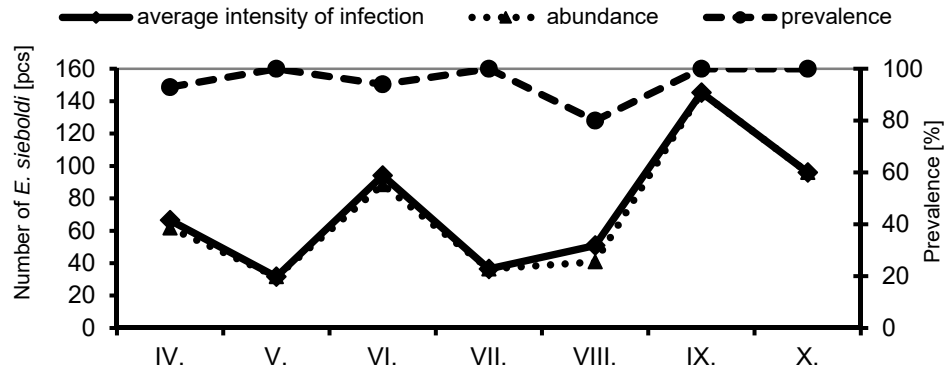
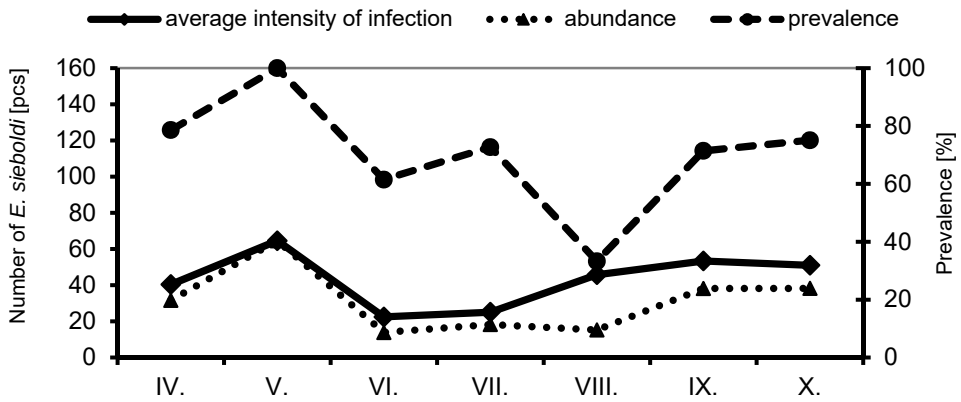
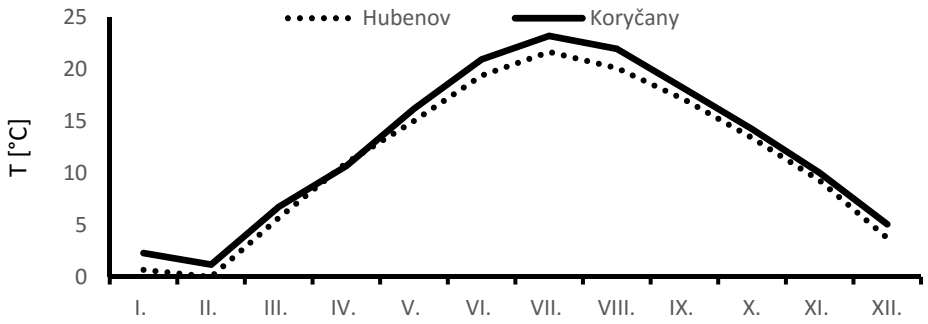


Figure 2 Mean intensity (ind.), abundance (ind.), and prevalence (%) of Ergasilus sieboldi in the Koryčany reservoir from April (IV) to October (X) 2014



Temperaturesat the Hubenov reservoir tended to be lower than at Koryčany over the same period (see Figure 3), with highly significant differences between the reservoirs during between January to March, June to September and in December ($p < 0.001$), and during May and October to November ($p< 0.05$).There was no significant difference in temperatures between the reservoirs in April ($p> 0.05$).

Figure 3 Water temperatures in the Hubenov and Koryčany reservoirs over 2014¨



The two peaks in *E. sieboldi* infection intensity and abundance recorded in June and September at Hubenov were most likely indicative of parasitisation by first (June) and second (September) generations of the parasite. At Koryčany, however, the first peak (first generation attack) appeared earlier (May), most likely due to the higher water temperatures in this reservoir promoting faster egg development (see Piasecki et al. 2004). Similarly, higher water temperatures may also have been responsible for extending the second generation attack, the increase in infection intensity and abundance extending through September into October. These values all decreased in the summer months at both reservoirs. These results correspond with those published by Lester and Hayward (2006) and Piasecki et al. (2004).

Prevalence reached relatively high values at both reservoirs throughout the monitoring period, though values were generally higher at Hubenov (80–100%) compared to Koryčany (33–100%). Similarly, values of the other epidemiological characteristics monitored also tended to be higher at Hubenov. These differences are almost certainly related to differences in zooplankton development, which in turn is impacted by the fish stock in the given reservoirs. Stocks of predatory fish are higher at Hubenov and this is likely to have suppressed the numbers of zooplanktonophagic fish to a greater degree than at Koryčany. Consequently, the abundance of *E. sieboldi* developmental stages is likely to have increased as a result of the drop in predation pressure on zooplankton (Beisner and Peres-Neto 2009). It is also possible that higher spring water levels at Koryčany may also have played a role, the flooded reservoir margins providing suitable conditions for the spawning of photophilic fish while also providing shelter for the fry. As a result, the subsequent increase in small-sized fish may have suppressed zooplankton to a larger degree at Koryčany. Differences in the seasonal dynamics of epidemiological characteristics may also be related to differences in water temperature, with generally higher temperatures at Koryčany speeding up parasite development.

CONCLUSION

In conclusion, our results suggest that each reservoir represents a unique ecosystem for which it is difficult, if not impossible, to generalise seasonal development of ergasilosis. More arthropod nauplius and copepod stages, which form part of the zooplankton assemblage, are likely to be found in reservoirs with low predatory pressure on zooplankton, i.e. where predatory fish suppress zooplankotonophagic fish. Hubenov supported a higher number of predatory fish species, which presumably helped reduce the number of zooplanktonophagic cyprinids. In this respect, while the stocking of predatory fish is desirable for maintaining water quality, it may also result in a steep increase in ergasilosis development. To date, no massive fish kills from *E. sieboldi* infestation have been recorded in either reservoir; nevertheless, the occurrence of this parasite needs to be continuously monitored as, unlike other fish parasites in reservoirs, it can occur at relatively high intensities.

ACKNOWLEDGEMENTS

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ZOOPLANKTON COMMUNITY DEVELOPMENT DYNAMICS DURING A YEAR IN A RESERVOIR WITH IMPLEMENTED BIOMANIPULATION OF FISH STOCK

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Abstract: This paper deals with the relationship between zooplankton, phytoplankton, and fish communities in a water reservoir, with implemented long-term biomanipulation measures to improve water quality. The Water Reservoir Hamry is located near the village of Hlinsko in the Czech-Moravian Highlands. It has a meso-eutrophic character and it is one of the smaller and shallower water reservoirs with an area of 42 ha of water surface and average depth of 2 meters. Bio-manipulation measures in this reservoir have been implemented and monitored since 2008. Assessment of zooplankton, phytoplankton, and fish community monitoring was conducted in the 2015 season, from March to November. Simultaneously, catches of unwanted fish were carried out in order to promote top-down effect in the reservoir. The resulting values of planktonic communities have proven to be mostly negative – almost a monoculture of cyanobacteria in the phytoplankton communities for much of the growing season, negative size ratio of zooplankton, and small overall density of organisms. These values for zooplankton indicate that when the bottom-up processes dominate, the effect of biomanipulation does not have to be demonstrably successful. On the contrary, due to excessive eutrophication of the reservoir associated with inappropriate structure of phytoplankton, the filtration pressure of zooplankton is prevented. As a result, process of improving the water quality in the reservoir via biomanipulation cannot effectively take place.

Key Words: Biomanipulation, fish stock management, top-down effect, bottom-up effect, zooplankton, eutrophication

INTRODUCTION

The subject of obtaining quality drinking water is very relevant today, not only around the world but on the European continent, as well. This problem affects even more the Czech Republic, as this is the headwater area of Europe. All water flows into neighbouring countries, so our resources are therefore dependent on the management of collected rainwater or groundwater resources.

Dangers threatening the availability of raw surface water for further processing are not only due to the lack of it caused by the relatively drier summers of the past seasons, but also because of very excessive nutrient impact (Randák et al. 2013).

Eutrophication of water has an adverse impact on the aquatic ecosystem in the form of reduced transparency, fluctuating hydrochemical parameters, and in the final stage oxygen deficit and contamination of the aquatic environment by toxins (Rulík et al. 2014). In reservoirs for drinking water affected by the higher trophy, it exacerbates the process of obtaining drinking water and makes it more expensive (Kočí et al. 2000).

Biomanipulation measures, which in recent decades have also been applied to combat eutrophication of water reservoirs, are trying to replace the chemical route with the biological one that is close to nature. It includes acting on the elements of the food chain in aquatic ecosystems in order to improve water quality in an indirect way (Randák et al. 2013). Their disadvantage is both a relatively lengthy process of application, because a successful biomanipulation must be repeatedly performed for several years, and relatively lower efficiency. On the contrary, their advantages include the already mentioned respect for the natural environment.

The effect of biomanipulation should be based on intervention in the food pyramid of the aquatic ecosystem by affecting the top-down and bottom-up processes. This includes the elimination of fish that are undesirable from the water supply point of view, namely consumers of zooplankton. It subsequently increases the abundance and size of organisms, so it can effectively exert a filtration pressure on phytoplankton community. With the decline of algae and cyanobacteria, water quality and its usability for further treatment improves (Rulík et al. 2014).

Zooplankton, as a key component of food webs of aquatic ecosystems plays an irreplaceable role in the ecological evaluation of the ecosystem quality. Its collection and subsequent evaluation is relatively an inexpensive, although time-consuming method. The resulting structure projects the image of the quality of the water reservoir, about the state of fish stocks, the presence of suitable abiotic conditions, as well as the representation of phytoplankton. Optimal structure of zooplankton communities in reservoirs is its high abundance in hundreds of pc/l, with the dominant component size of >0.7 mm. Thus we are talking about "coarse daphnia" zooplankton. In contrast, small amounts of generally small individuals are undesirable. This may be caused, for example, by increased pressure of the fish community.

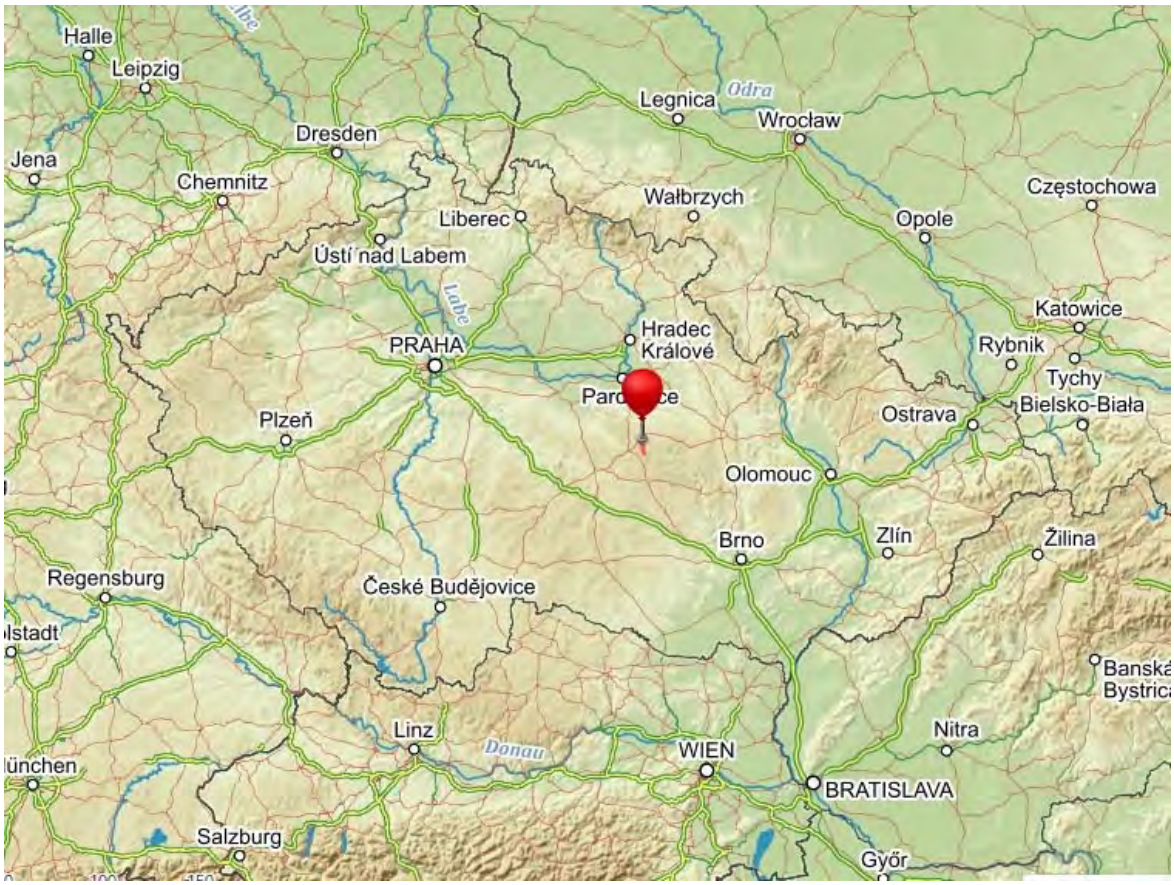
The aim of the study was to evaluate, by using analysis of zooplankton samples, to what extent the on-going biomanipulation influenced the abundance and structure of zooplankton, its effect on phytoplankton, and hence on the quality of water in the reservoir. It also tried to assess the overall sense of biomanipulation measures carried out in the Hamry water reservoir.

MATERIALS AND METHODS

Location of the study area

The Hamry Reservoir is located approximately in the middle of the Czech Republic, near the town of Pardubice, as shown in Figure 1.

Figure 1 Location of the Hamry water reservoir in the Czech Republic



Collection and processing of samples

Samples for determining the structure of zooplankton were collected during the growing season of 2015 in two to four week intervals between March and November, on a total of 13 dates. For the sampling, eight sampling points were identified throughout the area of the water reservoir to represent different habitats of zooplankton occurrence. A total of 104 zooplankton samples were obtained for the quantitative analysis.

For obtaining and processing of these samples, the methodology for collecting and processing samples of zooplankton in stagnant waters was followed (Přikryl 2006).

Zooplankton was collected by using throwing plankton nets with mesh size of 40 μm with a volume of approximately 5.5 litres. The length of the draw through the water profile was six meters. Still in the terrain, the samples were fixed with concentrated (36 to 38%) formaldehyde in a ratio of about 1:10 for later analyses in the laboratory.

Determining the number of organisms was carried out in Sedgewick-Rafter chamber at 40 x magnification. Representative results were ensured by repeated counting of chambers, for at least 6 ml of the sample, or at least 300 pieces of organisms in the sample. In a classical manner, the organisms were divided into four groups: Cladocera (all water fleas), Copepoda (adult copepods and their copepodite stages - cyclopoids and calanoids), Nauplii (developmental stages of copepods), and Rotifera (rotifers). The first two groups were further divided into four size categories, namely <0.5 mm, 0.5 to 1 mm, 1 to 2 mm, and >2 mm. This division has been adopted primarily due to the nourishment focus of particular groups of organisms and thus for the most accurate detection efficiency of influence of various groups on phytoplankton. For comparing the size groups with the works of other authors the values were converted only to two sizes, with a limit of 0.7 mm.

Both of our predatory daphnia species (Cladocera), were recorded, namely *Leptodora kindtii* and *Polyphemus pediculus,* which were present in the reservoir. Their total was minimal, so the resulting values did not significantly distort the counts of filtering zooplankton. Similarly, this procedure was applied to the group Rotifera, where we have also counted the predatory rotifers of the genus *Asplanchna*, although we are aware that they do not filter phytoplankton. In any case, the sample included only a few of them. Colonial rotifers of the *Conochilus* genus were recorded in individual units in the colony, or from the determined average number of individuals in the colony. Data from Wulfert (1969) for this genus were different from the numbers recorded in the Hamry water reservoir. For Nauplii, we did not differentiate into which group of copepods they belong, as it has no significant influence on their diet.

We have then recalculated the detected number of organisms for the actual volume of water obtained from a given sampled point in the reservoir, by screening of known volume of water.

Removal of undesirable fish

Catches of unwanted fish species were carried out by different methods at all ontogenetic levels. It included collecting eggs of common perch *Perca fluviatilis* L., catches of juvenile stages of species of fish feeding on zooplankton, as well as catches and elimination of unwanted adult fish. The catches also included trapping of spawning flocks of common bream *Abramis brama* L. and common roach *Rutilus rutilus* L.

RESULTS AND DISCUSSION

Structure of zooplankton

The final values of the structure and abundance of zooplankton varied both in terms of time and place. Differences were also in percentages of individual zooplankton groups depending mainly on the season. So it was probably due to the water temperature, phosphorus content, and filtration pressure of other zooplankton, and possibly even fish. Another important factor was the flow of water when the increased flow may have caused flushing of inoculum and thereby reducing the real numbers.

Among the sampling dates, July 1, 2015 (Figures 2 and 3) sticks out, when all catches, both in terms of locality and time, had the largest share of zooplanktonic organisms <0.7 mm, especially due to the abundance of rotifers. In several locations, this difference was more than 50 per cent. It is interesting

that on this sampling date, for the first time, there was a dominant presence of cyanobacteria (blue-green algae) in a sample of phytoplankton in a sharp jump from 14 to 80 per cent. Larger filtering zooplankton apparently failed to respond quickly enough to this rapid decline of filterable phytoplankton as a food source, which led to the success of rotifers. Their maximum observed number was more than 160 pieces of individuals per litre. In contrast, the coarser daphnia zooplankton (>0.7 mm) began to develop in the later period, namely at end of April and its percentage never exceeded 50 per cent of the total zooplankton.

In terms of numerical representation of zooplanktonic organisms indicated in individuals per litre of water, we can talk about very low levels. Normally, in open waters there are hundreds to thousands of pc/l (Sukop and Kopp 2003). In the Hamry water reservoir, their average number was 50 pc/l, while the determined minimum number was 10 pc/l and maximum was 120 pc/l. These low figures are disappointing. They are approaching the values detected in flooded mine pits formed after mining of mineral resources (Kallistová 2014).

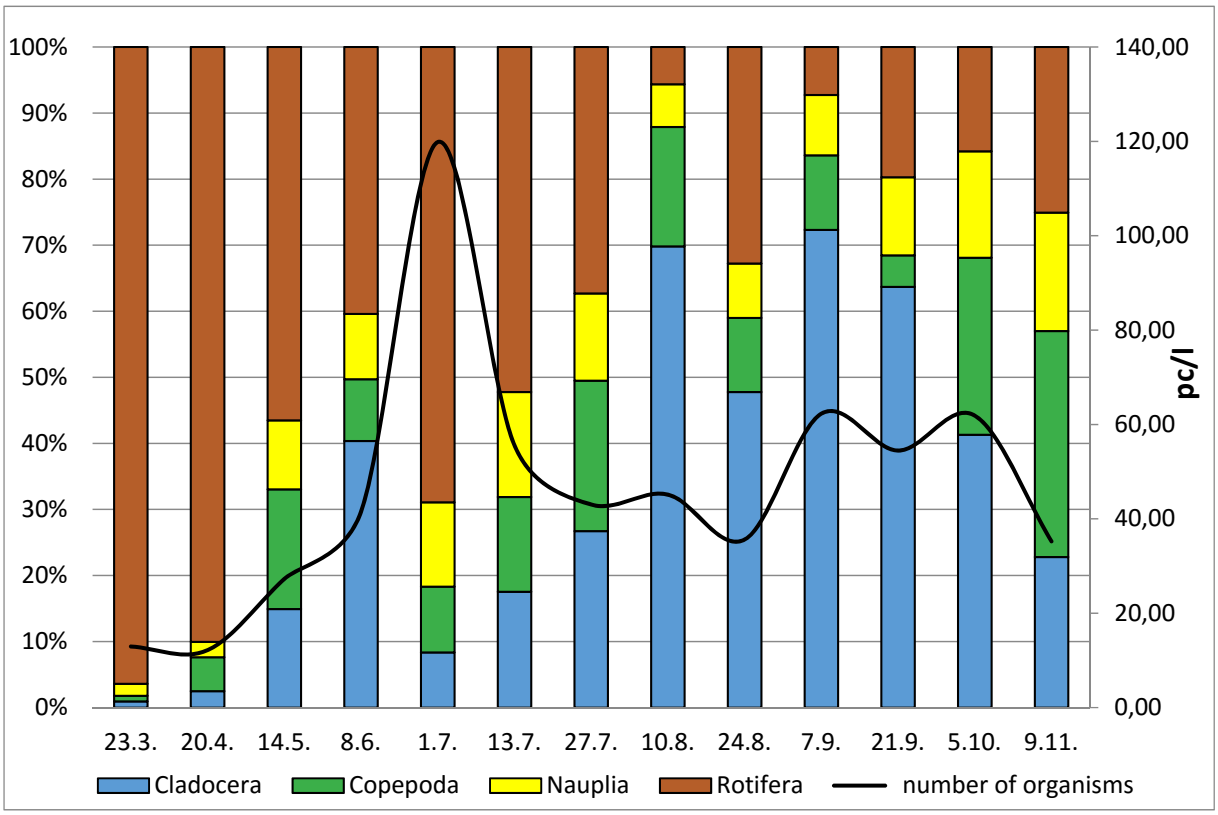
Figures 2 and 3 show detailed comparisons of temporal and spatial distribution of zooplankton, as well as the proportions of large and small zooplankton.

Catching of fish

The total amount of fish caught from the reservoir, namely common bream and common roach, exceeded 500 kg. Daily control catches using dragnets confirmed, via the relative size of catches from one drag, that there is a reduced fish density compared to previous years. However, there was no significant increase in the proportion of predatory fish. On the positive side, there was occurrence of juvenile asp, as a result of its successful reproduction in the reservoir (Jurajda et al. 2015).

Due to the number of eliminated fish feeding on plankton in the reservoir, it seems that the lack of zooplanktonic organisms greater than 0.7 mm is a consequence rather than the cause of excessive quantities of colonial cyanobacteria in the reservoir. Low density and the generally small size of the zooplanktonic organisms indicate an unbalanced ecosystem.

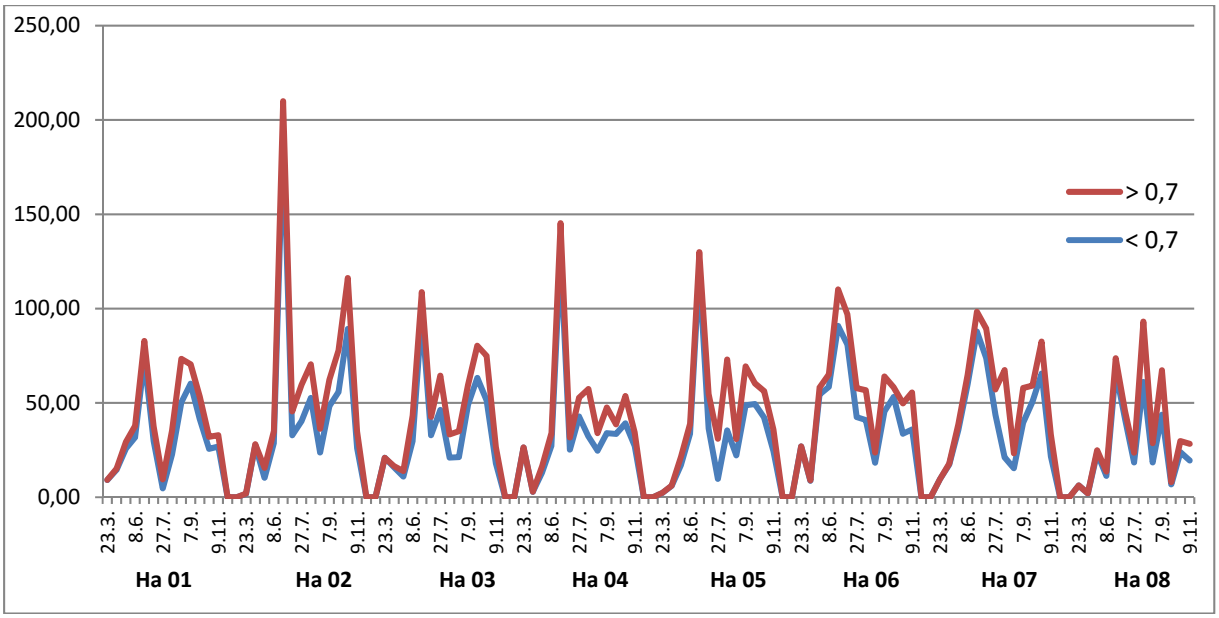
Figure 2 Main groups of zooplankton and the number of organisms in the Hamry reservoir during 2015



Legend: The black line shows the summary number of individual organisms in 1 litre of water; Colourful columns - each group of zooplankton; Dates of catching under the column at the bottom of the chart.

One of the reasons why biomanipulation is failing may be the fact that the water reservoir does not have a good enough "top down" control system when feeding pressure has an unfavourable ratio of zooplankton to phytoplankton. This is because for most of the growing season in a sample of phytoplankton, the monoculture of colonial cyanobacteria which are not accessible as food for zooplankton, basically dominated.

Figure 3 Size and numerical structure of zooplankton in different locations in Hamry water reservoir during 2015



Legend: Red line – zooplankton >0.7 mm, blue line - zooplankton <0.7 mm; Number of individuals in pieces/l, is given on the y axis; Sampling locations are at the bottom of the chart.

CONCLUSION

The collected and evaluated data on the occurrence and structure of zooplankton from the Hamry water reservoir during the growing season of 2015 point to two conclusions. Although it turns out that the performed biomanipulation measures are helping to reduce the number of carplike fish in the reservoir, yet they have no significant impact on the structure of plankton. This is probably due to the fact that if the "bottom-up" processes, in other words the flow of resources going "from the bottom up", dominate, then their effect is marginal and the impact of reduction of fish stocks on the increase of the zooplankton biomass is almost negligible. This is true even in the current conditions, when biomass of fish feeding on plankton is successfully reduced to a minimum through fish catches. In any case, it remains desirable to reduce the plankton feeding fish in the water reservoir, but the meaning of such a biomanipulation is a question of efficiency.

Much more significant factor of improving the quality of raw water in the reservoir for subsequent treatment for drinking purposes is to reduce the flow of nutrients into the reservoir from the basin and thereby to prevent eutrophication. Thus, in the case of the water reservoir Hamry, it means to eliminate phosphorus (organic pollution) in the inflowing water from the Chrudimka River, which probably comes from a combination of pollution in the river basin. This may be primarily from agricultural and municipal pollution, or secondarily from fishpond management.

If the reservoir continues to receive the current amount of nutrients, the phytoplankton situation will probably remain steady. That means that for most of the year, there will be more than 90% cyanobacteria in the sample. This structure of phytoplankton is for zooplankton uncontrollable and the filtration pressure of zooplankton on phytoplankton cannot be effective.

This study therefore implies that successful biomanipulation can be applied only under appropriate conditions and thus the findings of other authors, such as Hansson et al. (1998) are confirmed.

In the future, it will be necessary to focus more on the primary cause of eutrophication, namely nutrient input into the reservoir, than to deal with its result. If an appropriate method to restrict the supply of nutrients in the inflowing water (in particular phosphorus) is applied, then this should be reflected in the amount of phytoplankton. This restriction, together with the continued regulation of stock of unwanted fish species, should lead to a successful stabilization and improvement of water quality in the Hamry water reservoir.

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INVASIVE POTENTIAL OF DIKEROGAMMARUS VILLOSUS (SOWINSKY) BASED ON CLIMATE-MATCH SCORE

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Abstract: Killer shrimp (*Dikerogammarus villosus*) is omnivorous amphipod native to the Ponto Caspian region. When human-mediately or spontaneously invaded new area, it can rapidly reproduce and spread, prey on wide spectrum of benthic macroinvertebrates and fish, and affect the entire ecosystems. Killer shrimp spreads in Europe and no effective eradication methods are available. Since the temperature is limited factor for survival of this species, we processed climate matching to evaluate its establishment probability on the world. Based on this analysis the world's most at risk regions were highlighted. It follows that killer shrimp is most risky especially in temperate zone within Europe, North America and Asia including Japanese Archipelago.

Key Words: invasive species, biological invasion, killer shrimp, temperature

INTRODUCTION

Biological invasions by alien species often lead to niche competition between exotic and indigenous species and cause species replacement and biodiversity loss over the world (Lodge 1993). Eradication of invaders is in many cases difficult or impossible and hence prevention of new introductions is crucial (Kolar and Lodge 2001). One way to identify potential invasive species is modelling of climatic similarity between region of origin of these species and potentially threatened area (Bomford et al. 2009); this method is known as climate matching.

Amphipod gammarid crustacean *Dikerogammarus villosus* (Sowinsky) also called the killer shrimp is omnivorous species native to the Ponto Caspian region (Casellato et al. 2006). It undergoes rapidly spread over large distances in a short time and affects entire freshwater ecosystems by alternation of food webs through its exceptional predatory capabilities (Dick et al. 2002). According to its common name, this species is able to voraciously and extremely aggressively prey upon and replace other amphipods, isopods and other benthic macroinvertebrates and also previously introduced non-native species on localities which invaded (Dick and Platvoet 2000, Buřič et al. 2009). It exhibited predatory behaviour also towards small fish (Casellato et al. 2007). Furthermore, killer shrimp frequently dominate benthic faunal assemblages by its early maturation and high fecundity (Pöckl 2009) and can be perceived as “perfect invader” (Panov et al. 2009, Rewicz et al. 2014).

Killer shrimp established population in many European waterbodies occurring usually in high densities (e.g. Bij de Vaate and Klink 1995, Jazdzewski et al. 2002, Casellato et al. 2006, Bącela et al. 2008, Berezina and Ďuriš 2008) and it is one of the 100 worst alien species in Europe (DAISIE 2009). Killer shrimp can spread spontaneously but human-mediated translocations also exist (Martens and Grabow 2008, Bącela-Spychalska et al. 2013). Moreover, Bruijs et al. (2001) demonstrated that killer shrimp survived at salinities up to 10‰ and adapted to salinities of up to 20. Moreover, it is able to survive for circa one week out of water (Martens and Grabow 2008). Thus not only inland regions are endangered but also islands including Great Britain where this species was recorded for first time at the River Great Ouse catchment in eastern England in 2010 (MacNeil et al. 2010). Since methods of its effective eradication are not available (Madgwick and Aldridge 2011), the continuous spread in regions like North American Great Lakes is expected (Ricciardi and Rasmussen 1998, Rewicz et al. 2014).

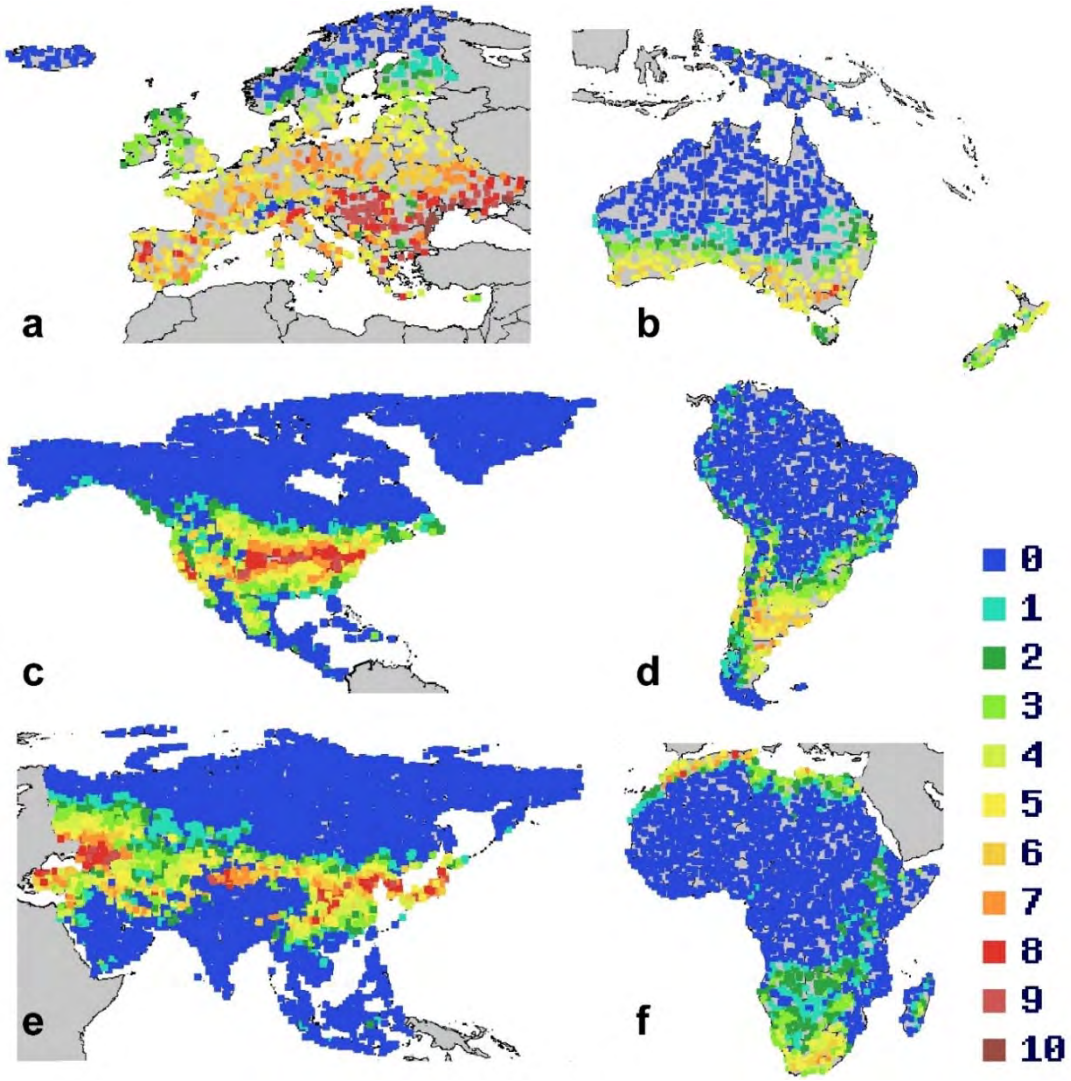
Killer shrimp does not exhibit a wider tolerance range for temperature and conductivity than indigenous gammarid species (Wijnhoven et al. 2003). Nevertheless, the prediction of its spread according to climatic conditions has been evaluated locally (see Gallardo et al. 2012). Therefore, in this study, we considered to process climate matching in world context to highlight the most at risk regions with high probability of killer shrimp establishment.

MATERIAL AND METHODS

Climate matching

Climatic conditions were represented in our analysis by temperature during the coldest, warmest, driest and warmest quarter of the year as variables and we opted Euclidean algorithm. The climate match between source and target area was compared using the Climatch tool (v.1.0; Invasive Animals Cooperative Research Centre, Bureau of Rural Sciences). We used region of native geographic range of killer shrimp as the source area. The target region was defined as rest of the world. For this purpose, data from 18,967 meteorological stations from database of WordClim project (Hijmans et al. 2005) were utilized. Where the climate match between the source area and the climatic station in the target area reached score ≥ 7.0, this was interpreted as there is high probability to establishment of killer shrimp.

Figure 1 Maps showing climate match of killer shrimp (Dikerogammarus villosus). Scores of ≥ 7 are interpreted as regions with high probability of its establishment.



Legend: **a** = *Europe without Russia,* **b** = *Oceania,* **c** = *North America,* **d** = *South America,* **e** = *Asia with Russia, and* **f** = *Africa and Madagascar*

RESULTS AND DISCUSSION

In the total of all evaluated meteorological stations, score of 7 reached 477 stations, score of 8 reached 310 stations, score of 9 reached 132 stations. Score of 10 reached 10 stations and most of them are located in the defined source area. Obviously, most threatened worldwide regions where killer shrimp has a high probability to become established when introduced lay in temperate zone (Figure 1).

With not numerous exceptions, Africa, South America and Oceania seem to be sub-optimal according to climatic suitability for killer shrimp establishment (Figure 1b, d, f). Only small regions in North Africa and South-East Australia can be perceived as more threatened in this regard.

As was expected, highest suitability for the killer shrimp represent Europe and North America (Figure 1a, c), the continents where killer shrimp is already present or was previously predicted to be introduced in future (Rewicz et al. 2014). In Europe, the most threatened region hitherto without occurrence of killer shrimp are Spain and Portugal. Moreover, there are overlooked regions with high probability of killer shrimp establishment in Asia including the Japanese Archipelago (Figure 1e).

Besides biological and genetic features, successful invasion of alien animal species depends not only on climatic conditions, but is determined by complex of various factors like resource availability, stability or disruption of the environment, propagule pressure, and many other biotic and abiotic factors (Stohlgren and Schnase 2006 and citations therein). It follows that climate matching is to a certain degree an estimation and therefore the reality can occasionally be different. This is the reason why the killer shrimp successfully invaded England (MacNeil et al. 2010) which seems not serving so much possibilities to establish its wild populations in our modelling. Despite mentioned limitation, the method of climate matching is widely used and respected as appropriate tool to prediction of species invasions (Bomford et al. 2009, Kalous et al. 2015).

CONCLUSION

In light of the fact that killer shrimp is listed as one of the 100 worst alien species in Europe and has been cited as a species constituting a high risk in terms of spread, establishment and environmental threat in global scale, our findings poses a contribution for conservationists, policymakers and other stakeholders who engages in wild life management. We strongly recommend adding of killer shrimp into the Black List of European Union (Regulation 2016/1141) where this species surprisingly absents. Also aforementioned Asian regions should increase attention and ensure prevention of possible introduction of this “perfect invader”.

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FEMALE MORPHOMETRIC AND GENETIC ANALYSIS OF POTENTIAL NEW BRYCINUS SPECIES (TELEOSTEI: CHARACIFORMES: ALESTIIDAE) FROM CUANZA (QUANZA, KWANZA) RIVER CATCHMENT, BIE, ANGOLA

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Abstract: African characiform fish species from genus Brycinus Valenciennes (family Alestiidae) greatly vary in morphological characteristics. Moreover, recent genetic analyses suggest that validity and systematic position of certain species are uncertain and confused, and further revision of entire genus is required. Therefore, we considered to publish our finding from Cuanza River catchment in Angola. In 2007, we captured four adult females of Brycinus sp. in Cuquema River, one of the main tributaries of Cuanza River. These fishes were morphologically and genetically analysed and B. imberi (Peters) and B. lateralis (Boulenger) were identified as the most similar species. Both mentioned species can be easily distinguished from found Brycinus sp. by certain morphological characteristics and by genetic sequences. Since adult males absent in our data set, we recommend future collecting trip focused especially on mentioned males to confirm validity of suggested potentially new species.

Key Words: morphology, DNA, African characids, Cuquema River, taxonomy, new species

INTRODUCTION

The order Characiformes, one of the major lineages of ostariophysan fishes, is widely distributed in freshwaters throughout the most of South and Central America, and continental Africa. New World characiform fishes occur from the southern boundary regions of the United States to the central parts of Chile and Argentina (Lundberg et al. 1998, López et al. 2008, Froese and Pauly 2016). Characiform fishes inhabit freshwater ecosystems within broad regions of sub-Saharan Africa with the exception of the southern portions of the continent and the Horn of Africa (Orti 1997, Calcagnotto et al. 2005). Several species occur through the Sahara Desert along the length of the Nile River basin (Stewart 2009). Phylogenetic studies in recent decades determined three subunits among African characiform fishes: (i) the clade consisting of the families Distichodontidae and Citharinidae (Vari 1979); (ii) the clade formed by the putatively monotypic family Hepsetidae; and (iii) the assemblage recognized alternatively as 'African Characidae', Alestiinae, Alestiidae or Alestidae (hereafter referred to as Alestiidae) (Vari 1979). These studies revealed that mentioned three subunits of Characiformes do not jointly constitute a monophyletic unit, being instead dispersed across the entire order phylogeny. This conclusion is interesting both phylogenetically and in terms of the historical biogeographical relationships of the South American and African freshwater ichthyofauna (Zanata and Vari 2005). The latter mentioned subunit Alestiidae had been previously classified as subfamily of family Characidae (e.g., Greenwood et al. 1966, Géry 1977, Orti 1997, Weitzman and Malabarba 1998). Nevertheless, Buckup (1998) considered Alestiidae to be a valid family belonging to superfamily Alestioidea. Thus, this group of characiform fishes formed one of the few freshwater fish families occurring in both South America and Africa (Murray and Stewart 2002). This family currently comprises dwarf members generally grouped in the "Petersiini", and the genera *Alestes* Müller and Troschel, *Brycinus* Valenciennes, *Bryconaethiops* Günther and *Hydrocynus* Cuvier (Murray and Stewart, 2002). In total, 18 genera of the family (Froese and Pauly 2016) are actually divided into three tribes: the Hydrocinini; the Alestini and the miniaturized Petersiini (Hubert et al. 2005).

Circa 118 valid species from family Alestiidae greatly vary in body and fin sizes, shapes and occupied ecological niches (Froese and Pauly 2016). Alestiidae is the most speciose of all African characiform families (Paugy and Schaefer 2007, Arroyave and Stiassny 2011). Many morphological (Vari 1979, Buckup 1998, Zanata and Vari 2005) and molecular (Orti and Meyer 1998, Murray and Stewart 2002, Hubert et al. 2005, Calcagnotto et al. 2005) studies considered this family to be monophyletic. Contrary, certain recent studies focused on the Alestiidae phylogenetics this monophyly rejected (Arroyave and Stiassny 2011, Decru et al. 2016).

The genus *Brycinus* belongs to the tribe Alestiini and is characterized by presence of a rudimentary adipose eyelids, and by having two rows of pluricuspid teeth on the upper jaw (Paugy 2003). This genus (together with *Alestes*) is divided into three groups: (i) *B. longipinnis* (Günther) – small sized (maximum 140 mm standard length, SL), with fronto-parietal fontanelle always present; (ii) *B. nurse* (Rüppell) – medium sized (maximum 270 mm SL), with fronto-parietal fontanelle present in juveniles but closed in adults; and (iii) *B. macrolepidotus* Valenciennes – large sized (maximum 530 mm SL), with fronto-parietal fontanelle always absent (Paugy 1986, Decru et al. 2016).

In total, seven *Brycinus* species are native to Angola: *B. grandisquamis* (Boulenger), *B. humilis* (Boulenger), *B. imberi* (Peters), *B. kingsleyae* (Günther), *B. longipinnis*, *B. macrolepidotus*, and *B. lateralis* (Boulenger). The last species, *B. imberi*, is very widespread (Paugy 1986) with native range in coastal watershed of the Gulf of Guinea and in the Congo basin in West and South Africa (Paugy, 2003); in tributaries of the Great Lakes region in East Africa; and in the middle and lower Zambezi River (Balon 1971, Bell-Cross 1976) and in Phongolo River catchment (Skelton 2001) in southern Africa. Aforementioned *B. imberi* is perceived as a species complex (Paugy 1986).

Since numerous original descriptions and taxonomic statutes of African characids are to a certain degree confused in light of recent genetic analyses, the revision is strongly recommended by many authors (Murray and Stewart 2002, Hubert et al. 2005, Stiassny and Mamonekene 2007, Arroyave and Stiassny 2011, Decru et al. 2016). In order to contribute to the knowledge of fish from genus *Brycinus* we considered to publish our findings from Cuanza River catchment in Angola with suggestion of one potentially new species.

MATERIAL AND METHODS

Study locality and data collecting

During ichthyological survey in 2007 we sampled Cuanza River catchment on Bié Plateau in Angola (Figure 1). The fishes were collected mainly by seine, rod and hand netting. Fish were euthanized with overdose of the anaesthetic Phenoxy-2-ethanol diluted in water. All individuals were fin-clipped for later DNA analyses, and specimens were fixed in formaldehyde and deposited in the Aquatic Organism Collection at the Czech University of Life Sciences Prague, Czech Republic.

Figure 1 African characid (Brycinus sp.) from Cuanza River, province Bié, Angola

Morphological analysis

All specimen morphometric measurements were made with a digital calliper (accuracy of 0.01 mm). According to Boulenger (1900), Poll (1967) and Skelton (2001) following characteristics were measured: number of scales in lateral line (l. l.), number of perforated scales in canal on lateral line between the head and the base of the caudal fin; number of scale rows above lateral line (Squ. sup.), number of rows from dorsal fin base diagonally forward to the lateral line; number of rows below lateral line (Squ. inf.), number of rows from pelvic fin base diagonally forward to lateral line; number of unbranched dorsal fin rays (D-Du); number of branched dorsal fin rays (D-Db); number of unbranched

anal fin rays (A-Au); number of branched anal fin rays (A-Ab); standard body length (SL), distance between end of snout and end of scaled caudal fin base; total body length (TL), distance from front of snout to end of longest caudal fin ray; head length (lc), lateral distance from top of snout being closed to mouth to posterior margin of operculum without gill membrane; maximum body height (H), distance from highest point of ridge vertically down; predorsal distance (pD), distance from top of snout in straight line to beginning of dorsal fin base; preanal distance (pA), distance from top of snout in straight line to beginning of anal fin base; preventral distance (pV), distance from top of snout in straight line to origin of pelvic fin base; length of caudal peduncle (lpc), distance from to posterior end of scaled caudal part of body; height of caudal peduncle (hpc), distance vertically between end of anal fin base and upper margin of body; length of dorsal fin (lD); length of anal fin (lA); length of pectoral fin ray (lP), distance between beginning and end of longest base of pectoral fin ray; length of pelvic fin ray (lV), distance between beginning and end of longest base of pelvic fin ray; head width (lac), transverse distance between margins at widest area of the head; horizontal diameter of eye (Oh), corneal diameter.

Genetic analysis

DNA was extracted in two individuals from small pieces of fish pectoral or ventral fins using DNeasyTM Tissue Kit (Qiagen, Valencia, CA, USA). We amplified one mitochondrial gene - cytochrome oxidase subunit I. Following primers were used for COI: forward primers 5′-TCA ACC AAC CAC AAA GAC ATT GGC AC-3′ and reverse 5′-TAG ACT TCT GGG TGG CCA AAG AAT CA-3′. PCR was performed in a volume of 25 µl. An initial denaturation step for COI gene an initial denaturation step was of 94 °C for 2 min, followed by 36 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min and elongation at 72 °C for 1 min. The terminal extension was at 72 °C for 10 minutes. PCR products were sent the Macrogen service in South Korea (www.macrogen.com).

The chromatograms obtained were assembled by hand and eye check for potential errors. The comparison specimens from Cuanza were obtained 61 sequences from the GenBank database. This dataset was aligned using Clustal W is done in the software package BioEdit (Hall 1999). Both sequences were submitted to GenBank (KX853170, KX853171). Family relationships were estimated using Bayesian methods of analysis (BI) using MrBayes ver. 3.0 (Huelsenbeck and Ronquist 2001).

RESULTS AND DISCUSSION

In total, we captured four adult females of *Brycinus* sp. in Cuquema River, one of the main tributaries of Cuanza River (Figure 2).

Detailed morphological measurements of analysed individuals are given in Table 1. Head twice longer than wide; head width 9.7 to 10.5 times in TL; snout shorter than eye; eye width 2.9 to 3.1 in head; depth of the body 4.6 to 5.0 times in TL. Head and body depressed dorsoventrally, eye pigmented. Jaw not reaching front edge of the eye. Two rows of teeth on the upper and lower jaws: 16 teeth (8/8) upper, 8 (6/2) lower. Number of dorsal-fin rays II-III 7; dorsal fin originating at the base of pelvic fins; predorsal distance 1.9 to 2.0 times and preventral distance 2.1 times to SL. Number of anal-fin rays II 15. Pectoral fin 1.1 to 1.2 times of the head length, reaching ventral fin base. Caudal fin deeply forked. Caudal peduncle 1.3 to 1.5 times longer than deep. Scales cycloid. Lateral line scales without arborescent or anastomosing canals, 20 to 22 in total, 2 scales between lateral line and ventral fin base and 5 scales between lateral line and dorsal fin base. Body colouration of live individuals silvery-greenish with prominent dark stripe on lateral line reaching posteriorly behind caudal fin base, large black blotch laterally on caudal peduncle not reaching the end of caudal fin. Individuals deposited at the Czech University of Life Sciences (No. AOCAO1, AOCAO2, AOCAO3 and AOCAO4).

Based on genetic and morphological matching, *B. imberi* and *B. lateralis* (occurring also in Cuanza River catchment) were identified as the most similar species to described *Brycinus* sp. Both mentioned species can be distinguished from found *Brycinus* sp. by: (i) different depth of the body which is 2.7 to 3.5 times in TL in *B. imberi* and 3.8 to 4.5 times in *B. lateralis*; (i) numbers of teeth in lower jaws which is 10 (8/2) in both *B. imberi* and *B. lateralis*; (iii) number of dorsal-fin rays which is II 8 in *B. imberi* and II-III 8 in *B. lateralis*; (iv) number of anal-fin rays which is II-III 14-16 in *B. imberi* and III-IV 15-16 in *B. lateralis*; (v) number of lateral line scales which is 30-33 in *B. lateralis*; and (vi) colouration: *B. imberi* has a dark spot laterally behind the head above lateral line and large black blotch laterally on caudal peduncle, and *B. lateralis* has a black lateral stripe extending to median rays of caudal

peduncle and large black blotch laterally on caudal peduncle reaching the end of caudal fin (Boulenger 1909).

The final result of genetic analyses of the COI sequences consisted of 578 characters. Phylogenetic reconstruction method recovered tree of genera *Brycinus* and *Alestes*. Both individuals from Cuanza River identified as *Brycinus* sp. belong in the same group of *B. imberi*. Detailed results of molecular analysis are shown in Figure 3.

Table 1 Detailed morphological measurements of analysed individuals

Characters	AOCAO1	AOCAO2	AOCAO3	AOCAO4
l.l.	22	22	22	20
Squ. sup.	5	5	5	5
Squ. inf.	2	2	2	2
D-Du	3	2	3	2
D-Db	7	7	7	7
A-Au	2	2	2	2
A-Ab	15	15	15	15
SL	66.43	79.4	77.14	69.77
TL	81.44	96.82	93.65	86.09
lc	16.94	19.24	18.8	18.6
H	18.92	21.17	19.92	18.48
pD	35.52	39.33	39.56	37.37
pA	49.82	56.58	54.92	49.77
pV	31,0	38.12	37.86	35.16
lpc	10.85	13.13	12.9	10.94
hpc	8.25	8.78	8.45	7.61
lD	5.88	8.65	8.57	6.82
lA	8.49	10.66	11.29	10.61
lP	15.58	17.78	17.43	15.85
lac	8.19	9.95	9.23	8.23
Oh	5.83	6.41	6.25	5.97

Figure 2 Map of Angola with locality where individuals of Brycinus sp. were captured (indicated by black triangle)

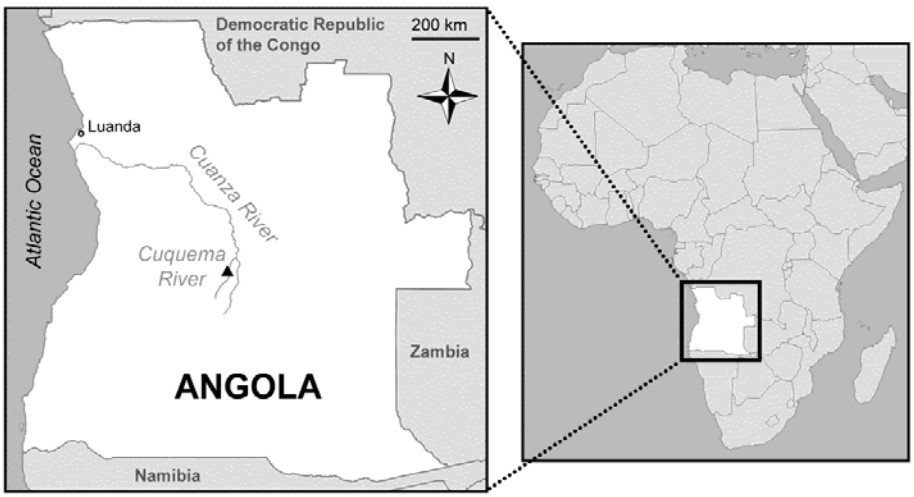
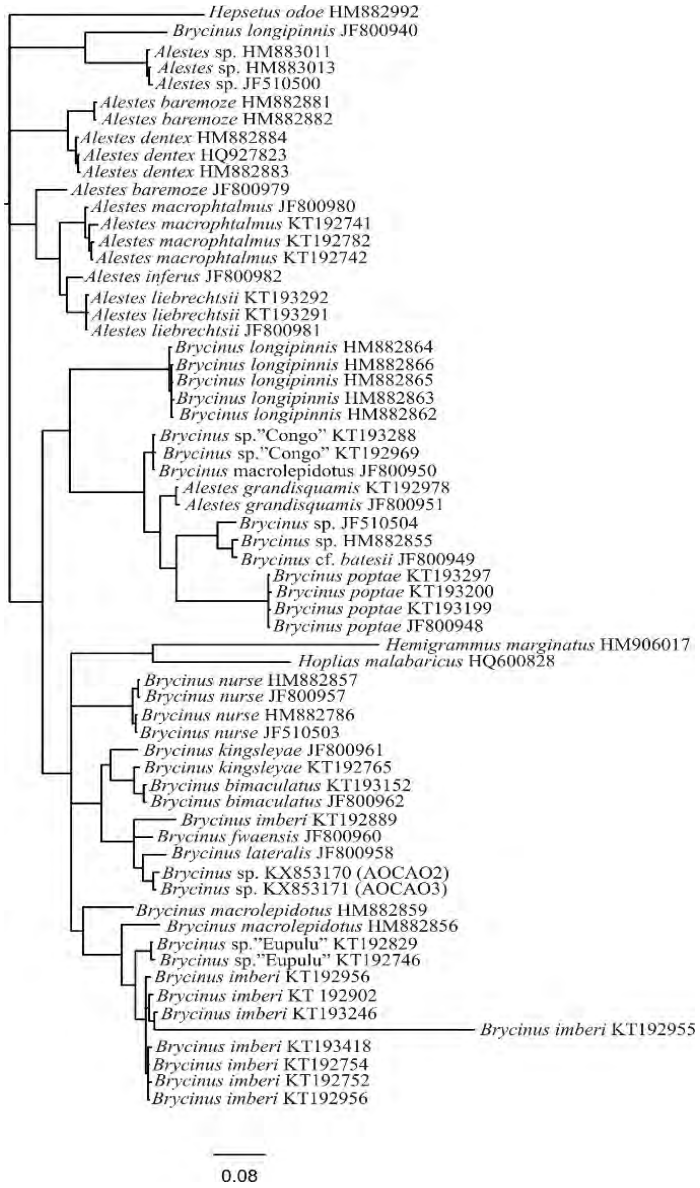


Figure 3 Detailed results of molecular analysis of the program MrBayes. Hepsetus odoe (Bloch) was used as the outgroup



CONCLUSION

Since genetic and morphological analyses suggested that found fishes belong to a new species of *Brycinus*, we cannot validate the new taxon due to the absence of adult males in our data set. Therefore, we recommend further collecting trip focused especially on mentioned males within the Cuanza River catchment.

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AFFECTING THE PHOSPHORUS RETENTION IN FISH BREEDING BY USING SPECIAL CEREAL VARIETIES

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Abstract: The aim of the study was to monitor the phosphorus digestibility of the feed used in the fish breeding. Phosphorus excreted by fish into the aquatic environment may be one of the potential sources of pollution of the aquatic environment by biogenic elements. The efforts to influence the amount of phosphorus leaving the fish breeding are divided into several directions, where the main reason is different physiology of fish digestion. In particular the pond breeding of cyprinids is becoming an increasingly addressed issue. The possibility of influencing phosphorus digestibility by the use of phytate enzymes in the carp breeding is almost impossible in practise; therefore it is important to focus on one of the other methods. Breeding of new varieties of cereals with reduced content of indigestible form of phosphorus for cyprinids is one of the potentially successful ways how to eliminate the amount of phosphorus leaving the fish breeding. 60 pc of common carp fry and four experimental diets were selected for this study. The first part of the test was focused on the feeding of wheat and the second one on the feeding of barley. Control varieties and varieties with a reduced content of phytate were used in both parts. Phosphorus digestibility of the feeds was determined through the indicator method where the crude fibre served as an indicator. In the case of wheat, there was determined a statistically highly significant increase ($P<0.01$) of phosphorus digestibility (by 11.21%) when feeding a low -phytate variety. In the case of barley, there was determined a statistically significant increase ($P<0.05$) of phosphorus digestibility when feeding a low-phytate variety. Feeding cereals varieties with a reduced content of phytate has a significant effect on reducing phosphorus loading of the aquatic environment.

Key Words: phytate, barley, wheat, digestibility, phosphorus

INTRODUCTION

Phosphorus is one of the most common elements on the planet. It is an integral part of all plant and animal cells. Phosphorus is contained mostly in the dental and bone tissue, as well as in fish scales. Phosphorus is also a part of proteins, sacharides, lipids, the nerve tissue and, not least, the blood (Jelínek et al. 2003, Chow and Schell 1980). Phosphorus is also a significant source of energy for the cell (Jelínek 2003) and a part of the nucleic acids (Pointillart 1987).

The issue of polluting the environment by phosphorus relating to agriculture and fish breeding is becoming increasingly important. In particular, feeding of carps by cereals in ponds may be a significant source of phosphorus in the aquatic environment. The most commonly used cereals for carps feeding are wheat and triticale or rye and barley (Mazurkiewicz and Przybyl 2004). In addition, maize is used, which, however, significantly increases the proportion of fat (Viola and Arieli 1983). Jirásek et al. (2005) states the phosphorus content in wheat kernel amounts to about 3 g, to 3.4 g in triticale. Decisive, however, is the rate of the phosphorus digestibility of plant components. Phosphorus is bound in plants in the form of phytic acid. Phytic acid is a complex cyclic hydrocarbon compound, to which residues of phosphoric acid are bound. The phosphorus contained in this compound is usable for cyprinids to a limited extent 8–38% (Jirásek et al. 2005) or almost unusable (Kumar et al. 2011). Phytate content in cereals varies depending mainly on the species of the cereal, the cultivated variety grown or the stage of ripeness (Malý 2015).

Phytate phosphorus digestibility can be influenced by several ways. The first one consists in the use of phytase enzymes which is very difficult in carp breeding. The pH of the GIT value of the carp is about 7, because the cyprinids do not have the stomach. However, most industrially produced phytases

exhibit the highest activity in pH about 2.5 (Cao et al. 2007). We can add one of the organic acids to the feeds in order to better utilize these phytases, whereby we create an ideal environment for the function of phytase (Baruah et al. 2005). Today, however, even neutral phytase, exhibiting the highest activity in neutral pH, is commonly available at the market. The other factor influencing the phytase activity is temperature. Most phytases lose their activity at the temperatures over 70 °C (Cao et al. 2007). This fact limits the use of phytases in particular in breeding of salmonid fish fed by extruded feeds where the temperature at the production of the feed exceeds the maximum temperature compatible with the function of phytase (Cain and Garling 1995). Therefore, spraying the preparation on the surface of the granules is considered the most effective way of application.

Another way through which phosphorus digestibility can be influenced is the use of specially bred varieties of cereals. The commonly used cereals for feeding carps contain a relatively high proportion of phytate phosphorus from total phosphorus. In the case of wheat, the phytate content ranges from 0.62 to 1.35% on dry weight basis depending on the cultivated variety, climate, soil etc. However, the proportion of the phytic acid of the total phosphorus is more important. Generally, the content of the phytic acid amounts to 61.7 to 79.9% of the total phosphorus. The higher this number is, the lower the phosphorus digestibility of feed is (Lolas et al. 1976)

Barley as a feed for fish, is used less frequently due to its worst intake by fish; therefore it is necessary to adjust the barley kernels. The content of the phytic acid is, depending on varieties, more balanced and ranges from 0.98 to 1.16%. Similarly, the content of phytate from the total phosphorus is more balanced and ranges from 66.1 to 69.6% (Lolas et al. 1976). In terms of utilization of the phosphorus of the feed, such a high proportion of phytate is ineffective. Special varieties of barley have been developed in order to utilize phosphorus more effectively and to reduce the phosphorus loading of the environment. These varieties were developed by hybridization with standard varieties with LPA (low phytic acid) varieties. LPA varieties are cereals with reduced content of the phytic acid which correlates with an increased proportion of the digestible component of phosphorus. Thus, the aim was to develop barley with a changed proportion of phytate from the total phosphorus. Vaculová et al. (2012) notes that the proportion of digestible phosphorus and the phytic acid in barley kernel is about 31%. While in LPA varieties this proportion amounts to from 138.4% to almost 770%, depending on the cereal variety. For barley, we can meet also with special hulless varieties. In the Czech Republic, it is the AF LUCIUS variety. The disadvantage of growing this variety consists in low yields in comparison with hulled varieties as well as higher bulk density. On the contrary, the advantage consists in the absence of hulls that are not suitable for feeding and a high nutrition value (Pechová 2015).

These factors may help us to facilitate to reduce the the phosphorus loading without using various kinds of chemical preparations and enzymes.

The study is therefore focused on monitoring of phosphor digestibility of specially bred varieties of cereals with a reduced content of the phytate component.

MATERIAL AND METHODS

Characteristic of test

60 pc of the general carp (*Cyprinus carpio* L.) stock fish with an average weight of 138.19 ± 36.88 g were selected in order to monitor phosphorus digestibility. The fish were divided into six groups of ten fish each and were planted in aquariums. Before starting the test, the fish were individually measured and weighted in order to set an appropriate feed ration for individual aquariums. The test was divided into two sub-parts, digestibility of wheat was monitored in the first part and digestibility of barley was monitored in the second part. Every parts of test were 14 days long and one week before both test was for acclimatizing for new feed. Every variant of feeds was fed in three tanks. Between the tests was one week long time lag.

- **TEST 1**

In this 14 days long part of the test, the fish were fed by a wheat-base feed. Fish in three tanks were fed by control diet and next three tanks were fed by experimental diet. Vánek wheat variety was selected as a control feed. Crossbreed variety JS-12/IDO 563 was selected as a tested feed in order to determine better phosphorus digestibility of the feed. It is a crossbred variety of Vánek variety with one

of the LPA varieties. Cereals samples for the feed test were provided by Ing. Vaculová of Agriculture Research Institute Kromeriz.

- **TEST 2**

In the second part of the test (14 days long), the fish were fed by a barley-base feed. Three tanks were fed by control and three tanks by experimental diets. Barley malting variety Bojos was used a control feed. LPA mutant designated as M 955 was used as a monitored feed. Both samples for phosphorus digestibility were provided by the Agriculture Research Institute Kromeriz.

Characteristic of the equipment

Six specially designed tanks with a volume of 106 litters, being divided into two parts, were prepared in order to monitor phosphorus digestibility in fish. The bigger part is the rearing part and the second smaller part is used for sedimentation of faeces. Sedimentation is achieved by proper adjustment of aeration and aquarium inflow. The sedimentation part is separated from the rearing part by a partition which is about 1 cm above the bottom. Thus, the principle consists in controlled fish feeding when the fish must consume all submitted feed which might contaminate the faeces samples leaving to the sedimentation part of the tank

Characteristic of used diets, collection and analyses of the samples

All four types of cereals used in the feed tests were analysed for the content of phytic acid and the content of digestible phosphate. The analyses were conducted in an accredited laboratory of the Food Preservation Department at University of Chemistry and Technology in Prague by the method of capillary electrophoresis and using conductivity and UV detection (Blatný et at. 1995). The aim of these analyses was to verify a higher content of phosphate and a lower content of phytate in newly bred varieties.

For better feed intake by the fish, these feeds were scrapped and subsequently granules were made of them. The composition of experimental mixes is reported in Table 1. All the mix components were mixed dry; compact dough was made and subsequently granules were made from the dough. The granules were then dried in a drying oven at the temperature by 50 °C for the period of 24 hours.

Table 1 composition of experimental diets

	TEST 1 - wheat control (K)	**TEST 1 - wheat** cross LPA	**TEST 2 - barley** control (K)	**TEST 2 - barley** LPA
Cereals	Vánek	JS-12/IDO 563	Bojos	M 955
Cr_2O_3	1%			
Pellet-dur	1%			
Mollases	1%			
Whey	1%			

The feed ration was determined individually for each aquarium up to 2% of the fish stock weight. The feed ration was divided into two up to three sub-rations during the day.

Hydro-chemical parameters of water were recorded regularly during the feed test. Always before the morning and evening feeding, the content of oxygen, temperature and pH value would be measured in each aquarium (multimetr Hach Lange HQ 40d, Germany) and each day before the morning feeding, the content of ammonium ions, chlorides, and nitrites would be measure (spectrophotometer WTW photoLab 6600 UV.VIS, Germany) with standard sets (WTW Germany), by the method Horáková (2007).

Faeces samples were taken in order to determine the phosphorus digestibility. Faeces were collected regularly each day before the morning feeding. Faeces were sucked off with a pipette into a plastic container and subsequently filtered through filter paper. After filtering water out, faeces samples were stored in a freezing box (-18 °C) in plastic sampling bottles. After completing the test, faeces and feed samples were analysed for the content of phosphorus, fibre and chromium hemitrioxide. Digestibility was determined using the indicator method (Figure 1) (MENDELU in Brno 2016) where first, crude fibre naturally occurring in the feed served as an indicator and second, chromium hemitrioxide added to the feed in amount of 1% served as an indicator.

Statistical evaluation of data was performed in Microsoft Excel 2010 program by the method of variance analysis (ANOVA) with entering one factor.

Figure 1 digestibility coefficient by indicator method

$$Digestibility\ coefficient = 100 - \frac{\mathbf{I}\ diet\ \times \mathbf{N}\ excreta}{\mathbf{I}\ excreta\ \times \mathbf{N}\ diet} \times 100$$

** I diet, I excreta – content of indicator in diet and excreta (%)*

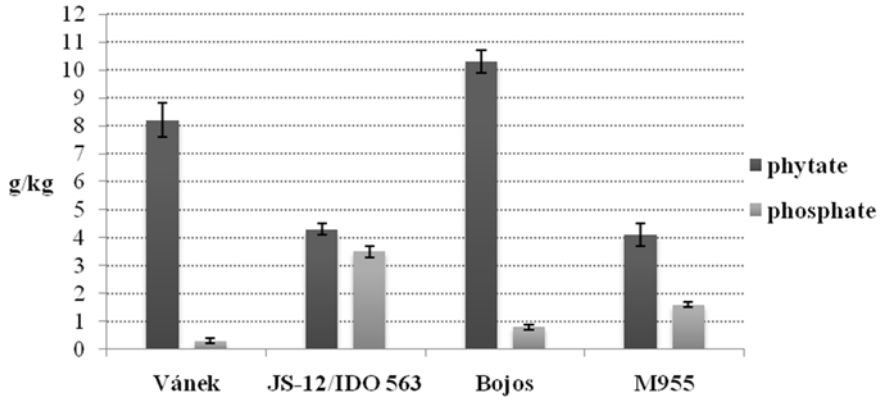
** N diet, N excreta – content of nutriment in diet and excreta (%)*

RESULTS AND DISCUSSION

Phytate and phosphate content in the main components of the feed

Figure 2 shows the results of cereal analyses for the content of indigestible and digestible phosphate. These primary results are important for other works focused on influencing phosphorus digestibility through LPA cereal varieties.

Figure 2 content of phytate and phosphate in experimental cereals



The graph shows that the cereals which were used as control ones, i.e. commonly grown cereals having a high proportion of indigestible phytate and a very small proportion of digestible phosphate. In the case of Bojos barley control variety, it amounts to almost 92% and Vánek wheat control variety it amounts even to 96.5%. In terms of phosphorus digestibility, these values are very negative, since phytate digestibility in plant components of feeds, in the case of the general carp, ranges from 8 to 38%. Percentage digestibility depends on the type of cereal, the stage of ripeness and the growing conditions (Jirásek el al. 2005). Unlike the control feeds, in tested varieties 71.9% of the phytate component was determined in barley and 55.1% in wheat. The results of these analyses correlate with the claim of Vaculová et al. (2012) who in her work presents reduction of the phytate content and an increase of the adjustable phosphorus component in LPA varieties.

The samples were analysed for the content of crude fibre as an indicator in order to finally determine phosphor digestibility. The results of the determination are evaluated in Table 2.

Table 2 content of crude fibre in experimental diets and excreta samples (%)

	Wheat K*	Wheat LPA*	Barley K*	Barley M955*	Wheat K	Wheat LPA	Barley K	Barley M955
CF (%)	**3.00**	**3.06**	**4.29**	**4.89**	**21.04** ± 1.04	**19.22** ± 0.23	**23.83** ± 1.00	**25.33** ± 1.17

** analyze of fishfeed, CF – crude fibre*

The average crude fibre content in wheat amounts to 3.03% and in barley to 4.59%. Similar crude fibre values in cereals are presented by Lee et al. (2016) who have determined the average crude fibre content in wheat amounting to 2.49% (2.38–2.62) and to 4.96% (4.46–5.81) in the case of barley. These slight differences are given especially by the variety and origin of the particular cereal.

The following table records the average values of the phosphorus content determined in the feed samples and faeces samples of individual groups. Feed phosphorus digestibility was calculated from the collected data by means of the indicator method.

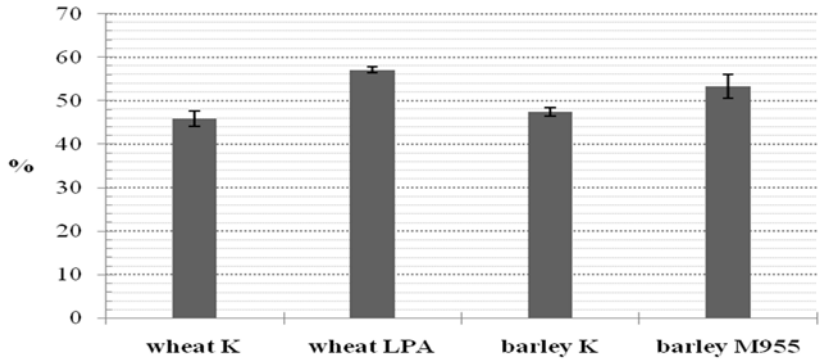
Table 3 content of total phosphorus in experimental diets and excreta samples (%)

	Wheat K*	Wheat LPA*	Barley K*	Barley M955*	Wheat K	Wheat LPA	Barley K	Barley M955
P (%)	**0.2622**	**0.3557**	**0.2875**	**0.3455**	**0.9951** ± 0.079	**0.9573** ± 0.027	**0.8397** ± 0.045	**0.8351** ± 0.035

** analyze of fishfeed, P - phosphorus*

In average, there was determined 0.309% of phosphorus in the feeds with the share of wheat and 0.317% of phosporus in feed with share of barley. The table shows that both monitored feed varieties contain a higer proportion of phosporus than the control groups. These results do not coincide with the results of Vaculová et al. (2012) who states that only the posphate and phytate proportion changes in LPA varietes but the content of phosporus remains the same. Lee et al. (2016) states the average content of phosphorus 0.30% (0.26–0.34) in wheat and 0.27% (0.25–0.28) in barley. Especially the control groups of cereals are comparable with these results.

Figure 3 digestibility of phosphorus from experimental diets



As shown in figure 3, both control groups of the feeds used exhibit a lower phosphorus digestibility than the experimental groups. In the case of groups of feeds made of wheat, a statistically highly significant ($P<0.01$) increase of phosphorus digestibility was determined in the monitored wheat variety JS-12/IDO 563 (57.10 ± 0.73 %) in comparison with the control group with the Vánek variety (45.89 ± 1.78%). In the case of groups made of barley, a statistically highly significant ($P<0.05$) of phosphorus digestibility was determined in the monitored low-phytate form of barley (M955).

CONCLUSION

The efforts to streamline the often reckless management of phosphorus in fish undertakings are going to be increasingly solved issue of global nature, as the fish breeding may be one of potential water pollution due to the excess of phosphorus. The physiology of digestion of the received food which is species-specific is becoming a considerable problem. In the case of salmonidae, feeds with the addition of phytase which supports the digestibility of phosphorus from the plant component of the feed are fully exploited today. Using these phytases in feed is possible due to the physiology of digestion of salmonidaes that have an acidic pH of the digestive tract which is one of the conditions for the possibility to use phytases. In the case of cyprinids, the use of phytases is negatively affected by the fact that the pH value in the digestive tract of cyprinids is around 7, which is insufficient for the utilization of phytases. Therefore, the efforts are to seek new methods how to increase the digestibility of phosphorus from plant components without using phytase. The aim of this study was therefore to verify the improvement of the digestibility of phosphorus using specially bred varieties of cereals that exhibit a lower share of the phytate component in favour of the phosphate component. Varieties of wheat and barley were used for monitoring. Vánek variety was used as a control variety in wheat and Bojos in barley. The selected monitored varieties (wheat JS-12/IDO 563, barley M955) different from the control varieties by the proportion of phytate and phosphate component. After partial feed test have been done, a statistically highly significant ($P<0.01$) increase of phosphorus digestibility among the wheat varieties used (by11.21%) and further a statistically significant ($P<0.05$) increase of phosphorus digestibility among the barley varieties used (by 5.89%). Through these results, we can state that using low-phytate varieties of cereals have a positive effect on the amount of digested phosphorus from the submitted feed.

In addition, this fact has a positive effect, thus decreasing the amount of phosphorus excreted in the water. Thus, it reduces directly water pollution. In the event these both newly bread varieties are used, the economics of breeding becomes the most significant issue. The price of these varieties differs significantly from the commonly grown varieties in the negative sense of the word. Comparing the efficiency of feeding LPA varieties depending on the improvement of phosphorus digestibility and economics of fish breeding is going to be a subject of further studies.

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ELIMINATION OF BRYOZOANS IN INTENSIVE FISH FARMING

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Abstract: The aim of this study was to establish a methodology for efficient elimination of bryozoans in fish farms and their water sources. Another point was to evaluate the economic effectiveness of the suggested measures. The presence of bryozoans in aquaculture may be unfavourable because they are hosts of myxozoa that cause proliferative kidney disease (PKD), accompanied by high mortality of fish and thus high economic damages. The second problem is that bryozoans' colonies may cover surfaces as pipes and biofilters. To test the elimination of bryozoans, agents commonly used in aquaculture, such as Savo, Persteril, and formaldehyde were used. Two species of bryozoans were selected as model species (*Plumatella emarginata* and *Cristatella mucedo*) and were tested using different concentrations of metioned substances. Colonies of species *C. mucedo* were more resistant against the chemicals than colonies of species *P. emarginata.* All individuals of species *P. emarginata* were killed in concentrations 0.025% for Savo, 0.0031% for Persteril, and 0.0063% for formaldehyde. All individuals of bryozoan *C. mucedo* were killed in concentrations 0.1% for Savo, 0.0063% for Persteril, and 0.0125% for formaldehyde. The concentrations of formaldehyde, as the only product used, killed bryozoan colonies at lower concentrations than quoted lethal concentrations for fish and selected aquatic invertebrates. Therefore, in devices with fish, the recommend concentration is 0.0125% (1.38 mg/l). Formaldehyde is the most expensive of all the tested agents at CZK 37.4/m^3, while Savo is the cheapest at CZK 30/m^3.

Key Words: myxozoans, bryozoans, *Tetracapsuloides bryosalmone*, PKD

INTRODUCTION

Aquaculture and fish farming represent in many countries the world's fastest growing livestock sector (FAO 2014). Aquaculture in the Czech Republic is primarily associated with fish farming in ponds, but recently, there are a constantly increasing number of specialized modern systems which are especially developed for intensive fish farms (Czech Ministry of Agriculture 2014). However, sometimes there is a spread of other organisms in the devices, common members of aquaculture systems are macroinvertebrates. Some species have no effect on the system. In some cases, their presence may be undesirable, because they can significantly affect the system. For example, they reduce the functionality of biofilters, or their colonies overgrow the device or are intermediate hosts for fish parasites (Canning and Okamura 2004, Lom and Dyková 2006, Hrabcová 2015) that often cause very serious diseases. Elimination of aquatic invertebrates from the system may be difficult and in some cases impossible.

One of the major diseases that threaten fish farms in Europe and North America is the proliferative kidney disease (PKD)(Hedrick et al. 1993, Okamura and Wood 2002). The disease occurs not only in aquaculture, but it also threatens fish in fresh waters (El-Matbouli and Hoffmann 2002, Feist et al. 2002). The course of the disease is chronic, as mortality of farmed fish may under certain conditions reach 90% (Hedrick et al. 1993, El-Matbouli and Hoffmann

2002), therefore it is often connected with huge economic demages. In the Czech Republic, the disease has been diagnosed for the first time in 1986 (Svobodová 2007). The causative agent of infection is a parasite *Tetracapsuloides bryosalmonae* (Myxozoa, Malacosporea). The disease occurs most frequently in young salmonids especially yearlings in summer, when the water temperature exceeds 15 °C. The development of the disease is primarily affected by increasing temperature but also by other environmental factors such as low amount of dissolved oxygen, or a poor water quality (Schmidt et al. 1999, El-Matbouli and Hoffmann 2002). Lifecycle of the myxozoan *T. bryosalmonae* takes place in two hosts, which are bryozoans (Phylactolaemata, Bryozoa) (Okamura and Wood 2002, Hrabcová 2015). Further hosts include salmonids (Hrabcová 2015).

Bryozoans are commonly occurring freshwater animals which make colonies composed of individual zoids that usually reach a size of only a few millimetres. The whole colonies can then reach several decimetres. They often grow on submerged objects, such as wood rocks, or ships (Wood 2005). Ten species of bryozoans were identified in the Czech Republic (Korábek 2009). Examples of potential hosts for *T. bryosalmone* previously included *Hyallinella punctata*, *Plumatella fungosa, Plumatella repens, Cristatella mucedo* or *Fredericella sultana* (Tops and Okamura 2005, Hrabcová 2015). Two species of commonly occurring bryozoans were used for tests, i.e. *Plumatella emarginata* and *Cristatella mucedo* that were detected as host species for *T. bryosalmone* (Canning et al. 1999, Longshaw et al. 1999, Henderson and Okamura 2004).

The aim of this study is to establish a method for efficient elimination of bryozoans in fish farming facilities and water sources (de Kinkelin 2002), which may be one way of prevention the occurrence of a serious fish disease and reducing the risk of economic losses. In the Czech Republic, these problems are currently not being solved. Finally, the assessment of the economic efficiency is important for the proposed solution results to be used in practice.

To test the elimination of bryozoans from aquaculture, three commonly used chemicals were selected, i.e. Savo, Persteril, and formaldehyde. Information about the influences of these agents on bryozoans is completely missing from literature. Sodium hypochlorite, the main compound of Savo, is commonly used in water treatment, disinfection of tanks, and other water systems. In aquaculture, it is used for disinfection for example to restrict bacterial infections (Anasco et al. 2008, Kim et al. 2008). Other used chemical included Persteril, with main compound peracetic acid, which is a strong antibacterial agent used for disinfection in the food industry, agriculture, or for the disinfection of waste water, etc. In the fisheries, it is mainly used in the treatment of bacterial (Meinelt et al. 2015) or parasitic diseases caused by *Ichthyobodo necator* (Jaafar et al. 2013) and *I. multifiliis* (Meinelt et al. 2009). The last used agent, formaldehyde, is utilised in many branches. In aquaculture, it is used mainly in the treatment of diseases caused by ectoparasites such as *Ichthyophthirius multifiliis* (Forwood et al. 2014) and monogeneans (Fajer-Avila 2003). Given the wide range of uses of these three agents, their effect on various species of fish was tested, for example, on rainbow trout (Tkachenko et al. 2013), *Danio rerio* and *Poecilia reticulata* and other fish (Buchmann et al. 2004, Straus et al. 2012, da Costa et al. 2014).

MATERIALS AND METHODS

The tests were carried out under laboratory conditions for two model species *Plumatella emarginata* and *Cristatella mucedo.* During testing, the water temperature was 22.4 °C and there were no significant fluctuations. The pH of the environment was moderately alkaline, ranging between 7.55 and 7.89. The percentage of oxygen saturation did not drop below 87%. Colonies of *P. emarginata* originated from the recirculating aquaculture system. The colonies

of other species *C. mucedo* were taken from pond at locality (Vysočina Region), which is used as water source for fish farm and where in the past years, an occurrence of PKD was recorded.

Bryozoan colonies were removed by scraping from solid surfaces with a scalpel. They were transferred to the laboratory, where they were divided into pieces of about 5 cm^2. Subsequently they were moved to Petri dishes with water and left for 24 hours for acclimatization. Then they were exposed to three chemicals (formaldehyde, Persteril, Savo) of different concentrations. Eleven concentrations were applied in the following sequence – 1, 0.5, 0.2, 0.1, 0.05, 0.025, 0.0125, 0.0063, 0.0031, 0.0016, and 0.0008%. Each concentration had five repetitions and a control sample, which used only water. Bryozoans were exposed to the agents for 30 minutes and then the solution was replaced with water. After that the colonies were allowed to rest for one hour without manipulation and then the first examination was carried out. After another hour, the second examination was carried out. During both controls, 50 randomly selected zoids were chosen from every sample and were microscopically monitored for reactions. Live zoids that showed no signs of disturbance were counted. Survided zoids were observed for next two hours.

RESULTS AND DISCUSSION

The first tested substance was Savo Original where the active compound is sodium hypochlorite. After a short-term exposure of species *P. emarginata* colony, the mortality of all zoids was observed at concentrations higher than 0.025%. Lower concentrations caused only slow reactions of zoids. After the use of low concentrations of Savo, the recovery was relatively fast, and within 2 hours more than half of *P. emarginata* zoids have recovered. In the 0.0125% variant, there was a statistically significant difference in zoid reactions ($p = 0.048$) between first and second control, for a variant of 0.0063%, there was no significant difference in zoid reactions ($p = 0.16$), and for lower concentration (variant 0.0031%) the difference was again statistically significant ($p = 0.001$). In these three variants, full recovery of all zoids occurred after four hours. At concentrations of 0.0016% or less, no changes in the responses have been observed for species *P. emarginata* and all individuals were active. Colonies of species *C. mucedo* were killed by Savo at distinctively higher concentrations, i.e. 0.1% or higher. Lower concentrations (0.05% and 0.025%) also caused a slow reactions and demobilization of few zoids, between the first and second control in both versions a rapid recovery of zoids occurred, but statistically significant difference was observed only at lower concentrations of 0.025% ($p = 0.018$). At concentrations of less than 0.0125%, no changes were observed in zoids of *C. mucedo*. Concentrations of sodium hypochlorite for bryozoans were converted to mg/l. The lethal value for species *P. emarginata* was 0.93 mg/l and for *C. mucedo* it was 3.72 mg/l. These concentrations significantly exceed the presented LC 50 for fish and other aquatic organisms. The toxicity of sodium hypochlorite for rainbow trout *(Oncorhynchus mykiss)* stated as 96 h LC50 0.2 mg/l and for the crustacean *Daphnia magna* the value is even lower at 48 h EC50 0.141 mg/l (Safety Data Sheet, Penta 2012).

The next tested product was Persteril. In tests, it killed the entire colony of *P. emarginata* already at concentrations of 0.0031% and higher. Lower concentrations, i.e. 0.0016 and 0.0008, caused a slow response and demobilization of certain portion of zoids. Recovery time of zoids was much longer, between the first and second controls there was a statistically significant difference in the zoid reactions only at concentrations of 0.0016%. Normal reactions were recorded in less than half of zoids after four hours at mentioned concentration. In the *C. mucedo* species, Persteril caused the death of colonies at concentrations of 0.0063% and higher. In the subsequent variant 0.0031%, for the species *C. mucedo* no changes were observed in the activity and the response of the controlled subjects were normal. The lethal concentration of peracetic acid, which is the main agent of Persteril, was for the species *P. emarginata* 2.37 mg/l and for

C. mucedo, it was 4.75 mg/l. Both of these values several times exceed the reported lethal concentration for fish and other aquatic animals. The toxicity of peracetic acid for trout *O. mykiss* is given as 96 h LC50 0.53 mg/l and for the crustacean *D. magna*, it is 48 h EC50 0.73 mg/l (Safety Data Sheet, EuroŠarm 2015). As very resistant to this acid appears to be signal crayfish *(Pacifastacus leniusculus)*, the lethal concentration for adults was determined to be 96 h LC50 77.3 mg/l (Kouba et al. 2012).

The last tested substance was formaldehyde. Its use has caused the death of the entire colony of species *P. emarginata* at concentrations of 0.0063% and higher. At a concentration of 0.0008%, a small portion of zoids was immobilized but in a relatively short time even these zoids did regenerate. The difference between the first and second control was not statistically significant ($p = 0.18$). Zoids of *C. mucedo* were more resistant and were killed at concentrations of 0.0125% and higher. At a concentration of 0.0063%, there has been no effect. The lethal dose of formaldehyde in our case for *P. emarginata* was 0.69 mg/l and for *C. mucedo*, it was 1.38 mg/l. For fish and other invertebrates, these values are not lethal for a short-term exposure. The toxicity of formaldehyde for rainbow trout *(O. mykiss)* is given as 96 h LC50 24 mg/l (Safety Data Sheet, Penta 2004). Formaldehyde, as the only tested substance, killed bryozoan colonies at lower concentrations than the lethal concentration for salmonids. However, if salmonid fish are exposed to high concentrations or prolonged action of formaldehyde, it may cause a decrease in density of mucous cells (Buchmann et al. 2004) or changes in heart activity (Tkachenko et al. 2013). For species *Daphnia magna*, the value given is 48 h EC50 42 mg/l (Safety Data Sheet, Penta 2004). Another important issue is effect of formaldehyde on bacterial community of the biofilter. This substance apparently has no negative effect on the ammonia-oxidizing bacteria even at a concentration of 90 mg/l. However, there was a significant interaction of nitrifying bacteria at concentrations higher than 40 mg/l (Keck and Blanc 2002). Another study has verified the value of 120h EC50 34.1 mg/l for a mixed bacterial culture (Tisler and Zagorc-Koncan 1997).

The results indicate that the *C. mucedo* colonies are more resistant to tested agents. Given that for employees in aquaculture it may be difficult to distinguish among the different bryozoan species, higher values needed to kill the more resistant species *C. mucedo* were taken into account. The cheapest option at CZK 30/m^3 turned out to be the use of Savo, however, concentrations necessary to kill bryozoans is lethal for fish, as well. Therefore this measure can be recommended for facilities, where the fish are not present during the application. The most expensive variant at CZK 37.4/m^3 was formaldehyde that killed bryozoans at concentrations of 0.0125%. This concentration is below the lethal concentration for fish. Therefore, the use of this substance is recommended for facilities with fish. Given the fact that the price differences for small amounts of water may seem minimal at first sight and therefore misleading, the cost of elimination of bryozoans were recalculated for the model facility with a volume of 1000 m^3. For a facility of this size, the resulting cost of one-time disinfection is on the order of tens of thousands of CZK (Table 1).

Table 1 The cost of eliminating bryozoans in a model system with a volume of 1000 m^3 and necessary volumes of agents per 1 m^3

		Savo Original (conc.)	**Persteril** (36%)	**Formadehyd** (37%)
P. emarginata	costs per 1000 m^3 (CZK)	7 500	17 700	18 700
	volume of agent on 1m^3 (ml)	250	86,3	170
C. mucedo	costs per 1000 m^3 (CZK)	30 000	35 400	37 400
	volume of agent on 1m^3 (ml)	1 000	172,5	340

CONCLUSION

To test the elimination of bryozoans, several concentrations of substances commonly used in aquacultural practice for cleaning and disinfection of devices, such as Savo, Persteril, and formaldehyde were used. The results indicate that colonies of *C. mucedo* taken from open waters were significantly more resistant than the colonies of *P. emarginata* derived from the model recirculation device. For elimination of the *C. mucedo* bryozoan, a double concentration of Persteril and formaldehyde had to be used. For Savo, the concentration needed was even four times stronger than for the elimination of species *P. emarginata.* Formaldehyde, as the only tested substance, killed off bryozoan colonies at lower concentrations than the lethal concentration for salmonids. For the complete elimination of bryozoans, formaldehyde comes out as the most expensive option. Savo and Persteril for bryozoans were lethal at concentrations that are lethal for salmonids, as well. The use of Savo was evaluated as the cheapest option, but it may only be used for disinfection of the systems temporarily without fish.

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ASSESSMENT OF NITROGEN IONS IN WATER - STABILITY OF THE RESULTANT VALUES

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Abstract: The basic parameters for the evaluation of managed aquacultural fish ponds include determining their chemical and biological composition. As the main indicators of water quality, we determine water temperature, pH, conductivity, dissolved oxygen, as well as compounds of phosphorus and nitrogen. The assessment of nitrogen compounds in aquacultural fish ponds is a daily routine. The experiment dealt with assessment of ions of nitrite, nitrate and ammonia nitrogen in samples of natural waters and in standard solutions. The aim of the study was to evaluate the effect of preservation of reagents used to determine nitrogen ions on the resultant values. Selected assessments of nitrogen ions were carried out between June 2014 and April 2015. Our results show that the preservation of reagents at a lower temperature significantly prolongs the shelf life for the assessment of ammonium ions. For the assessment of nitrite or nitrate nitrogen, this preservation is of no practical importance.

Key Words: nitrogen, chemical reagents, cold conservation, time

INTRODUCTION

Water is an indispensable part of human life and is an irreplaceable ingredient in the most varied fields of human activity. Therefore we require an accurate knowledge of its chemical composition (Adam 1977). Properly conducted analysis of water thus became the first condition for determining whether water is or is not suitable for a particular purpose. Analysis of water creates baseline data for the design of technology for water treatment. Without proper water analysis, it is not clearly possible to identify the originator of the deterioration of water quality and one cannot even correctly specify the path to remedy. Water analysis therefore constitutes an indispensable basis for decision-making at all levels and for all those who deal with water (Horáková et al. 1986).

Chemical analysis of water is a separate and demanding scientific discipline. Some components involved in the water content are often present only in very small quantities. Therefore it is necessary to treat water analysis with the utmost importance for an accurate execution. Due to the large variability in content of individual components, it is necessary that the analytical chemist is completely at ease with all analytical methodology, from classical methods to the state of the art methods (Adam 1977).

Nitrogen is one of the most important biogenic elements. Nitrogen compounds are of particular importance because they apply to all biological processes occurring in surface, underground, and waste waters. They also play a role in biological wastewater treatment processes, the treatment of surface water, and are important criteria in water quality (Heteša and Kočková 1998, Ahuja 2013).

Nitrogen in waters occurs in different oxidation states (-III, -I, 0, +I, +III, and +V), either in ionic or nonionic form (Žáček 1998). Its main forms that occur in waters include elemental nitrogen, inorganically bound nitrogen in ammonia, nitrites, nitrates, cyanides, cyanates, thiocyanates, hydroxylamine, and nitrous oxide, as well as organically bound nitrogen. These forms in nature are subject to various changes (Grünwald 1993).

Ammonia nitrogen is the primary product of decomposition of most organic nitrogenous substances of plant or animal origin. Anthropogenic source of ammonia nitrogen of organic origin in water are primarily domestic waste water, waste from agriculture, and sludge water from sewage (Pitter 2009).

Dissolution of ammonia in water produces $NH_3.H_2O$ hydrate, which dissociates into ions of NH_4^+ and OH^-. Ammonia is the main metabolite of fish, zooplankton, and other aquatic organisms, so it is mainly found in intensive fish farming, in recirculation systems, and in ponds (Valentová et al. 2013).

Ammonia nitrogen in water increases the corrosion of copper and its alloys and is toxic to fish. Toxicity is however greatly dependent on pH and temperature of water. The NH_4^+ ion does not have a toxic effect, but the undissociated molecule NH_3 does, as it more easily penetrates cell membranes. Undissociated ammonia has also a toxic effect on zooplankton (Pitter 2009).

Nitrites in water originate mostly through biochemical oxidation of ammonia nitrogen, or the so-called nitrification. They occur to a greater extent in waters with intensive fish farming that use biofilters. They may also arise via disinfecting water with UV radiation, which leads to reduction of nitrates.

Nitrites have a toxic effect on fish and may even cause mass death of fish. But toxicity depends greatly on the overall composition of water. It is assumed that undissociated molecule HNO_2 and not the NO_2^- ion is the main toxic substance. Nitrites penetrate the gill epithelium into the blood to form methaemoglobin, which no longer has the ability to carry oxygen. The value of the permissible concentration of nitrite nitrogen depends on the ratio of concentrations of chlorides and nitrites, and is on the order of hundredths of mg/l (Svobodová 1987).

Nitrates are mainly secondary products of nitrification of ammonium nitrogen and are the final products of decomposition of nitrogenous organic compounds in oxic environments. A major source of nitrates in the water is also fertilizing of farmland with nitrogen fertilizers. Nitrates themselves are less harmful, but may cause indirect harm. For example, they may reduce, through bacterial activity in the gastrointestinal tract, to more toxic nitrites. Nitrites then react with haemoglobin to methaemoglobin, which does not have the ability to transport oxygen in blood. If nitrates are not reduced to nitrites, they get excreted relatively rapidly via urine (Pitter 2009). For fish, nitrates are only very slightly toxic. Maximum permissible concentration of NO_3^- for carp is given as 80 mg/1, for rainbow trout it is 20 mg/l. Only at concentrations of NO_3^- above 1,000 mg/l, toxic and lethal effects occur (Svobodová 1987).

MATERIAL AND METHODS

In June 2014, we have prepared the following reagents:

Reagent 1: Colouring reagent for the assessment of ammonium ions.

Reagent 2: Sodium dichloroisocyanurate for the assessment of ammonium ions.

Reagent 3: 2,6-dimethylphenol for the assessment of nitrate nitrogen.

Reagent 4: Sulfanilic acid for assessment of nitrite nitrogen.

Reagent 5: Coupling solution for the assessment of nitrite nitrogen.

We have prepared the colouring reagent (1) by weighing 13 g of sodium salicylate in a 100-ml volumetric flask. Then we have added 13 g of dihydrate of trisodium citrate and 0.1 g of sodium nitroprusside and after dissolving, we have added distilled water to volume of 100 ml. We have prepared the solution of sodium dichloroisocyanurate (reagent 2) by dissolving 32 g of sodium hydroxide in about 500 ml of distilled water. After cooling, we have added 2 g of dihydrate of sodium dichloroisocyanurate and after dissolution added to volume of 1,000 ml.

We have prepared the solution of 2,6-dimethylphenol (reagent 3) by dissolving 0.12 g of 2,6-dimethylphenol in 100 ml of glacial acetic acid.

A solution of sulfanilic acid (reagent 4) was prepared by dissolving 3.46 g of sulfanilic acid and 27.2 g of potassium bisulphate in 1,000 ml of distilled water. Coupling solution (reagent 5) was prepared by dissolving 0.040 g of N-(l-naphthyl)-ethylenediamine dihydrochloride in 100 ml of distilled water.

Each reagent was divided into 14 50-ml plastic containers with screw caps. Subsequently, we have placed seven containers of each reagent in the refrigerator set to a standard temperature of 4 °C and seven containers in the freezer with standard temperature of -18 °C. Then we have prepared all listed reagents again and kept them in glass bottles in chemical storage room at a constant temperature of 23 °C.

Assessment of nitrogen ions

From July 2014 to January 2015, each month (seven times), we have determined the ions of ammonia, nitrate, and nitrite nitrogen in a chemical laboratory at the Institute of Zoology, Fisheries, Hydrobiology, and Apiculture. We have carried out the assessment of nitrogen ions on samples of natural water and on prepared standard solutions.

Samples of natural water came from different sources and each water sample had different concentrations of the tracked substances, while standard solutions were always prepared anew. From the standard solution for the assessment of ammonium ions (3.819 g of ammonium chloride NH_4Cl, dried at 105 °C and dissolved in 1 litre of distilled water) we have prepared a working solution with a concentration of 1 mg $N\text{-}NH_4$ per litre by mixing 1 ml of ammonium chloride with 1000 ml of distilled water. From the standard solution for the assessment of nitrate nitrogen (0.7216 g of potassium nitrate KNO_3 dried at 105 °C and dissolved in 1 litre of distilled water) we have prepared a working solution for the assessment of nitrate nitrogen by mixing 20 ml of potassium nitrate with 500 ml of distilled water at a concentration of 4 mg $N\text{-}NO_3$ per litre. For the assessment of nitrite nitrogen we have prepared a working solution by mixing 1 ml of standard solution of sodium nitrite (0.4926 g of sodium nitrite $NaNO_2$ dried at 105 °C and dissolved in 1 litre of distilled water) with 1,000 ml of distilled water. This solution had a concentration of 0.1 mg of $N\text{-}NO_2$ per litre.

We have carried out each assessment of nitrogen ions in five repetitions. Then we have excluded the highest and lowest values, and from the three remaining results calculated the average. First, we have carried out the assessment of nitrogen ions in individual water samples and in solutions of the standard by using freshly mixed reagents. After that, we have used reagents stored at room temperature of 23 °C. Next, we have used reagents stored in a refrigerator at standard temperature of 4 °C. And finally, we have used reagents stored in freezer at standard temperature of -18 °C. Prior to use, the preserved reagents were allowed to warm up to room temperature.

Assessment of ammonium ions ($N\text{-}NH_4^+$)

Ammonium ions were determined in water samples on the principle of reaction with sodium salicylate and hypochlorite ions in the environment of sodium nitroprusside to give a blue colour.

Using a pipette, we have put into each test tube 10 ml of water sample (or standard solution), 0.5 ml of colouring reagent (reagent 1), and 0.5 ml of dichloroisocyanurate solution (reagent 2). Then we have mixed the samples and let them stand at room temperature for 30 minutes. Subsequently, we have measured the resulting blue colour on the PhotoLab 6600 UV-VIS Series device. Before loading them into the machine, we have cleaned each test tube properly and gently mixed the content by flipping.

Assessment of nitrate nitrogen ($N\text{-}NO_3^-$)

We have performed the assessment of nitrates present in the water sample on the principle of reaction with 2,6-dimethylphenol in the environment of a mixture of concentrated acids to produce a brick-red coloured 4-nitro-2,6-dimethylphenol.

For each assessment, we have prepared new mixed reagents. Then we have used reagents from storage (23 °C), from refrigerator (4 °C) and finally the reagents from the freezer (-18 °C). Only the reagent of mixture of acids, namely sulphuric, phosphoric, and sulphamic acids, we have used those already prepared and stored in the chemical warehouse. This reagent has an unlimited shelf life.

Then using a pipette, we have added into the photometric test tube 3.5 ml of mixture of acids. To this, we have added 0.5 ml of a water sample (or standard solution) and gently stirred the mixture. Then we have added 0.5 ml of 2,6-dimethylphenol (reagent 3), closed the tube with a screw cap, and thoroughly mixed. After about 15 minutes, we have measured the absorbance on the PhotoLab 6600 UV-VIS Series device. Before inserting into the machine, each test tube was again thoroughly cleaned and carefully mixed by flipping.

Assessment of nitrite nitrogen ($N\text{-}NO_2^-$)

We have determined nitrites on the principle of diazotizing sulfanilic acid by present nitrites and coupling the diazonium salt with N-(l-naphthyl)-ethylenediamine dihydrochloride to form a red azo dye.

Using a pipette, to each tube we have added 10 ml of water sample (or standard) and 0.5 ml of sulfanilic acid (reagent 4) and 0.5 ml coupling solution (reagent 5). We have closed all test tubes with

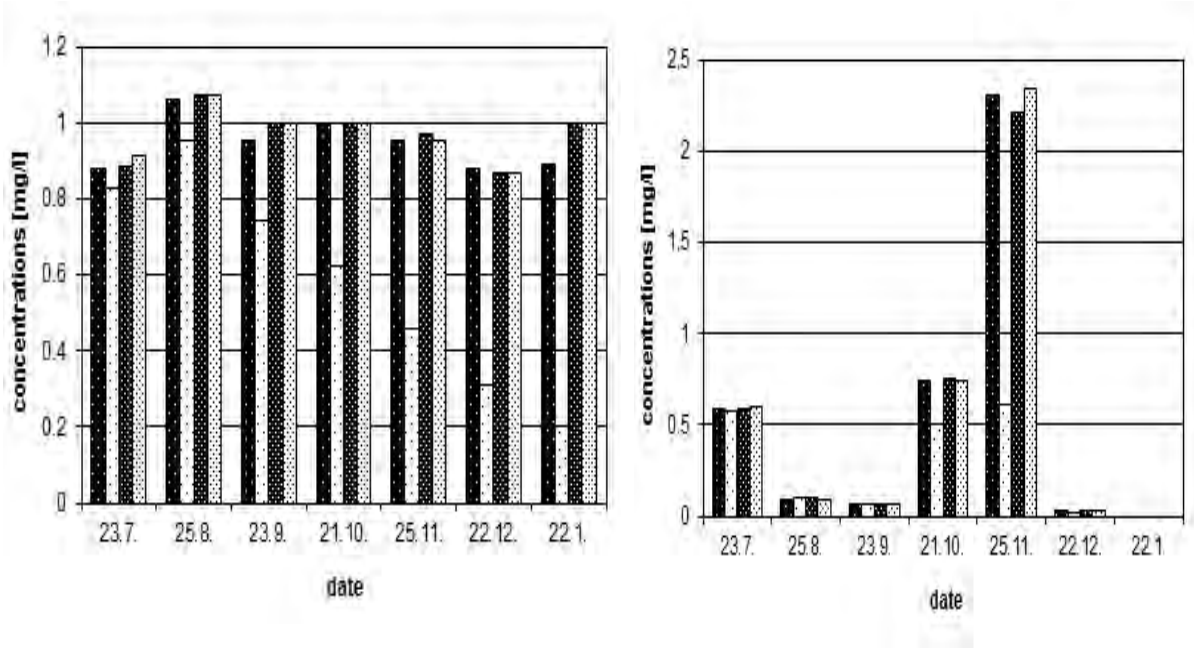
screw caps and properly mixed their content. Then we let the tubes stand for 20 minutes at room temperature. Next we have measured the absorbance on the PhotoLab 6600 UV-VIS Series device. Before loading into the machine, we have cleaned each test tube properly and gently mixed the content by flipping.

RESULTS AND DISCUSSION

Figure 1 Changes in the measured values of the standard N-NH_4 (A) and natural water (B) at different conditions of reagent storage

A) Concentrations of N-NH_4^+ in solution of standard

B) Concentrations of N-NH_4^+ in water samples



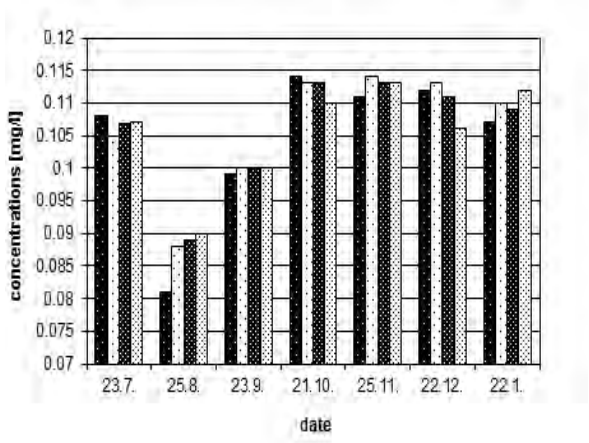
Legend: ■fresh □storage(23 °C) ■refrigerator (4 °C) ▣freezer (-18 °C)

Figure 1 suggests that for the assessment of ammonium ions using the reagents stored in the storage room at 23 °C, the measured values from the first measurement on are lower than the values determined by other reagents. At low concentrations of ammonia nitrogen in the sample, there is no noticeable difference, but at higher concentrations, there is a distinct difference in results based on the mode of preservation of reagent. After three months, the reagent stored at 23 °C shows the measurement results to be one fourth lower than with other reagents, and after five months, this difference increases by up to three quarters compared to other reagents. Stability of reagents for the assessment of ammonium ions, that is colouring reagent and the sodium dichloroisocyanurate solution, is given as at least two weeks (Horáková et al. 2007).

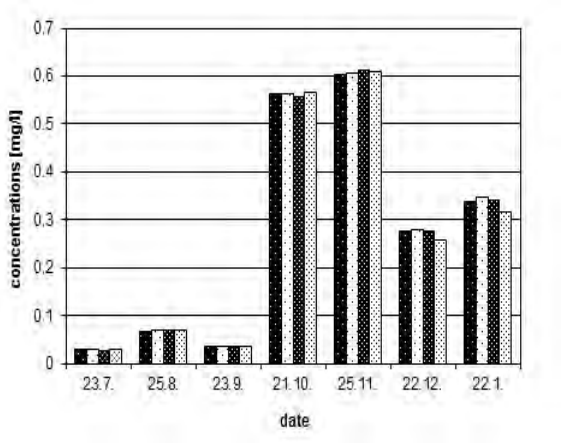
Our experiment suggests, however, that preservation has a great influence on stability of the reagents. If we keep reagents in a cold place, such as refrigerator (4 °C) or freezer (-18 °C), we can extend their applicability. The measured results from the assessments with the use of fresh reagent were almost identical with the measured results when using reagents from the refrigerator (4 °C) and the freezer (-18 °C) for a period of six months.

Figure 2 Changes in measured values of N-NO_2^- standard (A) and natural water (B) at various storage conditions of reagents

A) Concentrations of N-NO_2^- in solution of standard



B) Concentrations of N-NO_2^- in water samples

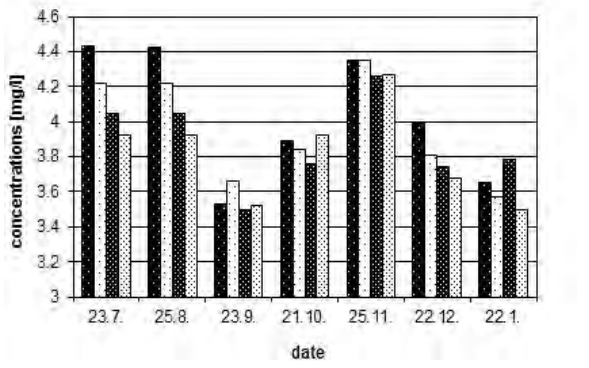


Legend: ■fresh □storage(23 °C) ■refrigerator (4 °C) ▣freezer (-18 °C)

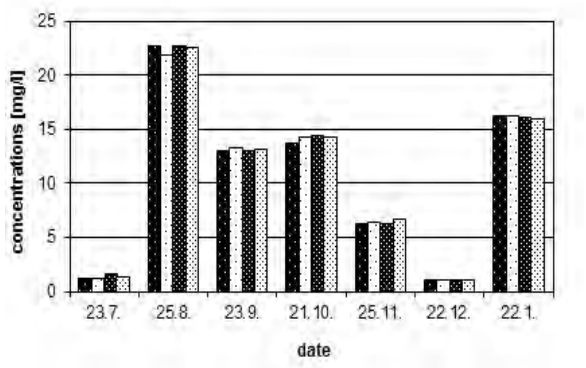
As we can see in Figure 2, when the first assessment of nitrite nitrogen took place on July 23, 2014, the measured values were nearly identical, which means that the mode of preservation of reagent did not have any greater influence on the result of the assessment. In assessments of nitrite nitrogen in the following months, the measured values were relatively balanced and varied only within the error context of the used photometric method. Based on our results, we can assume that the impact of preservation of reagents does not have much influence on the outcome. Reagents for the assessment of nitrite, such as the coupling solution and the sulfanilic acid solution, according to the literature data, are stable for about one month (Horáková et al. 2007). Our results indicate that the usability of reagents while maintaining the relevance of the test results is considerably longer and that reagents do not need to be stored at lower temperatures.

Figure 3 Changes in measured values of the N-NO_3^- standard (A) and natural water (B) at various storage conditions of reagents

A) Concentrations of N-NO_3^- in solution of standard



B) Concentrations of N-NO_3^- in water samples



Legend: ■fresh □storage(23 °C) ■refrigerator (4 °C) ▣freezer (-18 °C)

For the assessment of nitrate nitrogen in the standard solution, the most commonly measured lowest values in the assessment, were reagents stored in the freezer (-18 °C) or refrigerator (4 °C) and the maximum values were most often measured using fresh reagent, or reagents stored in the warehouse, as shown in Figure 3 A). The measured values of $N\text{-}NO_3^-$ assessment in water samples are relatively balanced, as seen in Figure 3 B). The cited usefulness for a solution of 2,6-dimethylphenol is at least one week (Horáková et al. 2007). Our results show that the method of storing reagents for the assessment of nitrate nitrogen does not affect the result of the assessment. The usefulness of 2,6-dimethylphenol is even under standard temperature conditions not more than one month.

CONCLUSION

Stability of reagents used for the assessment of ammonium ions in water, namely the colouring reagent and a solution of sodium dichloroisocyanurate is, according to our experiment, not more than one month. Our findings correspond with the published data, which indicate the minimum durability of these reagents to be at least two weeks. However, their usefulness can be greatly affected by cold storage since reagents from the refrigerator (4 °C) and the freezer (-18 °C) show a minimum difference in their results compared to the newly mixed reagents, for a period of six months.

Reagents for the assessment of nitrites, namely the coupling solution and the solution of sulfanilic acid, are stable according to our experiment for six months. Literature states the stability of the reagents to be about one month. In contrast to reagents used for the assessment of ammonium ions, the effect of preservation of reagents used to determine the nitrite nitrogen does not influence much the measurement result. The preservation of reagents neither plays any important role in the assessment of nitrate nitrogen. Regents used to determine the nitrate nitrogen is a solution of 2,6-dimethylphenol and a mixture of acids. A solution of a mixture of acids is stable indefinitely, but a solution of 2,6-dimethylphenol is stable according to literature for at least one week, and in our experiment for maximum of one month.

ACKNOWLEDGEMENTS

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DOES RECREATIONAL FISHERIES CONTRIBUTE TO SPREADING OF PUMPKINSEED (*LEPOMIS GIBBOSUS*, L.) IN THE CZECH REPUBLIC?

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Abstract: We attempted to answer the question whether the pumpkinseed (*Lepomis gibbosus*, L.) is used by anglers as a baitfish. We search the angling webforum and analysed relevant posts of the anglers. We found that anglers use pumpkinseed as bait but discussions reflect the awareness of potentially invasive species.

Key Words: anglers, baitfish, recreational fisheries, invasive, internet

INTRODUCTION

The introduction and spread of invasive species is considered as one of the major drivers in biodiversity loss worldwide (Lodge 1993, Strayer 2012). High rates of introductions, which occur in freshwater ecosystems, are caused by both intentional and accidental releases (Copp et al. 2005). Fishery enhancement practices and escapes from aquaculture facilities are responsible for the majority of non-native fish introductions (Villéger et al. 2011) but the release of unwanted ornamental and bait organisms into the wild by hobby keepers and anglers plays important role as well (Kalous et al. 2013, Patoka et al. 2014). When introduced non-native organism become established and spread, the complete eradication is costly and difficult to achieve (Gherardi and Angiolini 2004). The prevention of invasion is therefore of high importance and the proper management of potentially invasive species as well as potential vectors must be taken in consideration. This means that the knowledge of the dispersal mechanisms of particular species is of essential importance (Crooks et Soulé 1999, Bohonak et Jenkins 2003).

In the Czech Republic is registered 42 non-native fish introductions, from which 20 fishes is still present in the wild and 14 species became established (Musil et al. 2010).

One of the introduced species with invasive potential is pumpkinseed (*Lepomis gibbosus*, L.) from the North America. It is a small-bodied omnivorous fish from the family Centrarchidae that was introduced into Europe in the late 19th century for ornamental as well as for recreational fishing purposes (Copp et al. 2002). It inhabits both lentic and lotic environments in majority of European countries reaching as far as to Norway (Naspleda et al. 2012). It is considered invasive at some southern and central European locations (Copp et Fox 2007).

On the territory of the Czech Republic pumpkinseed was accidentally introduced in 1929 with fry of common carp (*Cyprinus carpio* L.) from Yugoslavia (Volf 1929, Šanda 2006). Since that time it has spread to new localities (Lohniský 1973, Peňáz and Jurajda 1995, Lohniský and Lusk 1998, Hanel and Lusk 2005).

In the present study we focused on potential of recreational fisheries as a vector of uncontrolled expansion of *Lepomis gibbosus*.

MATERIAL AND METHODS

We made a web search on the most visited Czech angling forum (http://www.mrk.cz, online since 1996, 78355 registered members). The website includes 75901 photos; 39899 questions in forums; 2648 article topics. The web page was accessed 10th August, 2016. We performed full text

search by using the common name of *Lepomis gibbosus* in Czech (slunečnice) as the keyword. By using Google Chrome searching tool we analysed in detail all the forum posts, including discussions, photos, videos and articles. We exclude all items regarding sunflower that in Czech language share the same common name with *Lepomis gibbosus*. The criteria we set up for analyses were: 1) posts with negative reaction, 2) posts with positive reaction, 3) posts with information about transportation, 4) posts mentioned locality, 5) posts with no information.

We aim to answer the question if *Lepomis gibbosus* is used as bait fish or by any other way by anglers and if it is transported into new localities.

RESULTS AND DISCUSSION

In total, as a result of the search on the angling web forum, we obtained following records for the keyword "slunečnice":12 articles, 24 question in forum, 40 photos and 7 videos. After selection to avoid synonymous sunflower, we ended up with 9 articles, 39 photos; 7 videos and 10 questions in forum. There were several questions (5) posted that asked if the pumpkinseed could be used as bait. If we skip the unimportant answers/comments (140), the majority of informative answers (21 from 29) were consistent with the following statement "do not use the fish as bait due to its non-nativeness and invasive potential". Although the usage of bait fish seems to be legislatively complicated (Kalous et al. 2013), the approach of majority of anglers regarding non-native pumpkinseed is in agreement with the environmental policy (Hickley and Chare 2004). We found also posts that confirmed the use of *Lepomis gibbosus* as bait and even confirm the transport of the fish from pond near Kardašova Řečice to the Nežárka River in South Bohemia.

From posts on the webforum, it seems that transportation of pumpkinseed can be attributed to fish hobby keepers and garden pond owners as it is the case of ornamental crayfish (Patoka et al. 2014). Some questions (5) ask directly for the source of the fish due to stocking of garden ponds. It is not surprising since the fish has very decorative appearance and it is already a object of the pet trade (Papavlasopoulou et al. 2013). Interestingly the majority of answers for these questions were positive (29 of 31) offering the information where to obtain fish for translocation.

In general angling web forums are very informative in regards of fish species occurrence, places of capture, photos etc. It is also highly advisable to use information and reports posted on the angling forums for planning field sampling and especially for monitoring of invasive or potentially invasive species (Banha et al. 2015). Recreational fisheries can serve as an important source of information about the vast area of rivers, small streams and other waterbodies (Roy et al. 2015). On the other hand awareness of the public in regards of invasive species is the crucial step for control and eradication (Gherardi and Angiolini 2004). We think that the spreading of pumpkinseed in the Czech Republic is under better control then other non-native fishes. This could be due to its attractive coloration unusual in the waters of the Czech Republic.

CONCLUSION

We noted records on the web forum, which explicitly confirm the usage of pumpkinseed as a bait fish by anglers. Anyway, we reflect also the awareness of potential invasiveness of this fish by other users in the angling web forum discussions. We conclude that *Lepomis gibbosus* is relatively well known as a pest and in this regard self-regulation towards to good practices in angler's behaviour could be expected. On the other hand translocation from wild to garden ponds and vice versa is likely.

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TOXIC EFFECT OF FLUORESCENCE PIGMENT ON ZEBRA FISH (*DANIO RERIO*)

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Abstract: The aim of the study was to determine the toxic effects of organic pigments Alizarin Red S and Alizarin Complexone on zebra fish (*Danio rerio*). For short-term acute toxicity tests on zebra fish concentration of 150; 300 and 600 mg/L were chosen for both dyes. Toxic effect of dyes was observed even in the variant with 10 g/L of sodium chloride. Addition of sodium chloride increases the deposition of dyes in the bone structures of the fish. LC50 values were analyzed graphically by using probit analysis. There was no mortality during the acute toxicity test with Alizarin Red S even at the highest concentration. Toxicity value (72hLC50) for zebra fish with a combination of Alizarin Red S + 10 g/L of sodium chloride is 546.42 mg/L. Mortality for Alizarin Complexone was 100% in 24 hours at concentrations of 300 and 600 mg/L, with concentrations of 150 mg/L there was no mortality. In Alizarin Complexone supplemented with 10 g/L of sodium chloride was 100% mortality at all concentrations up to 72 hours.

Key Words: Alizarin Red S, Alizarin Complexone, LC50, fish, marking

INTRODUCTION

Toxicity tests are used to detect or estimate the potential toxic effects of tested compounds on living organisms (Kočí 2006). Using of model fish species Zebra fish (Danio rerio) is recommended for testing of chemicals in toxicology. It is possible to use other types of freshwater, marine or brackish fish, provided that the appropriate adjustments are made, for example the quality of the dilution water and temperature conditions during test (ÚNMZ 1999).

Marking of fish (individual, group) is a normal part of scientific work or breeding handling (Rodina and Flajšhans 2008). In order to recognize in the water tanks fish from natural spawning from fish planted, marking of planted fish should be carried. When choosing a method of marking it is necessary to take into account a variety of circumstances and requirements. Especially the number of marked fish, durability of marking, laboriousness of application, reading of marks, the possibility to automate these activities, the price and availability of marks and ultimately the rate or risk of damage to fish during application, mark reading and handling with fish (Rodina and Flajšhans 2008). Fish in the early stage are not easy to mark using an external mark (Baer and Rösch 2009). Additionally, handling with fish during the marking can cause large losses due to stress. One way of marking a group of fish is the use of fluorescent dyes that can produce detectable marks in otoliths and other skeletal structures (Liu et al. 2009). Marking using fluorescent dyes has many advantages, marked may be a large number of individuals in a short time at low cost, marking lasts for several months to years, the dye can be used on fish of all ages (from larvae to adult), the labeling process is quite simple and there is no excessive stressing of fish. Dyes Alizarin Red S and Alizarin Complexone demonstrated good efficiency of fluorescent pigment marking in bone structures with minimal negative effect on the survival of marked fish (Lü et al. 2015). The best results were obtained during an experiment on the embryo whitefish (*Coregonus lavaretus*) 28 days after fertilization with a concentration of Alizarin Red S 1000 mg/L, and on rainbow trout (*Oncorhynchus mykiss*) with a concentration of Alizarin Complexone 10 mg/L (Eckman 2003, Walt and Faragher 2003).

MATERIAL AND METHODS

Characteristics of the tested substances

Alizarin Red S ($C_{14}H_7NaO_7S$) and Alizarin Complexone ($C_{19}H_{15}NO_8$) are fluorescent dyes which are used for marking of fish. Alizarin Red S was previously used for textile dyeing. Alizarin Red S forms a complex with calcium that is stored in the bones (Puchtler et al. 1968, Eckmann, 2003). Adding sodium chloride will increase the osmotic pressure, which improves the dye transport to the bone structures (Baer and Rösch 2009).

Alizarin Red S is a dark red powder. Alizarin Complexone is dark yellow powder with a boiling point of 190 °C. They have poor water solubility, better solubility in ethanol (Safety Data Sheet, Alizarin Red S Alizarin Complexone 2013).

Acute toxicity test on fish

Acute toxicity tests were performed on aquarium fish zebra fish (at the age of 4 months, the total body length of 20±5 mm). 7 days before testing fish were acclimatized to the medium, in which the test was performed. Dilution water was prepared according to methodology ČSN EN ISO 7346 1 (ÚNMZ, 1999). Fish were exposed to various concentrations of Alizarin Red S and Alizarin Complexone for 96 hours. Toxic effect of dye was evaluated even in the variant with 10 g/L of sodium chloride. Temperature during laboratory testing was constant (25°C) with controlled lighting mode – 13 hours light, 11 hours dark. For all the tested substances were selected concentrations of 150; 300 and 600 mg/L. Tested concentrations were chosen on the basis of other authors, who tested the same concentration of dyes on other fish species. Tests were carried out in glass tanks with 3000 ml of water without aeration with 10 fish per concentration and control group in triplicate. Fish were not fed during the test. Mortality of the fish was determined every 24 hours during the test. Temperature, pH and dissolved oxygen content in water were measured using device HACH HQ40d and conductivity using device Hanna combo.

Data from acute toxicity test on fish were processed using probit analysis to calculate 72h LC50.

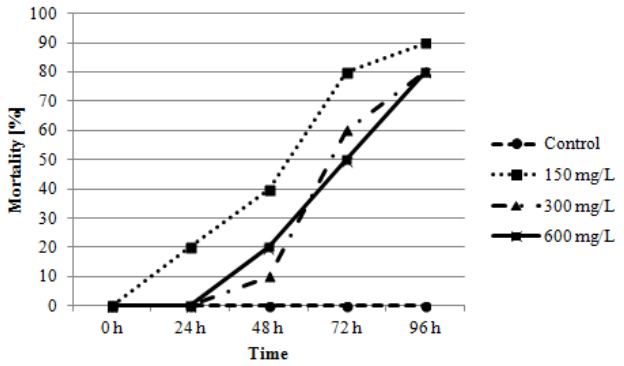
RESULTS AND DISCUSSION

Values of hydrochemical parameters are presented in the Table 1. During the acute toxicity test with Alizarin Red S and Alizarin Complexone the temperature and conductivity throughout were steady without significant fluctuations. The pH in all tested tanks ranged from 6.00 to 8.22. The value of the percentage saturation of oxygen in the test solutions during the whole test did not fall below 60% of saturation. Decrease of oxygen saturation under 60% was monitored only in the concentration of 600 mg/L during the test with Alizarin Complexone + 10 g/L NaCl. The decrease in oxygen saturation occurred at 48 h. The high value of the percentage saturation of oxygen (129.8 and 126.4%) was at the beginning of testing, which was caused by aeration of dilution water 24 hours before testing. Differences in pH among the aquaria were probably caused by different concentrations of fluorescent colors. There were no pH differences in control group (pH 7.86 to 8.09). Oxygen saturation in control group did not felt under 60% (84.6 to 99.3 %) for whole duration of the test. Temperature and conductivity did not fluctuated too.

Table 1 Values of physical and chemical parameters during the acute toxicity tests (min.–max.).

Parameters	Units	Control	Alizarin Red S	Alizarin Red S 10 g/L NaCl	Alizarin Complexone	Alizarin Complexone 10 g/L NaCl
Oxygen saturation	%	84.6–99.3	73.1–99.6	71.3–129.8	80.9–100.3	59.4–126.4
Values of pH		7.86–8.09	7.49–7.91	7.19–8.22	6.97–7.86	6.08–7.68
Temperature of water	°C	25	25	25	25	25
Conductivity	mS/m	49.2–54.9	49.8–56.6	>400.0	49.9–57.6	>400.0

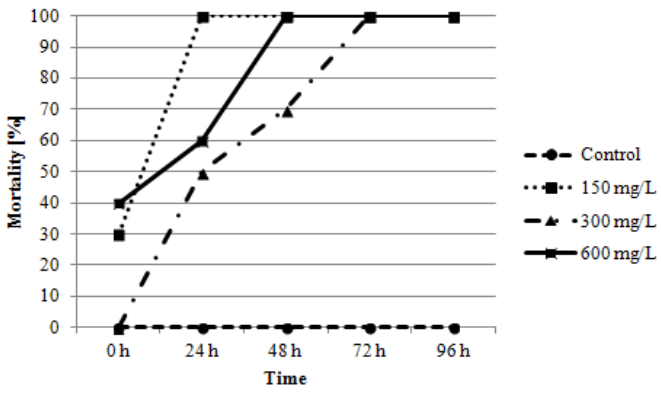
Figure 1 Mortality of zebra fish in certain concentrations of Alizarin Red S + 10 g/L NaCl during the acute toxicity test.



For Alizarin Red S mortality did not occur at any tested concentration. The process of mortality in different concentrations Alizarin Red S in combination with 10g/L is shown in Figure 1. Median lethal concentration at 72 hours (72hLC50) was calculated by probit analysis. Toxicity value (72hLC50) for zebra fish with a combination of Alizarin Red S + 10 g/L of sodium chloride is 546.42 mg/L. The highest mortality at a concentration of 150 mg/L and 10g/L was caused most likely by NaCl. The NaCl is probably fixed into the complex by fluorescent color which is the cause of lower mortality at higher concentrations of color. More tests will be conducted to support this hypothesis in a future. LC50 is calculated only from three values so it is just approximate. More tests with larger scale of concentrations will be conducted to specify the LC50 value more precisely.

Mortality was 100% at concentrations of 300 and 600 mg/L for Alizarin Complexone in 24 hours. At the concentration of 150 mg/L, there was no mortality. Mortality in all tested concentrations was 100% for Alizarin Complexone + 10g/L NaCl in 72 hours (see Figure 2). Mortality was monitored at the concentrations of 150 and 600 mg/L in a few minutes after test begun. This is the reason why the curves start at 30 and 40% in Figure 2. There was no mortality in the control group during the test.

Figure 2 Mortality of zebra fish in certain concentrations of Alizarin Complexone + 10 g/L NaCl during the acute toxicity test.



Baer and Rösch (2008) states, that the best dyeing was obtained using a concentration of 300 mg/L of Alizarin Red S. However, the concentration caused the death of 95% of the tested brown trout (*Salmo trutta*). Fluorescent marking with good quality and low mortality of brown trout, which did not differ from the control group (0 mg/L), was observed at concentrations of 150 mg/L of Alizarin Red S with the addition of 10 g/L of sodium chloride. Dying of brown trout with Alizarin Red S was carried out at a water temperature of 12 °C for 3 hours. The initial pH value was 7.6. In concentration of 50 and 150 mg/L of Alizarin Red S the pH was relatively stable (7.6 and 7.7). At a concentration of 300 mg/L of Alizarin Red S pH decreased rapidly. For this reason1 mg/L of NaOH was added to the highest concentration to maintain the pH constant. Throughout the test the fish were fed by commercial feed (57% protein, 17% fat). During the test with Alizarin Complexone supplemented with 10 g/L of sodium

chloride Baer and Rösch (2008) indicates, that the environment temperature was 8 °C. After addition of the dye there was an immediate drop in pH to 5.8. By adding 20 mg/L of NaOH they were able to increase the pH to 6.8. After stabilization of the pH they added brown trout to the tested tank. During the test the tanks were aerated. Liu et al (2009) tested the Alizarin Red S concentrations from 200 to 400 mg/L and Alizarin Complexone concentration from 50 to 300 mg/L on juvenile (20–30 mm) of japanese flounder (*Paralichthys olivaceus*). Deaths were avoid except for one fish at a concentration of 300 mg/L of Alizarin Red S, and for one piece with the concentration of 300 mg/L of Alizarin Complexone.

Environmental conditions were the same in both tests. Testing was carried out in seawater at a temperature of 20±5 °C, water was heavily aerated to increase pH, which was maintained in the range of 7.23 to 7.86. Eckman (2008) carried out testing for 15 minutes in a solution of a fluorescent dye on the fertilized roe of whitefish (*Coregonus lavaretus*). The experiment was performed in the recirculation water, where 5% of the total of 250 l of water was exchanged every other day. The water temperature was 4.5 °C and increased to 12 °C during 3 days. In the initial experiment with dye Alizarin Red S in a concentration of 400 mg/L were immersed embryos 5 days after fertilization. In this test dying of otoliths was avoided. Marking quality was increased on older embryos (38 days after fertilization) at the time of immersion. During immersion of embryos (28 days from fertilization) into a concentration of 1000 mg/L of Alizarin Red S the mortality was 35%, which was considered tolerated dose for multiple marking of whitefish. Marking was visible for 8 months. In our experiments with Alizarin Red S there was observed no mortality in 96 hours in any concentrations tested. Mortality was recorded in combination Alizarin Red S with 10 g/L of sodium chloride. When testing Alizarin Complexone there were no mortalities only at concentration of 150 mg/L, from concentration of 300 mg/L mortality was 100% within 24 hours. When testing Alizarin Complexone supplemented with 10 g/L of NaCl the mortality at 72 hours was 100%. Causes of different results of toxicity dyes effect on different fish species are likely to be caused by the different susceptibility of each species, different life stage of the experimental fish and different environmental conditions during testing.

CONCLUSION

Based on our results, marking of zebra fish by immersion in Alizarin Red S can be recommended as a simple and secure method. Dyeing using dye Alizarin Complexone is safe only to a concentration of 150 mg/L. Using fluorescent dyes in combination with the addition of 10 g/L of sodium chloride increased mortality on zebra fish. In comparison with the results of other authors who tested fluorescent dyes on other fish species (*Salmo trutta, Paralichthys olivaceus, Coregonus lavaretus*) the results are different in experiments with zebra fish, both when tested dye alone and in combination of dye and sodium chloride. Based on our results and the results of other authors is obvious different susceptibility of different species to the used dyes. In case of use of tested dyes for marking other species is therefore necessary to test susceptibility (toxicity) of given species.

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DYNAMIC OF THE PHYTOPLANKTON COMMUNITY IN EUTROPHIC FISHPONDS

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Abstract: Research was conducted in three fishponds, located in the area of the municipalities Šumice and Pohořelice in South Moravian region, in period from April to September 2015. Samples for phytoplankton analyses were collected twice a month, using plankton net and sampling bottles. At same time, by using portable field devices and Secchi disk, water temperature, dissolved oxygen, pH, conductivity and transparency were measured. A total of 153 taxa of the phytoplankton (27 taxa of cyanobacteria and 126 of algae) were documented in the studied ponds. The most abundant phytoplankton was in Šumický horní pond, in which big fluctuation in abundance was recorded over the study period. Cyanobacteria, over the entire year, were the most dominant group in this pond. Main representatives were from genera *Microcystis*. In Šumický dolní pond the most numerous were the green algae, with the genera *Desmodesmus, Pediastrum, Scenedesmus* and *Crucigenia tetrapedia,* which was the most abundant species in July and August. In Pohořelický pond, after a short period at the beginning of the season when low diversity and number of phytoplankton was documented, a dominance of green algae occurred, subsequently followed by the dominance of trichal cyanobacteria *Planktothrix agardhii, Cuspidothrix issatschenkoi* and *Aphanizomenon flos-aqua*. In all three ponds algae and cyanobacteria which correlated with the eutrophic and hypertrophic water systems were registered.

Key Words: cyanobacteria, chlorophyta, abundance, season, functional group

INTRODUCTION

Cultural eutrophication is considered to be one of the main causes of the deterioration of aquatic ecosystems. Perhaps the most obvious consequence of eutrophication is the increased growth of algae and aquatic weeds, which interfere with the use of water for fisheries, recreation purposes, industry, agriculture, and as drinking water (Carpenter et al. 1998).

The type of water ecosystems which are subject of our interest and the most common type of the stagnant water ecosystems in Czech Republic are fishponds. Fishponds are artificial water systems maintained by man, used primarily for fish breeding. In ponds, in addition to the external load of nutrients from the surrounding agriculture fields and inflows, inadequate water management can occasionally have an additional effect on water eutrophication. Fertilizers are very often overused in aquaculture to enhance the growth of primary producers, followed by the increase in biomass of the stocked fish. Consequently, fishpond water is often defined as eutrophic and hypertrophic, with the frequent emergence of water bloom.

Algal blooms in fishponds and other aquatic ecosystems have a number of consequences, e.g. limiting light penetration, the dominance of cyanobacteria, which are inadequate food source for the majority of zooplankton grazers (Tillmanns et al. 2008), hypoxia, caused by the decomposition of a great number of dead organisms over a short period. One of the biggest problems related with water blooms is the presence of potentially toxin-forming cyanobacteria (*Microcystis, Aphanizomenon, Planktothrix, Cuspidothrix*), with negative effects on humans and animals, including fish (Palíková et al. 2007).

Because of the above-mentioned, it is necessary to continuously monitor the phytoplankton in every water body type, including the fishponds.

MATERIAL AND METHODS

Studied localities

The studied area included three, intensively managed, fishponds: Šumický horní (18.34 ha), Šumický dolní (6.54 ha) and Pohořelický (5.60 ha) (Figure 1), located in a shallow valley along the flow of the Šumický potok stream between the municipalities of Pohořelice and Šumice (South Moravian region, the Czech Republic).

Figure 1 Satellite map of ponds and places of sampling

Sampling and analysis

Samples from the chosen localities were taken during the 2015, in the vegetative period from April to September, twice a month, with the exception of April, when the samples were taken only once.

Water temperature, dissolved oxygen and pH were measured *in situ* using portable device HACH HQ40d (Hach Lange, USA), conductivity using portable device Hanna Combo HI98130 (Hanna Instruments, USA) and transparency using the Sechhi disk. Cyanobacterial and algal biomass were evaluated by chlorophyll a concentrations, measured using a heated ethanol extraction (Lorenzen 1967).

Samples for the qualitative analysis of the phytoplankton were collected using the 20 μm mesh planktonic net and putted in 50 ml plastic bottles. Live samples of the phytoplankton were analysed in the first 24 hours from sampling using optical microscope Olympus BX51 and keys for the determination of algae and cyanobacteria. The determined taxa were divided into four groups: cyanobacteria (Cyanobacteria), green algae (Chlorophyta), diatoms (Bacillariophyceae) and group of other algae, which included Dinophyta, Cryptophyta, Chrysophyceae, Xantophyceae and Euglenophyta.

Samples for the quantitative analysis of the phytoplankton were taken from the surface water layer with 50 ml plastic bottles and preserved in Lugol's solution. After concentrating the sample by the ultrafiltration method (Marvan 1957), the cells were counted in Bürker's chamber (at least 300 cells) and then recalculated to a number of cells per 1 ml. When the colonies of the *Microcystis* were not disintegrated, the breaking of colonies with ultrasound was conducted. Approximately 25 ml of the sample was exposed to ultrasound SONOPULS HD 2070 (Bandelin electronic, Germany) for 3 minutes with 20% strength.

The most abundant taxa were classified in some of the seven functional groups: Group I: small organisms with high S/V, Group II: small flagellated organisms with siliceous exoskeletal structures, Group III: large filaments with aerotopes, Group IV: organisms of medium size lacking specialized traits, Group V: unicellular flagellates of medium to large size, Group VI: non-flagellated organisms with siliceous exoskeletons and Group VII: large mucilaginous colonies (Kruk et al. 2010).

RESULTS AND DISCUSSION

Values of *in situ* measured physico-chemical parameters in the studied fishponds (Table 1).

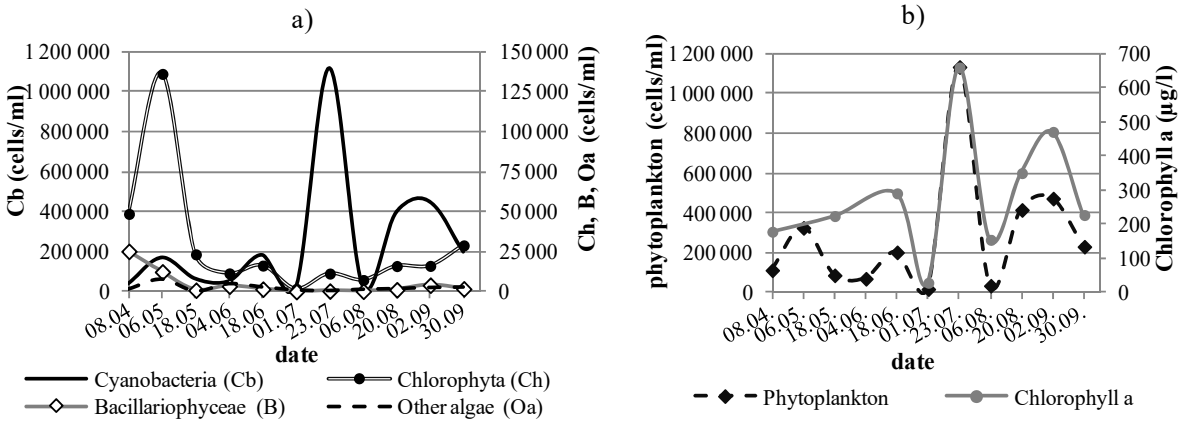
Table 1 Physico-chemical parameters in fishponds

Area	Šumický horní			Šumický dolní			Pohořelický		
	Average	Max	Min	Average	Max	Min	Average	Max	Min
Temperature (°C)	20.1	26.6	7.2	20.5	25.5	7.5	20.4	27.4	7.7
Dissolved oxygen (%)	55	117	9	61	87	26	36	99	9
pH	8.00	8.77	7.51	8.12	8.46	7.62	7.89	8.36	7.29
Conductivity (mS/m)	91.0	116.5	78.0	99.7	122.6	87.6	113.4	119.1	107.6
Transparency (cm)	34	60	10	31	50	20	54	200	20

A total of 153 taxa of the phytoplankton (27 taxa of cyanobacteria and 126 of algae) were documented in the studied ponds.

A total of 108 taxa (19 cyanobacteria and 89 algae) were documented in Šumický horní pond. Over the entire year, with the exception of April, the cyanobacteria were the most abundant group (Figure 2a) in this pond. At the beginning of the vegetation season, the most abundant group was the green algae, with *Chlamydomonas* sp. (27% of the total number of cells) as the most numerous, belonging to the functional group **V**. However, the most abundant species was *Aphanizomenon gracile* (33%), a potentially toxic (Wiedner et al. 2008, Kokociński et al. 2013) trichal cyanobacteria, categorized in the functional group **III**. A noticeable number of diatoms was also recorded, primarily *Stephanodiscus* sp. (group **VI**). In May, the most abundant were the trichal cyanobacteria *Aphanizomenon gracile* (**III**), *Pseudanabaena limnetica* (**IV**) and *Limnothrix redekei* (**III**) and green algae *Scenedesmus acuminatus* (**IV**). During this period none of functional groups have been dominant. Groups III and V are mostly dominant at temperatures less than 20 °C, which can explain their abundance in April. On the other hand their demands for nutrients are different (Kruk and Segura 2012). In May, the most abundant taxa were the ones belonging to the group IV. This could be a consequence of total nitrogen decrease and the rise of temperature.

Figure 2 Šumický horní fishpond a) Change of abundance of different phytoplankton groups during the season; b) Comparison of phytoplankton abundance and chlorophyll a



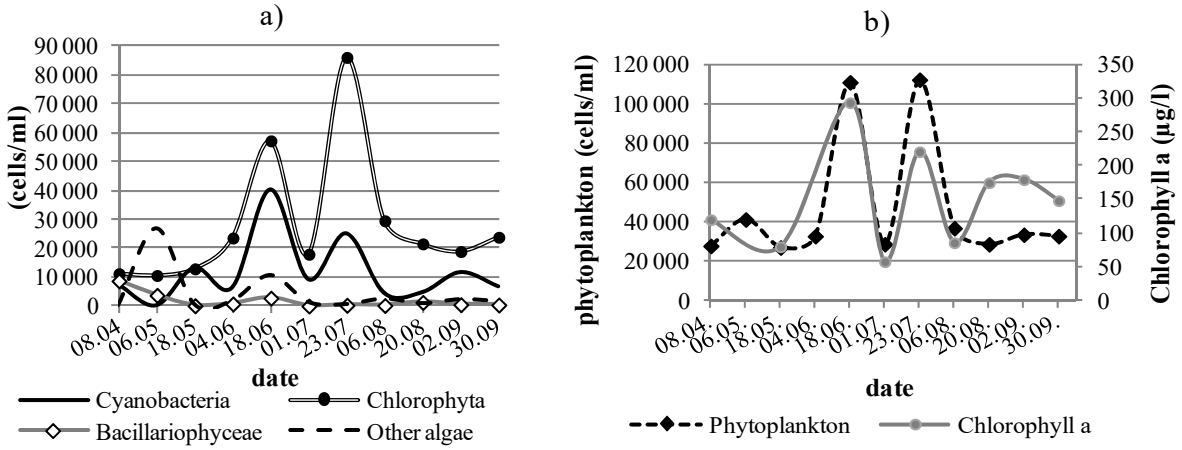
From June to the end of the vegetation season, phytoplankton was dominated by coccal cyanobacteria belonging to the genus *Microcystis* (group **VII**). All of the mentioned abundant taxa are correlated with eutrophic and hypereutrophic water systems (Padisák et al. 2009) which, in compliance with the chemical analyses, indicates that this fishpond is hypertrophic (Kopp et al. 2015). Unlike the first two months of the study period, in the period from June functional group VII was absolutely

dominant, which mostly attained the dominance between 24 and 28 °C of temperature, in high trophic water system (Kruk and Segura 2012).

The number of cells of the phytoplankton fluctuated throughout the year, reaching its maximal peak in July, which overlapped with the maximum measured values of chlorophyll a (Figure 2b). After the full development of the phytoplankton, a rapid decrease in its number occurred and was followed by the death of a part of the fish stock, due to the oxygen deficit.

A total of 108 taxa (18 cyanobacteria and 90 algae) were registered in Šumický dolní fishpond. The most abundant group was the green algae (Figure 3a), with the exception of May, when at the beginning of the month the most abundant was the group of other algae, primarily Cryptophyta *Chroomonas caudate* (36% of total amount of cells) and *Rhodomonas pusilla* (22%), both classified in group **V** (Padisák et al. 2009). At the end of the month green algae and cyanobacteria were equally present. Diatoms (mostly genera *Stephanodiscus* and *Nitzschia,* group **VI**) were documented during every month, but only at the beginning of the season in a considerable number (Figure 3a). Cyanobacteria were present during the entire study period, and its main representatives were from the genus *Microcystis* (group **VII**) (30% in May, 20% in June and September, July 10–16%, August 6–15% of the total amount of cells) and *Pseudanabaena limnetica* (**IV**), with the appearance of *Oscillatoria* sp. (**III**) in June, *Cuspidothrix issatschenkoi* (**III**) in July and *Planktothrix agardhii* (**III**) in June and in September.

Figure 3 Šumický dolni fishpond a) Change of abundance of different phytoplankton groups during the season; b) Comparison of phytoplankton abundance and chlorophyll a



The main representatives of the green algae were the genera *Chlamydomonas* (group **V**), at the beginning of the season, followed by *Desmodesmus, Pediastrum, Scenedesmus* (group **IV**), *Tetrastrum, Crucigeniella* (group **I**) and species *Crucigenia tetrapedia* (**I**), which was the most abundant species in July (30%), during the period of the maximum development (Figure 3b) of phytoplankton, and in August (50%). However, it is necessary to mention that although *Crucigenia tetrapedia* was very abundant, its share in the total biomass would have been lower, given the dimensions of its cells. Except at the beginning of May, when we can say that group V was dominant, during the rest of the season, more functional groups it were presented during the same period.

Same as in the previously mentioned fishpond, the Šumický dolní was also characterized as hyperthrophic, based on the chemical analyses (Kopp et al. 2015). The registered phytoplankton species are also correlated with eutrophic and hypereutrophic water systems.

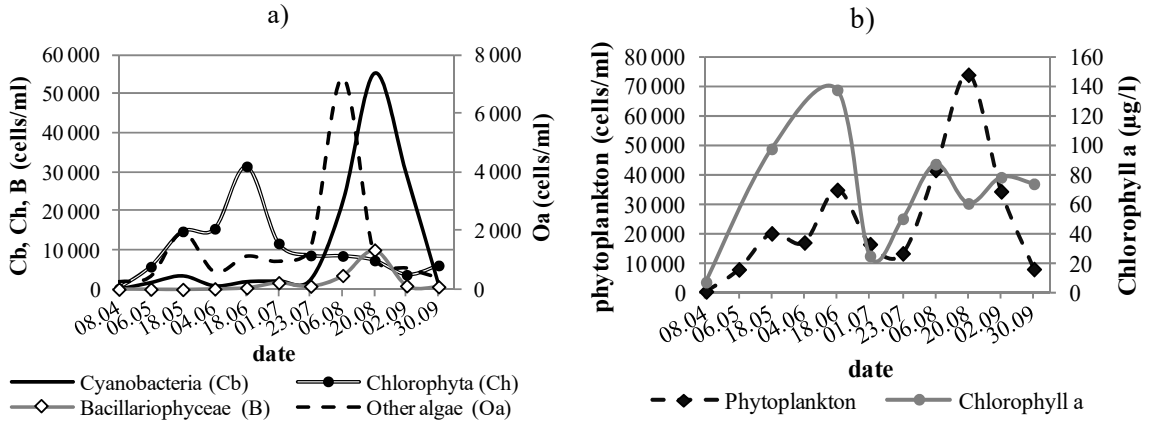
A total of 106 taxa (19 cyanobacteria and 87 algae) were documented in Pohořelický fishpond. After the harvesting and filling of the pond, at the beginning of the season, a very low diversity of phytoplankton was documented, with a very low abundance (Figure 4a, 4b).

From April to August, the most abundant representatives belonged to the group of green algae, with the dominance of *Crucigenia tetrapedia* (**I**), which was the most abundant species from May to the end of July, reaching 60% in the middle of June. The genera *Pediastrum, Scenedesmus, Desmodesmus* (**IV**) and *Tetrastrum* (**I**) were also registered in a noticeable quantity. An increase in the number of trichal cyanobacteria (group **III**) *Planktothrix agardhii* (26% at the beginning of August),

Cuspidothrix issatschenkoi (47% in August) and *Aphanizomenon flos-aqua* (23% in August and 73% in September), well known potentially toxic species, was recorded following the period of the green algae dominance. From the moment of filling the pond until the beginning of August, the most abundant group was group I, characterized by r-selected strategy, often dominant in transitional stages (Kruk et al. 2010). The group IV had significant share during this period, which can also be abundant during the transitional stages (Reynolds et al. 2002). This period was followed by the dominance of group III. The nutrient concentration was optimal for the dominance of this group during this period.

This pond was classified as hypertrophic, based on all of the measured parameters, same as the previous two.

Figure 4 Pohořelický fishpond a) Change of abundance of different phytoplankton groups during the season; b) Comparison of phytoplankton abundance and chlorophyll a



CONCLUSION

Research was conducted during the 2015. Almost equal diversity of phytoplankton was registered in all three ponds. Cyanobacteria and Chlorophyta were most abundant in all three ponds. Fluctuation in abundance was noticeable during the year. Tremendous fluctuation and abundance was recorded in Šumický horní pond, especially after a significant decline in phytoplankton at the beginning of July, followed by a large increase in phytoplankton biomass. In Šumický dolní and Pohořelicky pond fluctuation was also registered, but the abundance was not so high, as a result of the lower phosphorous content. Also, the presence of taxa more suitable for zooplankton consumption was noticeable in latter ponds. In all fishponds different functional groups were present, throughout the study period. While in Šumický dolni different functional groups were present over the entire period, in Šumický horní and Pohořelický some regularity between morfological caracteristics of the groups and environmental conditions was documented.

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INFLUENCE OF HUMIC ACID AND SODIUM CHLORIDE ON THE UPTAKE OF MERCURY BY THE COMMON CARP (*CYPRINUS CARPIO* L.)

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Abstract: The aim of the experiment was to determine the distribution of mercury in six selected tissues (skin, fish scales, kidneys, muscle, liver and gills) of common carp (*Cyprinus carpio* L.) and its relationship to humic acid and sodium chloride when they are added. Carp fingerlings weighing 47.67 ± 4.61 g were exposed to a water solution containing a defined concentration of Hg^{2+} (1.5 μg/l) and increasing concentration of humic acid (0 mg/l, 1 mg/l, 5 mg/l) and sodium chloride (29 mg/l, 300 mg/l, 1 000 mg/l) in fish tanks for 72 hours. The concentrations of mercury in fish tanks were continuously monitored and adjusted to the required value during the whole experiment. The fish were not fed during the experiment and mercury accumulated in the carp tissues from the fish tank water only. The total mercury content in the water and in selected tissues was determined by the atomic absorption spectrometer AMA 254. The increased concentration of both humic acid and sodium chloride caused a reduction (humic acid: -65% to -96% (exception for muscle 2%); sodium chloride: -8% to 21%) of mercury accumulated in carp tissues.

Key Words: common carp, mercury, humic acid, sodium chloride, atomic absorption spectrometry

INTRODUCTION

Fish is generally recommended for high protein content, low saturated fat and omega fatty acids, but water pollution leads to fish contaminated with organic and inorganic contaminants. The high attention is turned to toxic metals originated from many sources, which are accumulated in fish tissues. The most dangerous metal observed in fish tissues is mercury.

Mercury and its compounds are highly toxic substances (Syversen and Kaur, 2012, Zhou et al. 2008). The potential toxicity of mercury for humans and other organisms varies widely depending on the chemical form, the pathway of exposure, the amount, and the vulnerability of the person exposed (Robson 2003, Syversen and Kaur, 2012). An important factor about mercury is its ability to accumulate in organisms and move up in the food chain. Very low levels of mercury in surface waters can bioaccumulate to dangerous levels in the aquatic organisms (concentration increase by a factor of 10^7). Contaminated fish can pose a serious hazard to fish eating populations (Zhou et al. 2008, Amundsen et al. 1997, Watanabe et al. 2012).

An important constituent of the human diet in the Czech Republic is common carp (*Cyprinus carpio* L.). There are many studies in last years, which pay attention to mercury concentration in tissues of common carp fished in Czech ponds and reservoirs (Kruzikova et al. 2013, Cerveny et al. 2014).

It is generally known that mercury can be found in the aquatic environment in various chemical forms that include elemental, inorganic and organic mercury (Zhou et al. 2008). Mercury species build in aquatic environs strong complexes with some natural ligands. The formation of these complexes reduces content of the Hg free-ion form, which has greater chemical reactivity and has been shown to be more bioavailable (Roditi et al. 2000, Guo et al. 2001). In our work we choose humic acid and chloride ions as studied ligands.

The goal of this study was to determine the distribution of mercury in the selected tissues (skin, fish scales, kidneys, muscle, liver and gills) of common carp (*Cyprinus carpio* L.) in the presence of various concentration of humic acid (0 mg/l, 1 mg/l, 5 mg/l) and in the presence of various concentration of sodium chloride (29 mg/l, 300 mg/l, 1 000 mg/l).

MATERIAL AND METHODS

Common carp (carp fingerlings weighting 47.67 ± 4.61 g, 5 fish in each glass fish tanks) were fed by granules Screeting F1 PB 40 (2.5 mm) containing 0.018 ± 0.001 mg/kg total mercury 5 days before the start of the experiment. The glass fish tanks (Figure 1A, 1B), were filled with 85 l of tap water spiked with 1.5 µg/l mercury enriched in one series of experiment with tree various concentrations of humic acid (0 mg/l, 1 mg/l, 5 mg/l) and in the second series with sodium chloride (29 mg/l, 300 mg/l, 1 000 mg/l). During the whole experiment, the concentration of mercury, in the fish tank, were monitored by atomic absorption spectrometer AMA 254 (Altec, Czech Republic) (Figure 1C) and were adjusted in case of change to the required values. Ten carp fingerlings were deployed in every tank. Carps were not fed during experiment. Mercury was accumulated to the fish tissues only from water in glass fish tanks. After 72 hours carps were removed from tanks, killed (bleeding by syringe) and individual selected carp tissues (skin, fish scales, kidneys, muscle, liver and gills) were analysed.

Sodium chloride was chosen, because NaCl is in a long-term occurrence of chilodoneosis (*Chilodonella cyprini*) in fish kept in fish tanks or little handling ponds during autumn or spring. In such cases NaCl is used in the concentration of 1–2 g/l for 1–2 days. (Svobodova et al. 2007) Humic acid were chosen, because HA are presented in natural water and in sediment. Concentration 0 mg/l, 1 mg/l and 5 mg/l of humic acid was chosen, because this concentration was normally occurs in the natural water. (Zhang et al. 2012)

Figure 1 The glass fish tanks with sodium chloride (A), the glass fish tanks with humic acid (B), atomic absorption spectrometer AMA 254 (C)

Determination of total mercury content in the tissues of common carp

Atomic absorption spectrometer AMA 254 (Altec, Czech Republic) was used for the determination of total mercury content in the tissues of common carp and water in glass fish tanks. Homogenized solid samples of each fish tissues were directly weighted (10 ± 0.1 mg) into pre-cleaned combustion boat, and inserted into the AMA 254 analyser. Water in glass fish tanks was directly dosed (100 µl) into pre-cleaned combustion boat, and inserted into the AMA 254 analyser. Samples were dried at 120 °C for 60 s and thermally decomposed at 550 °C for 150 s under oxygen flow. Wavelength was 253.5 nm for analysis of total mercury. The limit of detection for the determination of mercury was 0.11 µg/kg for carp tissues and 0.11 µg/l for water in glass tank.

Method validation

The reference material DORM-4 (fish protein Canada) was used for method validation. Concentration of total mercury in reference material measured by AMA 254, 0.409 ± 0.007 mg/kg, was in very good agreement with certified value 0.410 ± 0.055 mg/kg.

Statistical analyses

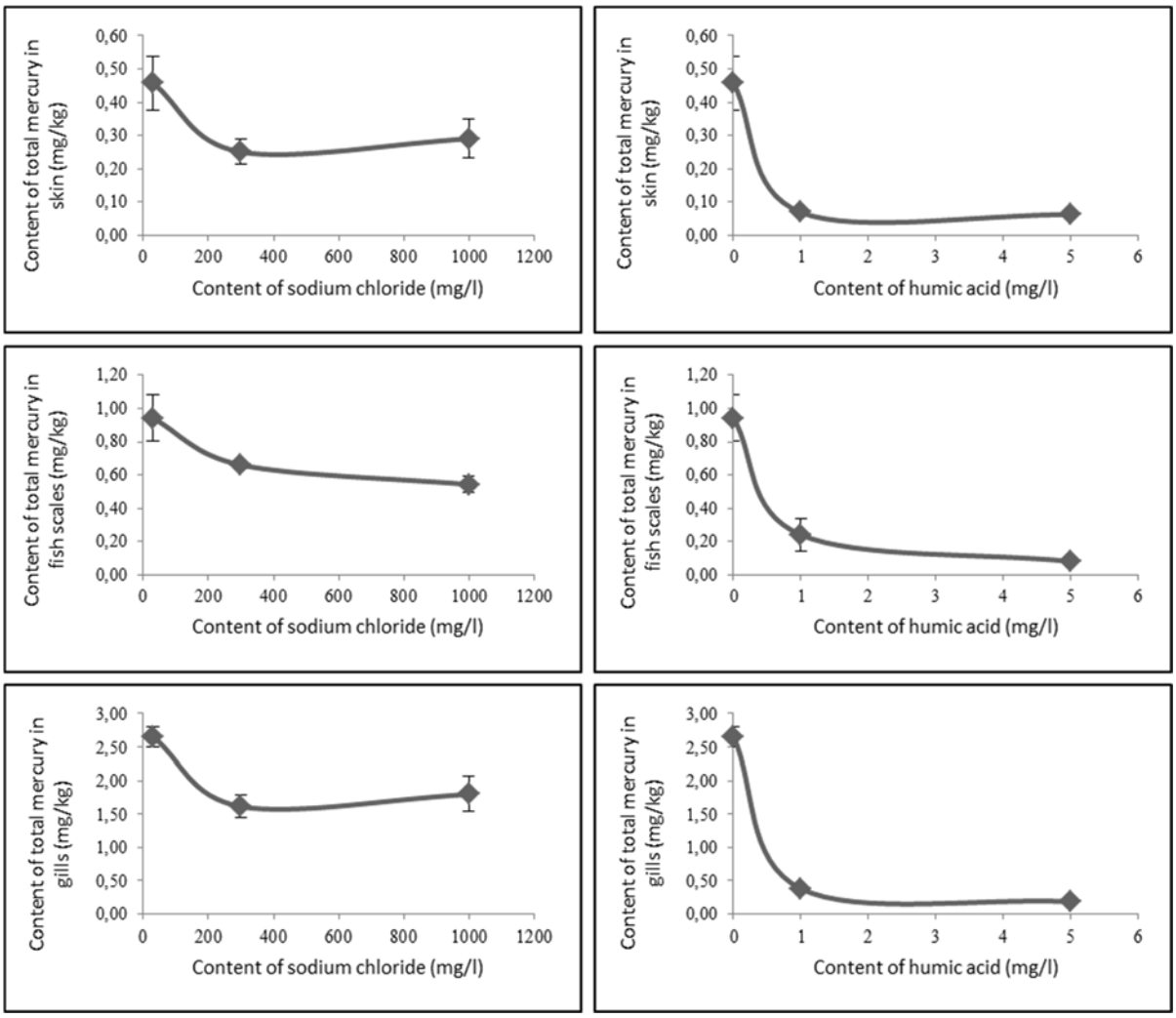
Statistical analyses of metal content in tissues were made using one-way analysis of variance (ANOVA) and statistical significance was declared when *p* value was equal to or less than 0.05.

RESULTS AND DISCUSSION

Content of mercury in selected tissues of common carp (*Cyprinus carpio* L.)

Mercury was determined in six selected tissues (skin, fish scales, kidneys, spleen, liver and gills) of common carp. The obtained results of mercury concentration (mg/kg) in tissues have been summarized in Figure 2.

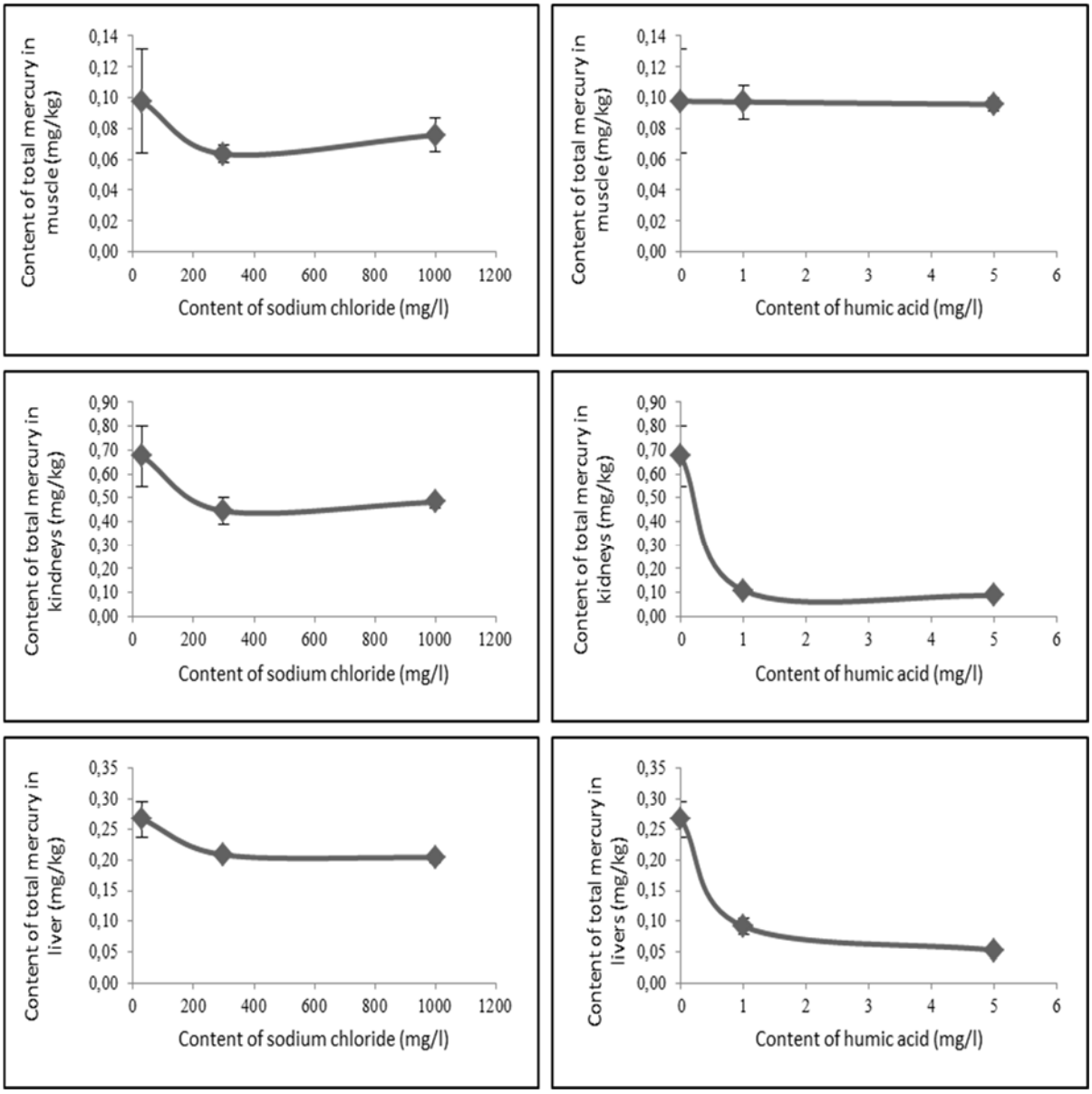
Figure 2 Concentration of total mercury in carp tissues (n = 30), commonly used for biomonitoring – skin, fish scales and gills



Skin, fish scales and gills were in direct contact with contaminated water. For this reason, higher concentration of mercury was in these tissues.

The concentration of mercury (mg/kg) in muscle and detoxification organs have been summarized in Figure 3.

Figure 3 Concentration of total mercury in muscle and detoxification organs (n = 30) of common carp – muscle, liver, kidneys



Higher concentration of mercury was in kidneys and liver, because these organs are detoxification organs for fish. (Watanabe et al. 2012) Concentration of mercury was lower in muscle, because experiment took only 72 hours and therefore the mercury was not accumulate to the muscle.

It is seen that concentration of mercury in all studied tissues was decreased with increased concentration of both of studied ligands, humic acid and sodium chloride. Increasing concentrations of sodium chloride was not had influence of humic acid on the uptake mercury. Difference between 300 mg/l NaCl and 1 000 mg/l NaCl are within the statistical error ($p < 0.05$). This observation corresponded with formerly published data of Kruzikova et al. (2011) and Marsalek et al. (2005) who reported decreased bioavailability of mercury for aqueous organisms in the presence of humic acid or sodium chloride. Dutton and Fisher 2012, studied influence of humic acid on the uptake of aqueous metals by the killifish (*Fundulus heteroclitus*) for 72 hours. The concentration of humic acid was range 0–20 mg/l. Content of mercury in tissues of studied fish was decreased with increasing concentration of humic acid. The same results were observed by Block et al. 1997, who studied influence of chloride on the uptake of mercury by the minnow (*Phoxinus phoxinus*). Content of mercury in tissues was decreased, when chloride was present. Decreasing concentration of mercury (%) in selected tissues (muscle, skin, fish scales, kidneys, gills and liver) of common carp, in the presence of chloride and humic acid, have been summarized in Table 1.

Table 1 Decreasing concentration of mercury (%) in selected tissues (muscle, skin, fish scales, kidneys, gills and liver) of common carp

Tissues	Concentration of NaCl (mg/l)	Reduction of total Hg (%)	Concentration of HA (mg/l)	Reduction of total Hg (%)
Muscle	300	-35.0	1	-0.50
	1 000	-22.4	5	-2.10
Skin	300	-45.2	1	-84.9
	1 000	-36.5	5	-86.4
Fish scales	300	-29.9	1	-74.4
	1 000	-42.3	5	-91.3
Kidneys	300	-34.1	1	-83.9
	1 000	-28.5	5	-87.1
Gills	300	-39.3	1	-85.7
	1 000	-32.4	5	-93.1
Liver	300	-22.0	1	-65.6
	1 000	-23.6	5	-80.0

HA = humic acid, NaCl = sodium chloride

Humic acid reduced the accumulation of mercury in the studied tissues highly significantly (by 65–86%) ($p < 0.05$)even in the case of low concentration of humic acid (1 mg/l). Muscles formed the only exception with mercury reduction of 0.5–2%. On the other hand, sodium chloride reduced mercury accumulation by a maximum of 45 %, even though the concentration of NaCl was by three orders of magnitude higher than that of humic acid. Humic acid are substances, which strongly reacts with mercury, thus creating strong and volumetric large complexes. On the other hand, sodium chloride is not creating so strong complexes than as humic acid (Fernandez-Gomez et al. 2015).

CONCLUSION

Increased concentration of both humic acid and sodium chloride in fish tank solution caused reduction of mercury accumulation in all studied carp tissues. Humic acids form with mercury strong and volumetric large complexes, which are absorbed in living organism much more difficultly than mercury complexes with chloride ions.

ACKNOWLEDGEMENT

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BIOMANIPULATING EFFECT OF GRASS CARP (*CTENOPHARYNGODON IDELLA* VAL.) IN ARTIFICIAL WATER CHANNELS

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Abstract: The grass carp (*Ctenopharyngodon idella* Val.) is an important species used to control invasive aquatic macrophytes in many parts the world. In this study, we assess the diet of grass carp introduced to two artificial channels overgrown with aquatic vegetation. We examined forty 7+ to 9+ carp (517–814 mm standard length) sampled in the summers of 1998 and 2015. Aquatic vegetation was the dominant dietary item, with insects, fruit and vegetable remains (stones of *Prunus sp*., potato remains) and detritus a minor part of the diet. Aquatic plant biomass declined significantly at the study sites after carp were introduced, with coverage reduced from 65–70% of the water's surface in summer 1998 to 10% in summer 2015.

Key Words: overgrown channels, aquatic vegetation, biomanipulation, carp, fish diet

INTRODUCTION

The herbivorous grass carp (*Ctenopharyngodon idella* Val.) has become an important species in many types of water body around the world due to its potential biomelioratory effect against invasive aquatic macrophytes (Adámek and Kokorďák 1982, Krupauer 1989). In general, its successful acclimatisation is only really limited by a mean temperature isotherm of around 5 °C (Opuszyński 1969, Carter et al. 1992). Grass carp are relatively well known to fish farmers in the Czech Republic (Krupauer 1967), having been introduced into Czech fish ponds in 1961 to provide biotechnological control of aquatic weeds and, at the same time, to increase fish production (Adámek et al. 1996, Kubů and Lusk 1962). Today, grass carp are an important element of fish pond stock. In some cases, however, the species can cause significant limnological changes to pond ecosystems due to its dietary preference for aquatic plants (Pípalová et al. 2009).

Between 1995 and 1998, around 1500 adult grass carp were stocked into two artificial channels (i.e. approx. 214 each year) in order to clear them of weed growth, the channels having become overgrown with aquatic plants in the early 1990s (Zapletal and Lohniský 2015). Since then, the accumulation of plant production in the channels has decreased dramatically.

Here, we assess whether feeding activity of grass carp was directly responsible for the decrease in aquatic plant biomass in the artificial channels. Secondly, we evaluate whether grass carp diet composition changed between 1998, when stocking ceased, and 2015, following the reduction in aquatic plant biomass.

MATERIAL AND METHODS

Study site

This study was carried out along two artificial channels, the Opatovicky channel near the village of Opatovice nad Labem and the Maly labsky millrace near the town of Hradec Králové, both in eastern Bohemia, Czech Republic. In each case, a 100m stretch of channel was fished upstream (method below) starting from 50°8′38.4 N, 15°47′28.1 E for the Opatovicky channel and 50°23′45.6″N, 15°82′42.0″E for the Maly labsky millrace. Before stocking, both channels were covered by a dense growth of contiguous riparian vegetation, with the surface community dominated by river water–crowfoot

(*Batrachium fluitans* Lam.), fan–leaved water–crowfoot (*Batrachium circinatum* Sibth.) and variegated red sweet–grass (*Glyceria maxima* Hartm.)–all taxa described according to Danihelka et al. (2012).

Percentage coverage of aquatic vegetation was evaluated in the second half of June in 1998, 2001, 2004, 2007, 2010, 2013 and 2015 (A three–year period was chosen as changes year–to–year were non–significant). At each site, Aquatic vegetation was sampled from 11 x 1 m^2 sites along the same 100 m stretch used for fishing and relative percentage coverage evaluated. The final result is an average of the 11 values (all methods according to Grulich and Vydrová 2006).

Fish sampling and analysis

A total of forty 7+ to 9+ carp (517–814 mm standard length [SL], 1300–5066 g total weight [TW]) were collected by means of electrofishing and angling, ten specimens at each site each year (i.e. Opatovicky 1998 and 2015, Maly labsky 1998 and 2015). After weighing (TW; nearest 0.1 g) and measuring (SL; nearest 1 mm), each fish humanely sacrificed and the gut contents removed and preserved in 4% formaldehyde for later laboratory analysis. Each sample was observed under a 40–450 x magnification binocular microscope and the remains separated into taxa. Unidentified plant remains were registered as "macrophyte fragments" only. Diet composition is presented as relative percentage biomass (%Wi; Hyslop 1980) and index of preponderance (IP; Natarajan and Jhingran 1961). Statistical relevance ($P < 0.05$) was assessed using one way ANOVA (Dohnal 1999) with post–hoc Tukey tests using the software provided in Microsoft® EXCEL 2010.

All aspects of this study were carried out in accordance with Czech regulations regarding animal welfare and protection.

RESULTS AND DISCUSSION

Aquatic vegetation coverage

In 1998, approximately 70% of the Opatovicky channel was covered in river water–crowfoot (70%), fan–leaved water–crowfoot (20%) and variegated red sweet–grass (10%). At the Maly labsky millrace, on the other hand, fan–leaved water–crowfoot only covered approximately 65% of the water's surface. These figures only dropped after 2006, following severe flooding (Q > 100). In 2015, seventeen years later, aquatic vegetation cover had been reduced to just 9% on the Opatovicky channel and 11% on the Maly labsky millrace, with fan–leaved water crowfoot the dominant species at both sites (Figure 1). Coverage in all years after 2006 was significantly lower than those before 2006 (ANOVA, all $P < 0.05$). This would suggest that grass carp had not been able to reduce macrophyte coverage in the channels prior to 2006. After large–scale scouring of the channels following flooding in 2006, however, the carp were able to prevent macrophytes from proliferating and covering the channels' surface once more.

Diet composition and biomanipulation effect

Macrophyte fragments; undeterminable parts of river water–crowfoot, red sweet–grass and remains of terrestrial vegetation; dominated in grass carp diet at both localities, both at the start and end of the study (Opatovicky channel, IP 1998 = 33.8, 2015 = 66.7; Maly labsky millrace, IP 1998 = 53.7, 2015 = 70.0). Water–crowfoot was sub–dominant in 1998 (Opatovicky, IP = 28.2; Maly labsky millrace, IP = 34.3), but was recedent 17–years later (Figure 2). Red sweet–grass was only found in one case in the Opatovicky channel in 1998 and has not been observed since in either study area. Fruit and vegetable remains (including stones of *Prunus sp*. and potato peelings from household/garden waste) and filamentous algae and aquatic invertebrates (intake associated with consumption of macrophytes; see also Pípalová 2009) were only recorded as recedent (IP < 10.0; Figure 2). While the relative percentage of identifiable remains changed between 1998 and 2015, there was no significant difference ($P > 0.05$) in the overall mean level of macrophytes eaten between the two periods (i.e. Opatovicky channel 1998 = 70% 2015 = 69%, Maly labsky millrace 1998 = 82%, 2015 = 79%)

Herbivory in grass carp is a well–known and widely–described phenomenon. Catarino et al. (1997), for example, noted that grass carp in a large Portuguese irrigation system fed primarily on the dominant species of parrot feather watermilfoil (*Myriophyllum aquaticum*), fennel pondweed (*Stuckenia pectinate*) and duckweed (*Lemna sp*.). Similar results were obtained at localities in the USA by Masser (2002). As such, grass carp are successful generalist feeders that can vary their feeding behaviour and

dietary choices depending upon the aquatic plant species available. Our own results clearly demonstrate, however, that while grass carp diet consisted almost entirely of the dominant aquatic plant species, they were unable to reduce aquatic biomass to any great degree until flooding apparently reduced aquatic biomass in the channels to between 55 and 60% of its former level. From then on, they were able to control any further growth to such an extent that the channels remain largely clear of plant growth to this day. In doing so, they played an important role in maintaining water discharge in these shallow artificial channels.

Figure 1 Aquatic plant coverage at the Opatovicky channel (white) and the Maly labsky millrace (grey) between 1998 and 2015

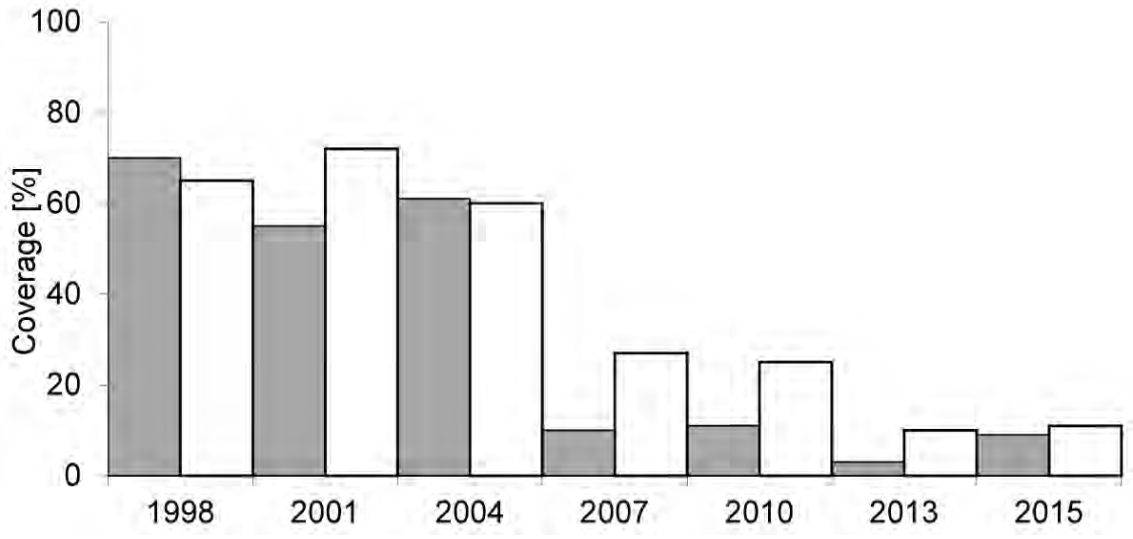
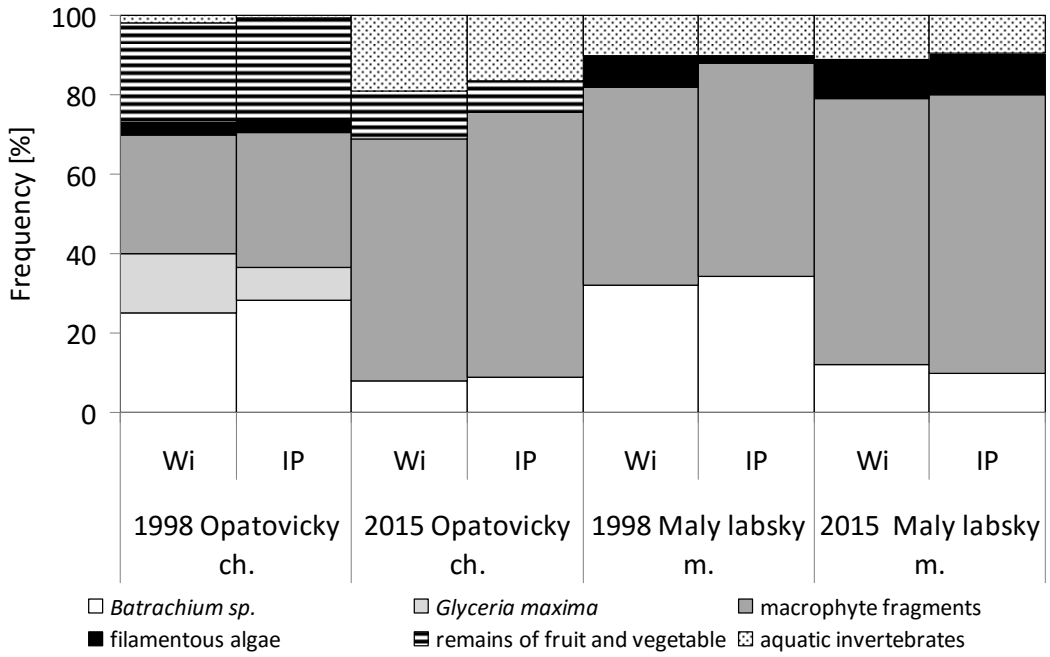


Figure 2 Diet composition of grass carp in 1998 and 2015; relative percentage biomass total food intake (Wi) and index of preponderance (IP)



CONCLUSION

At the stocking densities used in this study, grass carp in previously overgrown artificial channels in the Czech Republic did not have any clear bio–meliorating effect in overgrown channels until after flooding had reduced aquatic plant biomass by around 60%. Since then, the carp have proved highly

effective at controlling plant biomass in these channels, thereby playing an important role in maintaining water discharge. Future biomanipulation studies using grass carp, therefore, should either, use higher stocking densities, remove the bulk of plant material prior to stocking or stock carp during winter when the plant material has died back.

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Section – Agroecology and Rural Development

INVASIVE PLANT SPECIES IN THE ACCOMPANYING VEGETATION OF THE NITRA RIVER

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Abstract: This paper focuses on the evaluation of the species composition of accompanying vegetation adjacent to water flow in the of the Upper Nitra region and species diversity among communities attacked by invasive species and non-invasive communities. The research was carried out in the vegetation period during the years 2015 and 2016 on the 10 km long section with 48 phytocenological relevés. The results indicate that there is a high concentration of researched species in the area, which progressively push out the original, natural vegetation. A total of 133 plant species were recorded 111 species in non-invasive vegetation and 104 species in invasive vegetation, 78 species were the same in both types of relevés. The species with the highest average coverage were the following invasive taxa: *Helianthus tuberosus*, *Solidago canadensis* and *Amaranthus retroflexus.* The non-invasive species were: *Solanum nigrum*, *Urtica dioica*, *Rubus caesius* and *Humulus lupulus.*

Key Words: invasive plant species, the Nitra river, the Slovak Republic

INTRODUCTION

Invasive plant species cause problems and have negative impact on flora and fauna almost all over the world. They are one of the reasons for the change in the abiotic environment, and they affect human health and national economies (Křivánek 2006). The term "negative impact" conceals the suppression of native species in competition for resources (Melgoza et al. 1990), reduction of habitats, increased groundwater extraction and its subsequent lack for other species. There are also other changes in the hydrological regime, increased sedimentation and a subsequent change of rhythm to the whole ecosystem, e.g. riparian vegetation (Zavaleta 2000). Each non-native species changes the composition of natural diversity in a certain way. On the one hand, the introduction of non-native species can increase the overall number of species occurring in a particular place – at least in the short term. On the other hand, in the long term it will lead to a reduction in species diversity (the number and abundance of species). Another possibility is that it also leads to the displacement of native species from habitats or regions and to a total change in natural ecosystems (Shine et al. 2000, Nentwig 2014). On a municipal level, the crowding out of native plant species is a phenomenon resulting from the dominance of invasive plants, which prevail in contaminated locations. In general, the plant invasion causes homogenization of flora in which the originally different phytogeographic units become similar due to the massive invasion (Hejda and Pyšek 2006).

In his study of invasive species of the Czech flora, Pyšek et al. (1998) indicates that after settlements, the habitats connected with still standing and running water are places with the highest representation of non-native species. Some authors consider habitats along rivers are more prone to invasion. It is justified by the disturbance occurring in these areas. Because of this disturbance, these communities are receiving higher quantities of available resources (Stohlgren et al. 1998).

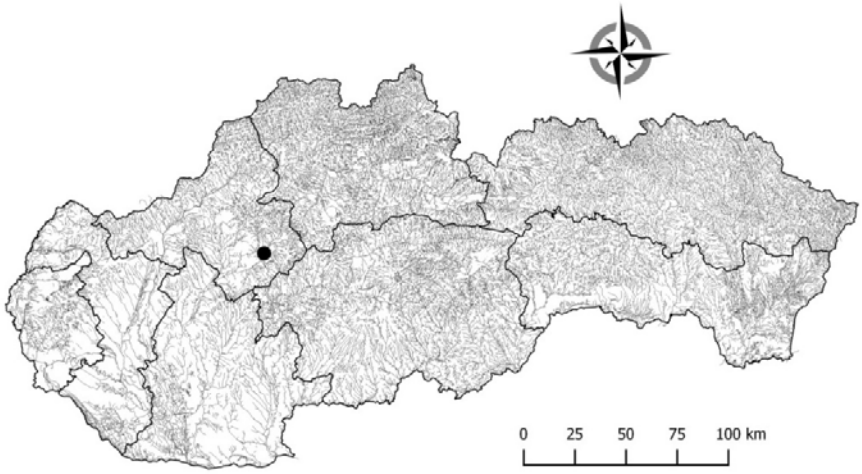
The aim of this paper is to assess the impact of invasive plant species on the composition and nature of the infested habitats, and to ascertain what the subsequent changes are to the composition of the community.

MATERIAL AND METHODS

Study area

The Nitra river rises on the southern slopes of the Malá Fatra Mts. After passing the Podunajská pahorkatina Upland it flows into the Váh river in the area of the Podunajská rovina Plain to the north of the city of Komárno. The flow length is 196.7 km and the total area of the river basin is about 5 144 km^2 (Mazúr and Lukniš 1980, Porubský 1991).The area belongs to the basin of the Nitra river with a left tributary of the Handlovka river and to the upland-lowland regions with a rain-snow runoff regime (Šimo and Zaťko 2002). The defined part of the river is 10 river kilometres in length, and passes through cadastral territory of Bojnice town and the villages Opatovce nad Nitrou, Diviacka Nová Ves and Zemianske Kostoľany (Figure 1).

Figure 1 Location of the study area in the Slovak Republic (Bencová 2016)



Methodology of work

The area of interest chosen was a 10 km long section from the confluence of the Nitra river with the Handlovka river (48° 44' 47.14" N, 18° 33' 32.70" E), up to the trigger point of the Zemianske Kostoľany village cadastre (48° 40' 42.27" N, 18° 30' 56.28" E). In this area a part of the river had transferred to a new river bed due to lignite mining. Also, settlements and a large number of cultivated areas occur in the area, where disturbance provides appropriate conditions for the occurrence of invasive species.

During the years 2015 and 2016, 24 pairs of phytocenological relevés were recorded on an area of 4 x 4 metres. Each pair had one relevé per locality with a minimum of 60% coverage of invasive species and in the nearby non-invasive vegetation. Non-invasive areas were selected to represent the same habitat conditions as the habitat conditions of invasive areas (Hejda and Pyšek 2006). Individual invasive plant species have also appeared in the relevés of non-invasive areas because it was difficult to find areas with the total absence of their occurrence. The areas of comparison were selected on the basis of the following criteria: (a) locations are strongly attacked with invasive species, while populations are homogeneous (b) non-invasive cover is linked as far as possible on the attacked localities in order to maintain the same habitat conditions of the environment (altitude, orientation, ... etc.). The presence of the species was evaluated on the basis of the Braun-Blanquet scale coverage (van der Maarel 2005):

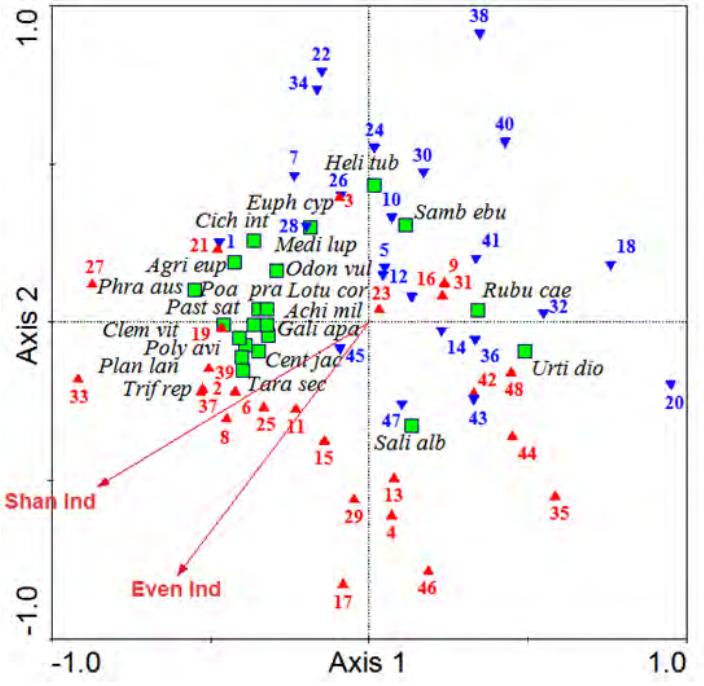
r – rare species, + – occasional species, coverage is negligible, coverage < 1%, 1 – coverage from 1 up to 5%, 2 – coverage from 5 up to 25%, 3 – coverage from 25 up to 50%, 4 – coverage from 50 up to 75%, 5 – coverage more than 75%.

Invasive taxa were selected and mapped according to Gojdičová et al. (2002): category 1 – invasive taxa and category 2 – potential (regional) invasive taxa. Invasive relevés were realized in the vegetation of the following species: *Helianthus tuberosus*, *Solidago canadensis*, *Impatiens parviflora*, *Tanacetum vulgare*, *Fallopia japonica*, *Fallopia x bohemica* and *Robinia pseudoacacia*. The key for determination of plants was used for verifying the taxa of vascular plants (Dostál and Červenka 1991, 1992). The nomenclature of taxa has been unified according to the work of Marhold and Hindák (1998). The relevés were processed in Excel and subsequently in JUICE 7.0 (Tichý 2002), with which we calculated the average coverage of species and Shannon diversity index H´. Subsequently we calculated the evenness index by using the formula: H′/In S, where S represents the number of species (Hejda and Pyšek 2006). The obtained data was processed in the CANOCO 4.0 program (Ter Braak and Šmilauer 2002), where DCCA (Detrended Canonical Correspondence Analysis) and RDA (Redundancy Analysis) analyses were carried out. A total number of 499 permutations were calculated in the Monte-Carlo test. Visualization was carried out using the CanoDraw program. The relation between invasive and non-invasive relevés was tested using the STATISTICA program (StatSoft Inc. 2007).

RESULT AND DISCUSSION

In the study area through the 48 phytocenological relevés we discovered the presence of 133 plant species. Data about species abundance and value indexes was processed by DCCA analysis. On the basis of DCCA analysis, we found that the length of gradient was 2.534 therefore in the next step we used RDA analysis (Redundancy Analysis). The resulting values were visualized through CanoDraw (see Figure 2). The diagram shows only species with more than 10% variability in the data.

Figure 2 RDA ordination diagram expressing relation between data and environmental variables



Legend: Agri eup – Agrimonia eupatoria, Achil mil – Achillea millefolium, Cent jac – Centaurea jacea, Cich int – Cichorium intybus, Clem vit – Clematis vitalba, Euph cyp – Euphorbia cyparissias, Gali apa – Galium aparine, Heli tub – Helianthus tuberosus, Lotu cor – Lotus corniculatus, Odon vul – Odontites vulgaris, Past sat – Pastinaca sativa, Phra aus – Phragmites australis, Plan lan – Plantago lanceolata, Poa pra – Poa pratensis, Poly avi – Polygonum aviculare, Rubu cae – Rubus caesius, Sali alb – Salix alba, Samb ebu – Sambucus ebulus, Tara sec – Taraxacum sect. Ruderalia, Trif rep – Trifolium repens, Urti dio – Urtica dioica.

For better differentiation, invasive relevés are represented by a blue triangle and non-invasive relevés are represented by a red triangle. The diagram shows the dependence between species, relevés and environmental variables. Relevés realized in areas of invasive vegetation were displayed in the opposite direction to the growing diversity of the relevés, while *Helianthus tuberosus*, *Sambucus ebulus* and *Rubus caesius* had the largest variability. The relevés located in the negative part of the diagram showed the greatest diversity and species richness. Ruderal and field species prevailed here as *Trifolium repens*, *Plantago lanceolata* and *Clematis vitalba*.

We used the Monte-Carlo permutation test for evaluating environmental factors. We found following values of Shannon-Wiener Index: P-value = 0.002, F-ratio = 2.29; evenness Index: P-value = 0.004, F-ratio = 1.71. This means that both variables have a significant impact on the variability of displayed data, while both of the factors according to RDA analysis explain 8% of the overall variability.

In the invasive relevés, the average occurrence of species was 14.41 species per relevé and in non-invasive it was 16.45. The difference in the number of species between the relevés is not too large. This may be due to the fact that some relevés were made in peripheral areas of invaded vegetation where the competitive struggle is not so significant. The average coverage of species in every relevé was assessed using the JUICE program. The species selected as the core taxa for creating the invasive relevés (that is, they had more than a 60% coverage in invasive vegetation) were removed from the table (Table 1). They were the following: *Helianthus tuberosus*, *Solidago canadensis*, *Impatiens parviflora*, *Tanacetum vulgare*, *Fallopia japonica*, *Fallopia x bohemica* and *Robinia pseudoacacia*. Out of them, the following species had the highest coverage in the area: *H. tuberosus* (inv. relevés: 50.9%, non-inv. relevés: 2.3%) and *S. canadensis* (inv. relevés: 37.4%, non-invasive relevés: 3%).

Table 1 Comparison of average coverage of selected species (> 10% in one category)

Species of plant	Life form	Invasive relevés	Non-invasive relevés
Amaranthus retroflexus	AH	32.5	-
Ambrosia artemisiifolia	AH	13	2
Brachypodium sylvaticum	PG	3	13
Cuscuta epithymum	AH	-	13
Echinochloa crus-galli	AH	3	20
Fallopia convolvulus	AH	-	13
Humulus lupulus	P	9.6	15.8
Jacea pratensis	P	13	3
Lysimachia nummularia	P	13	2
Phragmites australis	PG	-	13
Rubus caesius	S	7.9	13.4
Salix alba	T	3	10.5
Solanum nigrum	AH	38	3
Swida sanguinea	S	6.3	13

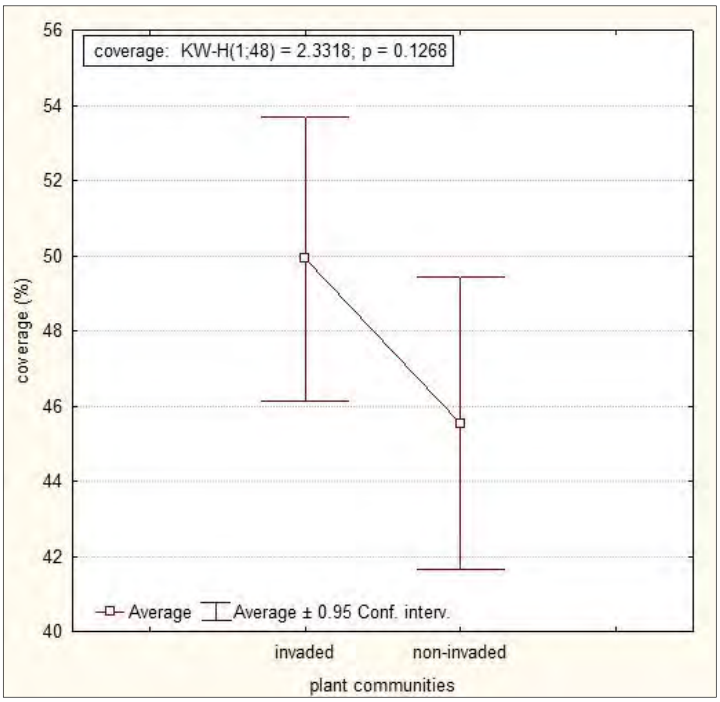
Legend: AH – annual herb, PG – perennial grass, P – perennial, S – shrub, T – tree (Dostál and Červenka 1991, 1992)

The data shows that of the invasive relevés the species with the highest average coverage was *Echinochloa crus-galli*, which is the characteristic species of roots and fallow lands. This species also occurred in invasive relevés although its coverage here has dropped to 3%. The loss of coverage for the species *Humulus lupulus*, *Rubus caesius* or *Salix alba* is also visible. The opposite effect was observed for species *Solanum nigrum* and *Amaranthus retroflexus*, where coverage was noticeably higher in invaded

vegetation (vegetation attacked by invasive species). They appear as ordinary weeds in fallow lands and disturbed areas that are typical habitats for the invasive species. *A. retroflexus* is classified as a potentially invasive taxon.

The relation between invasive and non-invasive types of relevés was subsequently tested in the STATISTICA program (see Figure 3). The hypothesis H_0 was specified: coverage of individual types in invasive and non-invasive relevés do not differ-pα = 0.05. Since the graph shows, that p is greater than pα, it means that a given hypothesis cannot be rejected at the 95% confidence level. The value of the standard deviation for invasive relevés is 10.75% and for non-invasive relevés is 3.98%.

Figure 3 Expression of the relation between invasive and non-invasive relevés



Legend: KW = Kruskal-Wallis test (difference between average values – the outliers were turned off), Conf. interv. – confidence interval, invaded – plant communities with invasive species, non-invaded – plant communities without invasive species

CONCLUSION

Watercourses with their accompanying vegetation represent in their natural form an extremely important landscape element, which forms an ecologically significant landscape segment. Riparian vegetation is an important element for the ecological stability of the landscape, where it forms important biocorridors and sometimes refuges for fauna in the surrounding mostly agricultural land (Benčať and Pažitný 2007).

Phytocenological relevés were located so as to document the nature of the accompanying vegetation of the Nitra river. They represent the vegetation and ecotone of riparian vegetation, as well as the edges of fields and ruderal habitats affected by humans to varying degrees. In the study area we recorded the presence of 133 plant species, out of them 103 species in vegetation with at least 60% coverage of invasive species and 111 species in non-invasive vegetation. *H. tuberosus* and *S. canadensis* showed a significant dominance in non-native taxa. We recorded a frequent occurrence of *S. nigrum* and *A. retroflexus* in uninvaded (without invasive species) vegetation from other species. *E. crus-galli*, *R. caesius* a *H. lupulus* prevailed in non-invasive relevés. The results show that the difference in diversity between invasive and non-invasive

vegetation is very high. There is a reduction of diversity and progressive crowding out of native species in the areas with a significant majority of invasive species.

ACKNOWLEDGEMENT
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MINING OF LIMESTONE AND ITS IMPACT ON THE ENVIRONMENT

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Abstract: The article deals with mining of limestone quarries in selected cadastral area of Brno (quarry Hády and quarry Stránská skála). In both quarries were found foreign substances. The risk elements were analyzed by XRF spectrometry. On the territory refractive Hády was found higher sulfur content. The highest amount of sulfur is in the small quarry - Růženin quarry. At the quarry Stránská skála was mainly found higher content of lead and zinc. Zinc has been found at higher concentrations, especially in the near factory.

Key Words: Mining, limestone quarry, contamination, environment

INTRODUCTION

Human activity since its formation disrupts the natural world. One of the major anthropogenic activities is mining in quarries. Resulting adverse conditions can manifest themselves in several characteristics: lack of water, lack of fine-gravel substrate and its own initial soils, excessive erosion of the walls of quarries or contamination of soil and bedrock with foreign substances due to mining and depositing waste. Foreign substances can then facilitate entirely new raid invasive woody species and the emergence of ruderal communities (Kabata-Pendias 1992).

The chemical composition of the soil is usually a reflection of two opposing elements operating cycle - the cycle of biological, geological, and this composition can be very diverse. Somewhat different processes act on the chemistry of the city and on anthropogenic significantly affected soils, when a person with their own accord completely exceeds the importance of natural forces, and especially if it is taken into account redistribution of heavy metals and toxic elements (Šarapatka et al. 2002).

The risk elements can degrade the quality of environmental compartments (Salmani et al. 2016). In so-called hazardous elements provided their critical quantities in the soil, which was determined based on the transfer of hazardous elements in plants. Soil analysis often determine the content of hazardous elements – chlorine, zinc, copper, cobalt, lead and cadmium (Kabata-Pendias 1992, Šarapatka 2002).

Basic legislative measures expressing the problems of contamination of soil Act. no. 334/1992 Coll. On the protection of agricultural land (as amended)., Act. No 156/1998 Coll. On fertilizer (as amended) and Act. No. 185/2001 Coll. On waste (as amended)., the relevant decree to these laws (Ambrožová 2015).

We have many established analytical technologies for determine risk elements in soil. Such as Inductively coupled plasma optical emission spectrometry (ICP-OES), Inductively coupled atomic emission spectroscopy (ICP-AES), Inductively coupled mass spectrometry (ICP-MS), Atomic fluorescence spectrometry (AFS), Neutron activation analysis (NAA) or X-ray fluorescence spectrometry (XRF) (Soodan et al. 2014). XRF technology offer easy use and results comparable to those of laboratory based instruments (Parsons et al. 2013).

MATERIAL AND METHODS

Chemical analysis of soil were analyzed using XRF spectrometry methods during the autumn of 2013. This method (with the proper use of hand-held X-ray analyzer Delta 50 from Olympus Innov-X

USA) provides quick and very precise information on the presence and amount of each element in the sample. XRF is a non-destructive method enabling qualitative and quantitative analysis of a wide spectrum of samples (Ambrožová 2015). All Measurement was done a total of three days.Before measurement was carried automatic calibration spectrometer. Measurements were performed directly in the field (quarry Hády, Stránská skála quarry). Under field conditions was formed a square grid of 50 x 50 meters, in which were measured. One measurement by analyzer lasted 60 second. Selected elements, which exceeded the set limits are shown on maps of the elemental anomalies. Maps were processed in ArcGIS Desktop Help 10.2 using Kernel Density for clear representation of outbreaks of contamination, or other values. The coordinate system was chosen - a uniform system of trigonometric cadastral network.

The principle of the method Kernel Density can be thought of as individual measurement points manually spectrometer, around which the system creates a circular pattern. These elements are the highest level inside and then declining values using a mathematical function defined towards the edge, where become zero (Krtička 2012).

For measuring the sample in situ the threshold humidity of 20%. After exceeding this value can expect significant inaccuracies in the result. Therefore recommended sample before analysis drain. To dry soil samples before analysis leans most authors XRF method ex situ (Ambrožová 2015).

For laboratory analysis samples (total 7 soil samples) were taken from surface horizon (in depth 5 cm) as the disturbed soil sample. Subsequently samples have been deposited into labeled sealable polythene bags. After samples were transported to the laboratory at Mendel University Brno. The samples were dried in air for longer than necessary. The drying took place in a furnace at 105°C to constatnt weight. Dry samples were homogenized and sieved through sieves with a mesh size of 2 mm of fine. Subsequently ventured sample weighing 2.8 grams. This samples were undertaken to XRF analysis. Proximity x-ray beam from the sample was 2mm. Time of one analyses were 60 seconds.

RESULTS AND DISCUSSION

In Stránská skála quarry were selected seven representative samples, which were measuring risk element values.

For samples 1, 3, 4, 6 and 7 were measured on the topsoil (depth of about 5 cm, only for samples 2 and 5 were measured from the subsoil (depth of about 10-20 cm, average 15 cm). Sample number 6 is specific since been removed from the cave. Table 1 shows higher content calcium in quarry Stránská skála (Muchová 2014).

Table 1 Measured elements in the quarry Stránská skála (Muchová 2014)

Sample	Ca [%]	K [%]	Si [%]	P [%]	Cu [%]	Pb [%]
No. 1	7.19	0.57	5.68	0.29	0.26	0.17
No. 2	15.56	0.55	6.20	-	0.03	0.01
No. 3	8.64	0.41	5.60	0.26	0.93	0.39
No. 4	9.71	0.55	10.38	-	1.03	0.29
No. 5	8.68	0.68	7.28	-	-	0.01
No. 6	11.69	0.68	9.23	-	0.19	0.09
No. 7	11.38	0.69	7.76	0.21	0.39	0.09

Figure 1 The incidence of lead fracture quarry Stránská skála (Burnog 2016)

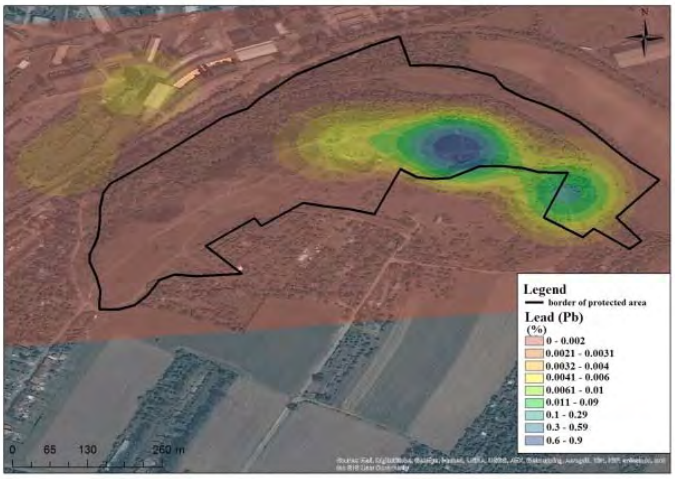
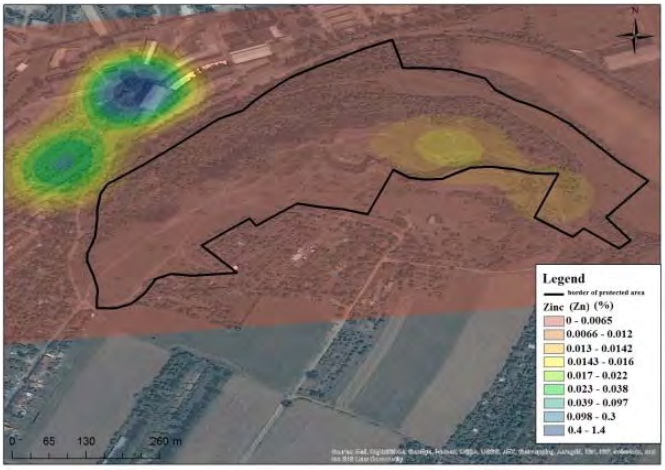


Figure 2 The incidence of zinc fracture in Zetors tractors a.s. (Burnog 2016)



On the territory refractive Hády were measured higher concentration of elements of copper, lead (Figure 1) and sulfur. Higher load territory sulfur was detected on parts of the quarry Hády called Růženin quarry, then the location at the tunnel connecting the quarry jungle and Růženin quarry (Gregorová 2003). The primary source of sulfur are sulfides, which are formed by weathering and sulphates. The most common is sodium sulfate, calcium (Šimek 2004).

Figure 3 Occurrence of sulfur quarry Hády (Burnog 2016)

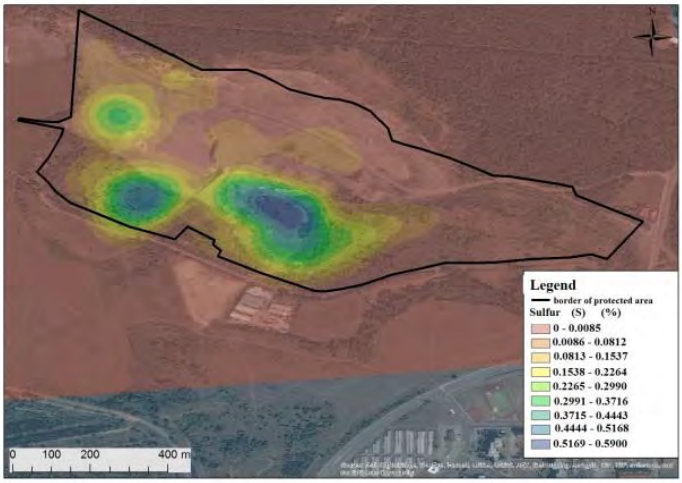


Table 2 Measured elements in the quarry Hády (Muchová 2014)

Sample	Ca [%]	K [%]	Fe [%]	Si [%]
No. 1	6.99	1.15	2.54	15.51
No. 2	5.30	0.98	2.97	14.16
No. 3	6.76	0.88	1.10	11.99

The chemical composition of the soil is usually a reflection of two opposing operating cycle of elements - the cycle of biological and geological, said composition can be very diverse (Rejšek 1999).

In so-called hazardous elements provided their critical quantities in the soil, which was determined based on the transfer of hazardous elements in plants. In soil analyzes the content of hazardous elements – chlorine, zinc, copper, lead or cadmium (Kabata-Pendias 1992, Šarapatka et al. 2002).

Both limestone quarries located extraneous elements that may be the remains of mining activities. In Stránská skála quarry may occurrence of contaminants subject to the proximity of the two companies - Waste Incinerator SAKO a.s. and Zetor traktors a.s. Picture (Figure 2) shows that the company's premises Zetor traktors a.s. in the pond is an excessive amount of zinc that can be transferred to the ground water fracture Stránská skála (Brzobohatý et al. 2001, Muchová 2014). The risk limits of zinc in soil is about 200 ppm (Ambrožová 2015). Zinc content in area of Zetor tractors a.s. company was average over 200 ppm.

On the territory of refraction were measured Hády excessive concentrations of copper, manganese, lead and especially sulfur. The primary source of sulfur are sulfides, which are formed by weathering and sulphates. The most common magnesium is calcium sulfate, which can be found near ponds (Šimek 2004).

CONCLUSIONS

On the area of national natural monuments Stránská skála were detected higher concentrations of copper, lead and manganese. To verify and identify the origin of higher concentrations of copper measurements were taken on the territory of the complex Zetor tractosrs a.s. These increased concentrations have been confirmed only by manganese. New discovered heavy metal was zinc, which are found in higher concentrations.

At the significant European locations southern slopes of Hades was an increased concentration of sulfur, specifically on the territory Růženin quarry and nearby tunnels linking Ruženin fracture and Džungle quarrry. On the territory of SLE Ruženin quarry also occurred elevated concentrations of copper and manganese.

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BIOINDICATION OF CLIMATE DEVELOPMENT ON THE BASIS OF LONG-TERM PHENOLOGICAL OBSERVATION

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Abstract: The aim of the study was to evaluate long-term observations of phenological phases „beginning of apricot flowering“ (BAF). Further to establish the level of dependence between the BAF Velkopavlovická variety and selected meteorological elements in the period before apricot flowering. For the evaluation the phenological and meteorological data from Velké Pavlovice station of Czech Hydrometeorological Institute Brno branch was used. Evaluated was a data from the period 1940–2008. The average onset of BAF phenophase in the Velké Pavlovice (Czech Republic, Central Europe) was found 11th April, i.e. 101st day of the year. The earliest flower of apricot was 18th of March 2007, the latest 6th May 1957. Linear trend-line shows an earlier onset phenophase approximately 13 days, i.e. 2 days per decade. To quantify the apricot demands on heat before the flowering the sums of effective air temperature from 0 to 10 °C for varying lengths of the period before flowering were calculated. The relationship between the accumulated growing degree days (AGDD) and BAF was assessed using correlation coefficients. Observed correlation coefficients varied from -0.46 to 0.50, suggesting a relatively weak dependence between the BAF and test meteorological characteristics. In connection with the assumption that flowering trees may be affected by heating of buds due to solar radiation was also performed an evaluation of the duration of sunshine. Significant effect on the flowering apricot, applicable for predicting of the onset of phenological stage, was not established. Nevertheless, the air temperature plays an important role on onset of apricot flowering phenophase is not its clear indicator. Methods based on AGDD calculations are quite successfully used in particular for determining of onset phenophases following the flowering. Attempts to forecast of the onset of the first phenophases, bud development and BAF are not entirely successful.

Key Words: air temperature, sum of temperature, apricot, flowering, prediction

INTRODUCTION

The prediction of the onset of phenophases of plants has significance especially in the area of agriculture, where it is used for the determination of an optimal term of application of means for the protection of plants, fertilizers and regulators of growth, during the selection (regionalization) of varieties, during the determination and forecast of the term of the harvest and the quality of the products, evaluation of the state of the stands, estimation of the impacts of the lack of moisture, and during the determination of the terms of sowing and planting. Phenology is finding its use also in medicine (determination of the term of occurrence of pollen allergens), in environmental sciences etc. In connection with the study of the change of climate, the knowledge of phenological data is proving to be a very important document of their impacts, mainly during the evaluation of the time series.

Long-term monitoring of phenological phases onset (forest species, fruit species and agricultural crops) used to be carried out by Czech Hydrometeorological Institute (CHMI). Sadly this monitoring, mainly of agricultural crops and fruit species, was significantly reduced in 2012. From the viewpoint of climate change, prolongation of vegetation season and occurrence of spring frosts (for instance in 2016) is interesting to observe and evaluate early phenophases of fruit trees species. This paper deals with the

data of apricot (*Prunus armeniaca* L.), Velkopavlovická variety. The variety had been known since 1931 and in 1954 was registered. Phenological data of genotype identical material are thus available.

Monitoring of apricot phenological phases is important mainly for varieties regionalization, the assessment of field operations terms and also for environmental monitoring (with regard to the length of the phenological observation). In recent years, also for preventive chemical treatments against the fungus *Monilia laxa* before flowering of apricot. Overview of stone fruits phenophasses is involved into the globally used scale BBCH – Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie (Meier 1997). Phenological observations on CHMI station are driven by methodology of instruction for phenological observers (in original: Návod pro činnost fenologických stanic, 2009) with its annex phenological atlas (in original: Fenologický atlas) by Coufal (2004).

Bažant et al. (1999) stated that biological activity of Velkopavlovická variety begins when mean daily air temperature exceeds 5.5 °C (from the end of dormancy). They found out 40day range of the earliest ant the latest „beginning of apricot flowering" (BAF) within 30year period of 1969–1998. As a driven factor they determined the sum of active temperature not length of interval in calendar days (amount of days). Pretty much identical range (37 days) for the same variety stated Vachůn (1986), who analyzed 37year period. Bláha (1990) and Morávek (1964) stated 7 °C as a biological minimum affecting BAF. According to Vachůn (1988) the biological minimum for genotypes of middle Asian group is 7 °C and for European eco-geographical group it is 8 °C.

MATERIAL AND METHODS

The subject of evaluation was onset of phenophase BAF, variety Velkopavlovická within the period 1940–2008 (longer homogenic database not available) at CHMI station Velké Pavlovice. Meteorological data were taken from CHMI station Velké Pavlovice – differences of meteorological conditions on climatological station and in orchard are usually minimal (Středa et al. 2011).

The data of BAF confronted with:

- Number of days with minimum, maximum and average air temperature above defined threshold.
- Sum of active or effective temperature above defined threshold.
- Number of sunshine hours.

Active air temperature is experimentally accessed value which is higher than i.e. biological minimum. It is the temperature which enables metabolic processes.

The effective temperature is the same value bud about the biological minimum lower. Sum of effective air temperature is cumulative value of effective temperature summed from defined term and is given in hourly or daily grades.

RESULTS AND DISCUSSION

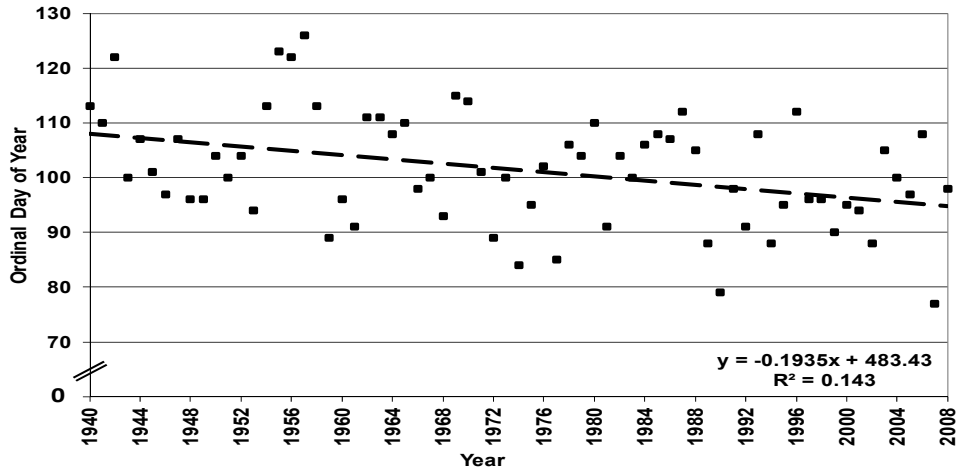
Figure 1 presents the onset of BAF in individual years from 1940–2008. Average onset of BAF is April 4 (i.e.101st day of the year). The earliest onset of BAF was in 2007 – March 3 and the latest was in 1957 – May 6. The difference between those two terms is thus 49 days. Even without the trend analysis the earlier onset of BAF towards the end of the period is obvious from the Figure 1. Linear trend line nevertheless proved earlier BAF onset about 2 days per decade, i.e. 13 days for whole period.

Vachůn (1988) states average date of phenophase "full apricot flowering" (FAF) as April 16–17 (i.e. 106th to 107th day of the year) in similar climatic condition (Lednice station, South Moravia, Czech Republic). The earliest FAF in this area was around March 25, the latest April 29. Totally latest FAF in South Moravia states Bláha (1990) – May 5 1942 for varieties Liabandova and Mandlová.

Extraordinary early FAF in open space observed Vávra (1963) in January 1 1956. This author also states that average onset of FAF in South Moravia is middle April. Rožnovský and Bauer (2004) analyzed data from 1961–2003 and found out 10% probability of FAF (Velkopavlovická variety, Velké Pavlovice) in March 27 and 90% in April 21.

Vachůn (1992) pointed out to certain degree of heterogeneity from flowering viewpoint. He discovered in frame of clones of Velkopavlovická variety the FAF of individuals varies about 1–5 days. This feature is vegetative maintained (Krška et al. 2005).

Figure 1 Beginning of apricot flowering in Velké Pavlovice during the period 1940 to 2008



Long-term trend of phenophases onset of 78 species of field and horticultural crops in Germany were evaluated by Estrella et al. (2007). The states average term of BAF as April 11. Trend analysis of 1951–2004 proved average annual shift of BAF of +0.074 day. The strongest relationship between BAF and mean monthly temperature was in March.

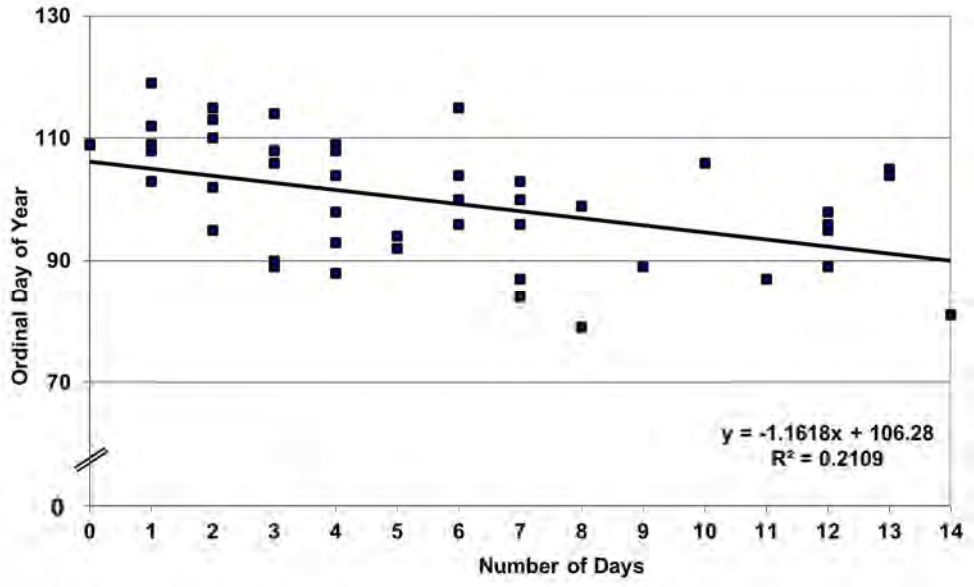
The next aim of the research was to quantify relationship between meteorological conditions of the year (expressed as SET) and BAF. Correlation analyses were carried out for BAF and:

- number of days with mean daily air temperature above 0, 3, 5, 6, 7, 8, 9, and 10 °C,
- SET of mean daily air temperature above 0, 3, 5, 6, 7, 8, 9, an 10 °C,
- number of days with minimum and maximum air temperature above 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 °C,
- SET of daily extreme values above 0, 1, 2, 3, 4, 5, 5, 5, 6, 7, 7,5, 8, 9, and 10 °C,

in all cases for all possible combination of differently long period before onset of BAF (i.e. several thousands of variants), when the starting point was always January 1.

The correlation coefficients reached up to -0.46 or 0.50. Those values evidence relative weak dependence between BAF and tested characteristics. The tightest and logically explainable relationship (correlation coefficient -0.46) was found out for BAF and number of days with minimum air temperature above 0 °C from 37th to 50th day before BAF (Figure 2).

Figure 2 Relationship between the number of days (horizontal axis) with minimum air temperature above 0 °C from 37th to 50th day before the date of flowering and term of BAF (vertical axis)



CONCLUSION

A significant shift in the onset of phenophase beginning of apricot flowering has been found out. Linear trend-line shows an earlier onset of phenophase beginning of apricot flowering approximately 2 days per decade.

Attempts to forecast of the onset of the first phenophases, bud development and beginning of flowering are not entirely successful. Main problem is determining end of the endogenous dormancy and significant weather fluctuation in winter and early spring.

From the observed characteristics number of days with minimum air temperature above 0 °C from 37th to 50th day before the date of flowering and term of beginning of apricot flowering can be recommended for predicting flowering apricot with a certain degree of imprecision.

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PUBLIC TRANSPORT SERVICEABILITY AS A FACTOR OF RURAL DEVELOPMENT

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Abstract: The paper is focused on the assessment of transport serviceability in rural municipalities in the Banská Bystrica district by public bus transport. Particular parameters of serviceability are able to reflect the level of rural development in terms of quality of life for inhabitants as well as offer of transport for visitors and tourists. Based on the categorization of municipalities into the size categories, the main differences among the groups and municipalities are highlighted. Very positive results were obtained in municipalities located at thoroughfares and municipalities with tourism potential, which creates wider range of connections with places of local, regional and national importance. The worst values were identified within the small rural settlements with less than 200 residents that are usually situated on the periphery and therefore they do not have conditions to create an increased demand for public transport. The presented results depict a municipal development in the light of public transport serviceability, which is a one of the factors affecting the migration of population.

Key Words: public bus transport, transport serviceability, rural municipalities, the Banská Bystrica district

INTRODUCTION

Serviceability in municipalities through public bus transport has significantly changed since the Velvet revolution. A successive privatization of the former branches of state-owned bus company has reflected in the qualitative and quantitative changes that have adapted to market conditions. One of the impacts has been a decrease in the proportion of public passenger transport at the expense of individual automobile transport (Horňák and Pšenka 2013). In spite of that, public transport has a relevant importance especially in the countryside. There is higher demand for this service compared to cities with wider offer of transport opportunities (Marada and Květoň 2006). The main function of transport serviceability is to ensure necessary connections for inhabitants mostly in the cases of commuting to work, school, health care facilities or public authorities (Poliak and Semanová 2013, Straková et al. 2016). This service has a large significance particularly for the non-driving category of people such as children, disabled persons or elderly population (Rahman et al. 2016). From the ecological and environmental point of view, public transport is more acceptable alternative to individual automobile transport. Just bus transport has a significant advantage in comparison with rail transport because of accessibility of network in any municipality, what makes serviceability by bus more flexible (Marada et al. 2010). From the viewpoint of quality and quantity of lines and connections, public transport depends a lot on bus transport as the prime medium of transportation (Wang and Qu 2015).

Legislatively, the process of ensuring of public transport in municipalities through bus transport up to 100 km comes under competences of self-governing regions that order this service from operators in public interest (Hejhalová 2009). Therefore, transport as a service presents a factor of rural development along with other indicators (Straka and Tuzová 2016), while transport connections affect the quality of life in rural environment (Kohutková and Baus 2012, Lee and Sener 2016). Moreover, the aspects of transport serviceability belong to social and economical characteristics of regional development (Mrázková 2004). The quality of transport infrastructure along with serviceability plays an important role in the municipal development for investors as well as for inhabitants, who make

decisions about their future place of permanent residence. Thus, transport serviceability may be the one of key attributes of quality of life in countryside (Boruta and Ivan 2010).

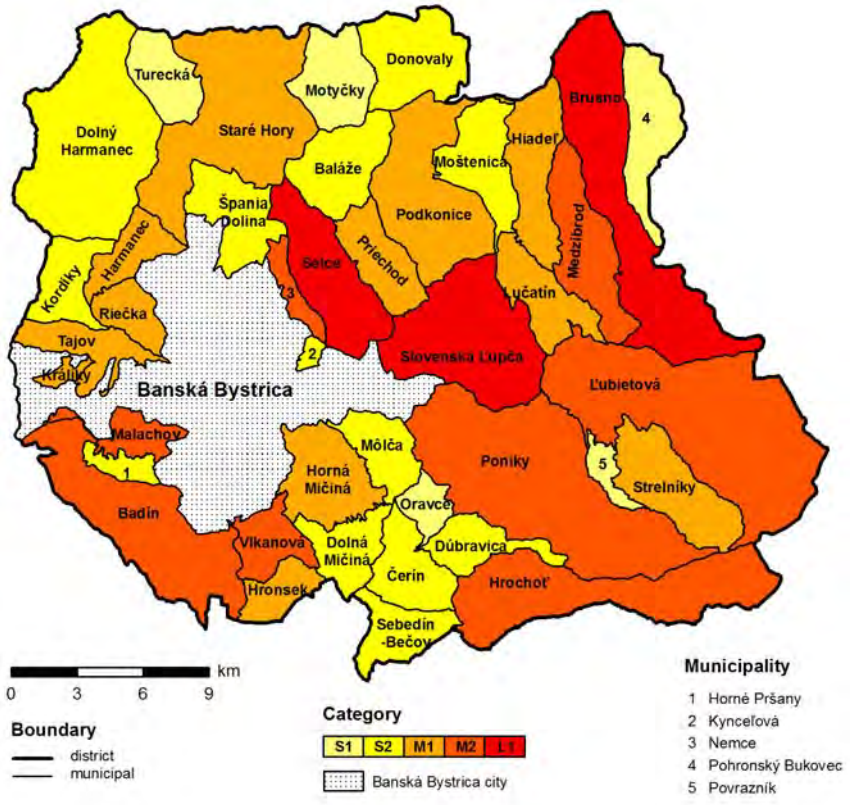
The aim of the paper is to present a comparative analysis of size categories of municipalities as well as particular settlements in the district of Banská Bystrica. It is the example of a heterogeneous region due to the various natural and socioeconomic conditions, which have formed a wide spectrum of rural municipalities with different functions and levels of public transport serviceability.

MATERIAL AND METHODS

Basic characteristics of the Banská Bystrica district

The target area is composed of 42 settlements, while there is only 1 city (Banská Bystrica) and 41 rural municipalities. In accordance with the methodology by Baran and Bašovský (1998), municipalities are divided into the size categories depending on the number of population (Figure 1). There are just 5 (12.2%) small municipalities with population to 199 inhabitants (abbr. S1), whereas there are 13 (31.7%) small municipalities in the group from 200 to 499 residents (abbr. S2). The district is also formed by medium-sized municipalities, while 12 (29.3%) of them have the number of population from 500 to 999 (abbr. M1) and 8 (19.5%) municipalities are characterized by the level from 1,000 to 1,999 residents (abbr. M2). The last category is formed by 3 (7.3%) large municipalities with population from 2,000 to 4,999 inhabitants (abbr. L1).

Figure 1 Structure of municipalities in the Banská Bystrica district based on the size categories



Geographically, the formation of settlement and transport network in the study area was affected mainly by natural conditions in the form of terrain segmentations. Three main mountainous units overlap into the district – Veľká Fatra, Low Tatras and Kremnica Mountains. They have become a major limiting factor for the development of settlement compactness, what also affects transport serviceability (Květoň et al. 2012) and characteristics of routes (Dandapat and Maitra 2014). Primarily owing to these factors, two main transport axes has shaped – the first in the north-south direction from Banská Bystrica through Donovaly to Ružomberok (road No. 59 (E77)) and the second in the west-east direction from Banská Bystrica through Brusno to Brezno (road No. 66). A localization of municipalities along the mentioned thoroughfares creates a prediction of more favourable level of serviceability due to their higher degree

of centrality. Another stimulating factor for transport serviceability may be developed tourism in some municipalities (Bačík 2016).

Methods and data

The basic information about municipalities was acquired from the Statistical Office of the Slovak Republic. The initial data on transport were obtained from the National information system on timetables of public bus transport valid to 6 September 2016. According to the extended methodology by Civáň, Némethová and Krogmann (2014), the indicators for evaluation were selected and further divided into the following two groups.

The first group covers basic parameters – number of population to 1 June 2016 (abbr. A1), area in km^2 (abbr. A2), population density in inhabitants per km^2 (abbr. A3), number of bus stops in municipality (abbr. A4), number of population per 1 bus stop (abbr. A5), percentage of population of the total number of population per 1 bus stop (abbr. A6), area in km^2 per 1 bus stop (abbr. A7), percentage of area of the total area in km^2 per 1 bus stop (abbr. A8).

Through the further calculations, the final indicators of transport serviceability were set – number of suburban (abbr. B1), regional (abbr. B2) and international (abbr. B3) bus lines that have at least 1 stop in the municipality (only regularly operating lines were included into the evaluation), number of bus connections in school days (abbr. B4), Saturdays (abbr. B5) and Sundays (abbr. B6), number of population per 1 bus connection in school days (abbr. B7), Saturdays (abbr. B8) and Sundays (abbr. B9), percentage of population of the total number of population per 1 bus connection in school days (abbr. B10), Saturdays (abbr. B11) and Sundays (abbr. B12).

RESULTS AND DISCUSSION

Within the first group of parameters, there is a directly proportional tendency based on the rising number of population in the size categories of municipalities (Table 1). The highest average value of population density (over 100 inhabitants per km^2) is associated with the group of medium-sized municipalities with level from 1,000 to 1,999 inhabitants. The number of bus stops usually reflects real needs in terms of settlement structure, while there are 4 stops per municipality on average. Even 11 stops are located in Poniky, mainly because of its large area that covers more than 59 km^2. The above-average values were acquired also in Badín (8 stops), Tajov (7 stops), Staré Hory (7 stops) and Slovenská Lupča (7 stops). The last two municipalities are the places with developed tourism. On the other hand, just 4 municipalities (Povrazník, Oravce, Baláže, Horné Pršany) were typical for only 1 bus stop, while all of them belong to the category of small municipalities (S1 or S2).

Table 1 Basic parameters of transport serviceability (average values)

Category	A1	A2	A3	A4	A5	A6	A7	A8
S1	135	9.16	21.74	2.00	91	67.31	4.56	49.84
S2	330	12.72	56.86	3.00	144	43.72	4.80	37.71
M1	709	14.56	89.72	4.58	177	24.94	3.67	25.24
M2	1,395	28.05	107.01	5.63	287	20.56	4.90	17.46
L1	2,520	31.94	85.89	5.67	454	18.01	5.62	17.60
All municipalities	785	17.22	74.10	4.05	198	25.18	4.52	26.25

Within the A5 parameter, values are typical for increasing trend according to the number of population. The mentioned tendency is much more noticeable here than in the case of an areal indicator (A7). The average area per 1 bus stop oscillates from 3.67 to 5.62 km^2. Proportional indicators (A6, A8) point out differences more precisely, because they show the increasing level of transport serviceability in accordance with the growing number of population. The municipalities in the S1 category are characterized by more than the two thirds of population per 1 bus stop. The situation in the L1 group is opposite, because less than the one fifth of population falls on the stop. The designed trend is registered within the areal indicator (A8), too. Small municipalities with no more than 199 inhabitants are typical for the fact that almost the half of their area falls on 1 bus stop. Conversely, the best values were acquired

in the M1 (17.46%) and the L1 category (17.60%). Averagely, approximately the one fourth of population as well as area falls on 1 bus stop. The results at the municipal level were affected mostly by the number of bus stops, thanks to that Poniky reached the best value (9.09%) along with the other municipalities with above-average number of stops.

Closer differences among the size groups as well as particular municipalities are visible through the final indicators combining transport and geographical aspects (Table 2). Within the total number of bus lines servicing municipalities (B1 + B2 + B3), there is an increasing trend depending on the rising categorization of municipalities. On average, three bus lines service each municipality, while two of them are suburban and just one regional. In the whole district, there is just one international bus line (routed from Praha to Brezno) that has a stop in the municipality of Slovenská Lupča. Significantly above-average values were identified in Staré Hory (11 lines; M1), Donovaly (12 lines; S2), Slovenská Lupča (15 lines; L1) and Brusno (16 lines; L1). Despite the fact that these municipalities belong to the various size categories, their common denominator is called tourism. Donovaly is a year-long tourism centre for sport and recreational activities, while Staré Hory represents the destination of religious tourism. They both have a favourable geographical position on the axis from Banská Bystrica to Ružomberok. Brusno is a municipality focused on spa tourism and Slovenská Lupča is the municipality with the highest number of population (3,244) in the district. Thus, the high number of bus lines is understandable. Both municipalities have also a suitable location within the district, since they are situated on the axis linking Banská Bystrica with the neighbouring district city Brezno.

In terms of number of bus connections servicing the size groups of municipalities during school days (B4), Saturdays (B5) and Sundays (B6), the proportional increase is registered according to the rising category. The municipality of Donovaly reached the best results in the S2 category, because it is serviced by 42 connections in school days, 22 connections on Saturdays and even 30 connections on Sundays. This municipality with only 225 residents emphasizes its own position among neighbouring municipalities, what is the one of tourism impacts. Within the M1 category, the best values were identified in Lučatín (116 connections in school days, 45 connections on Saturdays and 41 connections on Sundays). This municipality is serviced by 7 suburban bus lines, especially due to its location at the axis connecting Banská Bystrica and Brezno. Generally, suburban lines are typical for higher density of connections, thanks to that Lučatín cumulatively reached so positive results. Conversely, municipalities of Baláže and Podkonice are placed at the endpoints of road network and due to that they reached the worst results. They are serviced by less than 10 connections during Saturdays or Sundays. Within the M2 category, the most positive values were identified in the municipality of Medzibrod (71 connections in school days, 33 connections on Saturdays and 32 connections on Sundays). It has a common border with Lučatín and it is also located along the road No. 66 connecting Banská Bystrica with Brezno. Within the scope of transport serviceability, the factor of geographical location plays an important role. Among all municipalities, the best results reached Slovenská Lupča as a largest municipality in the district. It is serviced by 197 connections during school days, 76 connections on Saturdays and 70 connections on Sundays, what creates a wide range of connections to various directions at different time.

Table 2 Final parameters of transport serviceability (average values)

Category	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
S1	1.60	0.40	0.00	19	9	9	8	16	16	5.93	11.85	11.85
S2	1.69	0.69	0.00	29	12	13	14	34	34	4.24	10.30	10.30
M1	2.00	0.75	0.00	40	17	17	22	51	53	3.10	7.19	7.48
M2	2.00	0.00	0.00	44	16	17	35	103	102	2.51	7.38	7.31
L1	5.00	5.33	0.33	118	48	46	31	79	80	1.23	3.13	3.17
All municipalities	2.07	0.88	0.02	41	17	17	21	54	54	2.68	6.88	6.88

Further recalculated indicators (B7, B8, B9) show an upward trend in the number of inhabitants per 1 connection during selected days. The best results are typical for the M2 category and the worst values were acquired within the S1 category. These findings are affected mostly by the number of population and number of bus lines servicing municipalities. Brusno is the typical example, because it has 2,166 inhabitants and therefore belongs to the L1 category, but just 18 residents fall on 1 connection

in school days, 40 on Saturdays and 39 on Sundays. This presents better results than any in the case of municipalities in the M2 category. Within the S2 group, the most favourable values were registered in Donovaly (5 residents per 1 connection in school days, 10 on Saturdays and 8 on Sundays). There is a wide range of bus connections despite of dominance of tourists using cars. In terms of the M1 category, the most positive results were identified in Lučatín (6 inhabitants per 1 connection in school days, 15 on Saturdays and 16 on Sundays) that benefits from its location. Favourable findings were acquired also in Staré Hory (12 residents per 1 connection in school days, 22 on Saturdays and 19 on Sundays) not only due to its location, but tourism potential as well.

Proportional parameters (B10, B11, B12) reflect an increasing quality of transport serviceability based on the rising size category of municipalities. While almost 6% of inhabitants per 1 connection are registered within the S1 group, the results in the largest category (L1) are much more positive, because there were identified just 1.23% of residents per 1 connection. This designed tendency is obvious also during Saturdays and Sundays. More noticeable differences were identified in transitions between the S2 and the M1 as well as between the M2 and the L1 categories. There are not registered any negative changes between Sundays and Saturdays. This fact presents a very positive finding, because transport serviceability in municipalities is ensured during the whole week. The negative results in school days were identified in the municipality of Špania dolina. Although it has only 208 inhabitants (S2 category), it is serviced only by 10 connections, what is the lowest number among the all municipalities in the district. In Saturdays and Sundays, an inappropriate level of serviceability is registered in the municipality of Horné Pršany. It is serviced just by 4 connections per a day and the one fourth of the population falls on each connection. These values come out from its peripheral position, road network and absence of other stimulating factors. On the other hand, the best results were typical for the largest municipality (Slovenská Lupča), as only 0.51% inhabitants were identified per 1 connection in school days, 1.32% on Saturdays and 1.43% on Sundays. Other municipalities with suitable geographical location (Lučatín, Medzibrod, Brusno) reached very positive findings, too. Favourable values were identified also in Donovaly and Staré Hory, since no more than 5% of residents fall on 1 connection in any day.

CONCLUSION

Transport serviceability of rural municipalities in the district of Banská Bystrica by public bus transport depends mainly on the two dominant factors. Firstly, the position of municipality within the transport and settlement network, what is a determinant for quantity of suburban bus lines that create a cornerstone of transport serviceability. The second important parameter is the development of tourism that is able to ensure direct connections with other parts of Slovakia through regional lines that complete the transport mosaic.

Generally, the level of transport serviceability rises with the increasing size category of municipalities. Settlements typical for the higher concentration of population have qualitatively and quantitatively wider spectrum of lines and connections. On the other side, mainly natural barriers has disabled further development of settlement and transport network, what had negative impacts to the level of serviceability in many peripheral municipalities. Therefore, their inhabitants have at disposal an insufficient offer of lines as well as connections. There may be predicted higher dependence of population on individual automobile transport, mostly because of time flexibility in the necessity of travel.

The support of public transport at the expense of individual transport due to ecological and environmental purposes may be expected in the future. The level of transport serviceability in municipalities may become one of the key factors in the process of selection of future place of residence. Altogether, it may reflect the level of municipal development in terms of transport. Sufficient serviceability by public transport in direction from the municipality to city or main central settlement is necessary for various classes of population with different possibilities of mobility.

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INFLUENCE OF HABITAT CONDITIONS ON ABUNDANCE AND DIVERSITY OF SHREWS (*EULIPOTYPHLA*, *SORICIDAE*) IN MORAVIA

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Abstract: In the years between 2005 and 2012, this study has evaluated the relative abundance and diversity of insectivores of the shrew family (*Soricidae*) in lowland, upland, and mountain forest habitats of Moravia. In each of these three different elevation levels, two types of habitats were further defined. They include old growth forests, with tall, fruiting trees and a limited herbaceous forest floor, as well as forest clearings with dense undergrowth of herbs and grasses, which means a total of six types of habitats. Shrews were captured using snap traps set up in lines. A total of 302 individuals belonging to seven species were found. The most abundant and most dominant species was *Sorex araneus* (rA = 0.313; D = 73.45%), while other species were present in much lower numbers. To evaluate the communities, the used ecological indices included diversity, equitability, and similarity. The highest number of species was found in mountain clearings (n = 5), while the lowest occurred in old upland forests (n = 1). The highest diversity was in old growth lowland forests (H' = 1.194), the lowest in upland forests. In terms of abundance and diversity, forest clearings were richer than old forests, while mountain and lowland forests were richer than uplands forests. In terms of species, the most similar were communities of old growth mountain forests and mountain forest clearings. Forest clearings as early successional forest habitats with rich herbaceous undergrowth in lowlands and mountains proved to be important environmental refugia for this group of small mammals in the landscape.

Key Words: shrews, *Soricidae*, diversity, abundance, forest ecosystem, forest clearings

INTRODUCTION

Seven species of insectivores of the shrew family (*Soricidae*) occur in the Czech Republic. The common shrew *(Sorex araneus),* Eurasian pygmy shrew *(Sorex minutus),* Eurasian water shrew *(Neomys fodiens),* and the lesser white-toothed shrew *(Crocidura suaveolens)* occur nationwide, while the alpine shrew *(Sorex alpinus)*, southern water shrew (*Neomys anomalus)*, and bicolored shrew *(Crocidura leucodon)* occur regionally. The species *Sorex araneus, Sorex minutus,* and *Neomys fodiens* replicate with their elevation distribution of the occurrence the diversity of the relief of the Czech Republic. The species *Neomys anomalus* and *Crocidura leucodon* concentrate their occurrence in the middle positions of uplands (200 to 600 m above sea level), while *Crocidura suaveolens* occurs in the lowlands to uplands (140 to 400 m above sea level). Only the species *Sorex alpinus* has shifted its occurrence into foothills and mountain areas (Anděra 2010).

Due to intensive agriculture that is taking place today, the environment is changing, causing a total loss of shrews due to loss of habitat and food supply (Ryszkowski et al. 1973, Kozakiewicz and Kozakiewicz 2008). Compared with agricultural landscapes, larger populations of shrews occur in forests (Suchomel et al. 2012, 2014). Forests became for them important refugia in intensively farmed landscape, because their presence in the agrocoenosis is minimal (Heroldová et al. 2007).

The aim of this study is to assess the importance of selected types of forest habitats for the abundance and species composition of shrews. Up to this time, studies were concerned separately either with lowlands or mountains and moreover were parts of comprehensive studies of entire communities of small mammals, including rodents (Suchomel et al. 2012, 2014). Overall comparison of abundance and diversity of shrews at the altitudinal gradient is still missing.

MATERIAL AND METHODS

This study used data from surveys of small mammal communities from the years between 2006 and 2011. These surveys were carried out in the mountain forests of Moravian-Silesian Beskids and Hrubý Jeseník (High Ash Mountains), 640 to 1200 m above sea level, in the upland forests of Drahany Highlands and Kelečsko Upland, 450 to 660 m above sea level, and in the lowland forests of the Lower Morava Valley and Dyje-Svratka Valley, 173–233 m above sea level in Moravia, Czech Republic. Forest habitats were divided into six groups: (1) old growth lowland forests (2) lowland forest clearings, (3) old growth upland forests, (4) upland forest clearings, (5) old growth mountain forests, (6) mountain forest clearings.

Old forests are stands more than 100 years old, with a strong canopy and less developed herbaceous layer.

Forest clearings (plantations of young trees) represent early successional stages of forest growths that are not older than seven years, with the least-developed canopy and strongly developed herbaceous layer with coverage up to 100 per cent.

In each group of habitats, regular trapping of small mammals was carried out in the spring and autumn seasons, using snap traps. Traps were placed in a line at distances of 3 to 5 meters apart. Kerosene lamp wicks were used as bait. They were wrapped in flour, fried in vegetable oil, and then smeared with peanut butter. Traps were left in place for four days or three nights (so-called trap-nights), and checked each morning (Pelikán 1976). The numbers of used traps in individual areas differed due to the fact that they were parts of earlier independent research trappings. Table 1 summarises the total number of trap-nights at individual locations. All aspects of capture were in accordance with the provisions of EU Council Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes.

The study evaluated basic synecological characteristics such as the number of species (n), dominance (D) (Tischler 1949), as well as species diversity and similarity. For evaluation of diversity, the Shannon & Wiener index (H') was used, and equitability (E), which expresses the degree of equitable representation of individual species in biocoenosis. To determine the species similarity of different groups of biocoenoses, Jaccard index (Ja) (Magurran 1988) was used.

Due to different trapping methodologies (different numbers of traps) at individual locations, it was necessary to determine the relative abundance (rA). This is expressed as: rA = 100 n/P, where n is the number of captured individuals and P is the number of trap-nights.

RESULTS AND DISCUSSION

A total of 302 individuals of seven shrew species were trapped. The most abundant species was common shrew *(Sorex araneus)* (n = 245, rA = 0.313, D = 73.45%), followed by bicolored shrew *(Crocidura leucodon)* (n = 34; rA = 0.051, D = 15.41%), both species were eudominant (D > 10%). Eurasian pygmy shrew *(Sorex minutus)* (n = 13; rA = 0.040; D = 7.01%) was dominant (D = 5–10%). The lesser white-toothed shrew *(Crocidura suaveolens)* was a subdominant species (D = 2–5%, n = 6; rA = 0.004, D = 3.18%. The alpine shrew *(Sorex alpinus)* (n = 2, rA = 0.002; D = 0.45%), Eurasian water shrew (*Neomys fodiens*) (n = 1, rA = 0.002; D = 0.40%) and the southern water shrew (*Neomys anomalus*) (n = 1, rA = 0.001, D = 0.10%) were the least abundant species and all were subrecedent (D < 1%). Table 1 shows the number of trapped individuals and their dominance at each of the locations.

Sorex araneus was the most abundant species in all monitored habitats. This is a very adaptable insectivore species. It lives in all forest types, meadows, bogs, windbreaks, in agrocoenosis and parks. However, it prefers wetter types of forests with deep soil and hygrophilic undergrowth, where it finds suitable living conditions, such as wide range of invertebrates and suitable microclimatic conditions. In contrast, it avoids the dry forest stands with the poor herb layer (Anděra and Horáček 2005, Baláž 2005, Anděra 2010). It seems that if the essential habitat requirements are met, then it may occur relatively frequently in a wide range of altitudes, both in mature stands, as well as in early successional stages such as forest clearings. It was most common in mountain forest clearings (n = 158, rA = 0.717) and least common in old growth upland forests (n = 9, rA = 0.027). This may be due to the fact that they are mostly same-age production monocultures of *Picea abies* and *Fagus sylvatica,* with the least developed

herbaceous layer, which are poorly suited for the presence of small mammals (Suchomel and Urban 2011). Table 1 indicates that other species of shrews were significantly less common, and in comparison with the common shrew occurred only marginally or occasionally.

Table 1 Values of Dominance (D), relative abundance (rA), number of individuals (n), number of trap-nights (NTP), Shannon index (H') and equitability index (E) among individual species on particular plots (1-6)

Species/Biotope	1			2			3			4			5			6			Total		
	n	D (%)	rA	n	D (%)	rA	n	D (%)	rA	n	D (%)	rA	n	D (%)	rA	n	D (%)	rA	n	D (%)	rA
Sorex araneus	10	52.63	0.024	10	23.81	0.099	9	100.00	0.027	15	78.95	0.714	43	89.59	0.299	158	95.75	0.717	245	73.45	0.313
Sorex minutus	2	10.53	0.005	1	2.38	0.010	0	0.00	0.000	4	21.05	0.190	3	6.25	0.021	3	1.82	0.014	13	7.01	0.040
Sorex alpinus	0	0.00	0.000	0	0.00	0.000	0	0.00	0.000	0	0.00	0.000	1	2.08	0.007	1	0.61	0.005	2	0.45	0.002
Neomys fodiens	0	0.00	0.000	1	2.38	0.010	0	0.00	0.000	0	0.00	0.000	0	0.00	0.000	0	0.00	0.000	1	0.40	0.002
Neomys anomalus	0	0.00	0.000	0	0.00	0.000	0	0.00	0.000	0	0.00	0.000	0	0.00	0.000	1	0.61	0.005	1	0.10	0.001
Crocidura suaveolens	3	15.79	0.007	0	0.00	0.000	0	0.00	0.000	0	0.00	0.000	1	2.08	0.007	2	1.21	0.009	6	3.18	0.004
Crocidura leucodon	4	21.05	0.010	30	71.43	0.297	0	0.00	0.000	0	0.00	0.000	0	0.00	0.000	0	0.00	0.000	34	15.41	0.051
Total	19	100.00	0.046	42	100.00	0.416	9	100.00	0.027	19	100.00	0.905	48	100.00	0.333	165	100.00	0.749	302	100.0	0.413
Number of species		4			4			1			2			4			5			7	
NTP		41385			10098			33000			2100			14400			22032			123015	
H'		1.194			0.760			0.000			0.515			0.433			0.207			0.518	
E		0.861			0.548			0.000			0.742			0.312			0.129			0.432	

The richest habitat in terms of species were (6) mountain clearings with 5 species, while the poorest habitat for species were (3) old growth upland forests with 1 species. Evaluation of dominance points to a greater or lesser extent disturbed habitats, because all habitats displayed superiority of eudominant or subrecedent species (Tischler 1949). The highest diversity index and the most balanced community was identified in (1) old growth lowland forests (H' = 1.194; E = 0.861), while the lowest values of both indices were in (3) old growth upland forests (H' = 0; E = 0). Overall, in terms of diversity and abundance of populations, lowlands and mountains appear richer than uplands. Even though shrews, mainly the common shrew, may in some forest habitats, especially in mountain forests with dense and species-rich herbaceous layer, achieve dominant values in the communities of small mammals (Suchomel et al. 2014), their abundance in forests, in comparison with other small mammals (mainly rodents), is relatively low (Suchomel et al. 2012, 2014). The same ratio is also in other types of habitats, including agrocoenoses (Heroldová et al. 2007). As the main cause of low abundance of shrews are quoted to be changes in the landscape due to human activity (intensive agriculture, climate change), leading to loss of suitable vegetation cover and food sources (Ryszkowski et al. 1973, Kozakiewicz and Kozakiewicz 2008). Despite the low proportion of insectivores in the community of small mammals, lowland forests (including isolated fragments) represent important habitats in intensively farmed landscape for the conservation of their populations and diversity (Suchomel et al. 2012). In this respect, mountain forests are then even more important refugia, especially in terms of their abundance (Suchomel et al. 2014).

In terms of species, the most similar habitats were (5) old growth mountain forests and (6) mountin forest clearings (Ja = 80 %). In contrast, the least similar habitats were (3) old growth upland forests and (6) mountain forest clearings (Ja = 20%). Table 2 shows the species similarity among other forest habitats.

Table 2 Jaccard index (%)

Biotope	(6) Mountain forest clearings	(5) Old growth mountain forests	(4) Upland forest clearings	(3) Old growth upland forests	(2) Lowland forest clearings
(1) Old growth lowland forests	50.00	60.00	50.00	25.00	60.00
(2) Lowland forest clearings	28.57	33.33	50.00	25.00	-
(3) Old growth upland forests	20.00	25.00	50.00	-	-
(4) Upland forest clearings	40.00	50.00	-	-	-
(5) Old growth mountain forests	80.00	-	-	-	-

Table 1 shows the important role of forest clearings in lowlands as well as uplands and mountains. Table 1 also indicates that the number of shrew species and their abundance are significantly higher in forest clearings than in the old growth forests. This is because their richly developed herbaceous layer provides the necessary protective cover, to which a number of insect species is tied as a food source. The most suitable in this respect are mountain forest clearings. Their herbaceous layer is richly diverse with many species of monocotyledon grasses and dicotyledon herbs (Suchomel et al. 2014). Regardless of the method of management, the presence of early successional stages of forest growths (plantations of young trees) is very important because as open habitats near forest, they serve as refugia for populations of both forest species and species of open habitats (Suchomel et al. 2012).

The current forest management, through creating clearings and clearcuts and their subsequent reforestation, creates appropriate living conditions for small terrestrial mammals, both in terms of their numbers and diversity. Although mostly dominant are species that come in conflict with forest management (Heroldová et al. 2012), especially the various species of rodents, the forest clearings may represent important refugia even for insectivores. This situation may change, for example due to prolonged exposure of negative factors, such as climate change, associated with harmful abiotic and biotic factors that would force the forestry operation to adopt different ways of management.

CONCLUSION

This study highlights the importance of forest habitats as refugia for shrew insectivores. Most important in this respect are mountain forests, especially the forest clearings, where shrews are most abundant. This may be related to local conditions, especially with plenty of moisture, which allows a rich growth of vegetation and sufficient amounts of invertebrates tied to it. Of considerable importance are lowland forests, where the frequency of shrews is significantly lower, but the detected diversity is the highest. For this reason, the importance of lowland forests in agricultural, intensively farmed landscape is considerable. Among the forest habitats, the forest clearings are more important than older forests, due to harbouring more species and larger populations. As for the shrews, the forest habitats are most important for the common shrew, which represented the most abundant and most dominant species (D > 70%) of all shrew insectivores.

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ACTIVATING BIOCHAR AND ITS INFLUENCE ON AERBUSCULAR MYCORHIZAE

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Abstract: Biochar is carbonized organic matter rich in carbon. Application of biochar into the soil is considered as way for mitigating climate change. Its influence on soil is controversial. According many studies, biochar has positive effect on soil properties, especially on chemical and physical properties. Assessing the impact on the biological component is problematic because biochar contains common aromatic substance which could be toxic for plant and microbiota. Our study wants to use simple fast and cheap process for so-called ageing (activating) of biochar. Biochar was ageing-activating in aeration water environment during two weeks. After this period, activating biochar was used the same way like conventional biochar. Six variants of experiment were established, including variant with compost and combination of different type of biochar and compost. Significant differences between individual kinds of biochar in plant biomass production were not detected, but the measured results indicated positive effect of compost application on decrease in phytotoxicity of biochar. On the other hand the application of activated biochar had significant positive influence on colonization of indicator plant roots by arbuscular mycorrhizal fungi about 50% in comparison with variant where conventional biochar was applied.

Key Words: biochar, arbuscular mycorrhizae, *lactuca sativa*, terra pretta, compost

INTRODUCTION

Biochar is a product of pyrolysis with high concentration of carbon. According to many studies (Atkinson 2010, Lehmann 2011), we can produce quality biochar from various organic materials, including waster whose removal could be very costly or dangerous for environment.

Biochar is organic fertilizer, which has a positive effect on the formation of soil aggregates, soil reaction and sorption complex. Its application to the soil is supported by the natural carbon cycle where carbon returns back to its natural reservoir (Busscher et al. 2010, Kammann et al. 2012).

The application of biochar into the soil unfortunately offers many problems, this material is formed under high temperature and pressure which means biochar in high probability contains toxic substances which could be very toxic to the soil microflora and the plant as well (Pietikäinen et al. 2000, Yin et al. 2000, O'Neill et al. 2009, Liang et al. 2010). Biochar is often compared to soils Terra Pretta, soils occur for example in the Amazon and they are one of the most fertile soils in the world. These soils originated hundreds years ago in the nearby Indian villages. Their basis is also charring organic matter, however, these soils were still left to the natural elements and, according to many studies (Lehmann 2011), their foundation was enriched by fresh organic matter (food residues, plants, faeces etc.). Land, which was supplemented by biochar in recent decades, cannot be the same quality as soil Terra Pretta. Fresh biochar still contains substance like pyrene of naphthalene, this substance used to be degraded, but it is very time-consuming (decades to centuries), this step is so-called ageing biochar (Sombroek et al. 2003).

Arbuscular mycorrhiza is a natural part of healthy and fertile soils. In terms of symbiosis, this is beneficial for both the host plant and the soil fungi**.** From the relation, the plant obtains inorganic substances and nutrients and mycorrhiza provides carbohydrates **(**Farooq 2009, Augé 2001). However,

excessive colonization of plant roots by arbuscular mycorrhiza points to the fact that the soils is stressed. Mycorrhiza increases root system up to 7 times. Moreover, it enables the plant to withdraw nutrients and moisture from distant microenvironment. If the root is surrounded by unfriendly soil, such as soil containing heavy metals, fungi can create sufficient distance from the pollutant and thereby they are able to ensure the survival of plants (Mukherjee 2011). For this reason, it is very difficult to determine whether the high mycorrhizal colonization by fungi is indicator of positive or negative effect of additives.

The main aim of the presented work was to found potential differences in effects on development of soil microbial communities and biomass production between fresh (conventional biochar) and biochar, which has previously been subjected to activation in water, aerated environment.

MATERIAL AND METHODS

Experimental soil

Experimental soil was removed from area Březová nad Svitavou in depth of 0–25 cm in accordance with ČSN ISO 10 381-6 (ČSN – Czech Technical Standard). Soil samples were sieved through a 2 mm mesh sieve, and left in thermostat for stabilization (4 °C).

Table 1 Variants of experiment

Variants	Fertiliser	Repetition
Bi + C	Activated biochar (50 t/ha) + compost (50 t/ha) 1 : 1	4
B	Biochar (50 t/ha)	4
C	Compost (50 t/ha)	4
B + C	Compost (50 t/ha) + Biochar (50 t/ha) 1 : 1	4
Bi	Activated biochar (50 t/ha)	4
K	Control	4

Design of experiment

Experiment was prepared by Elbl et al. (2014): Six variants of the experiment were established, each one in four repetitions. The experimental pots were placed in a phytotron at a day temperature of 20 °C and a night temperature of 18 °C, light intensity of 350 µmol/m^2/s. Length of the day was 12 h.

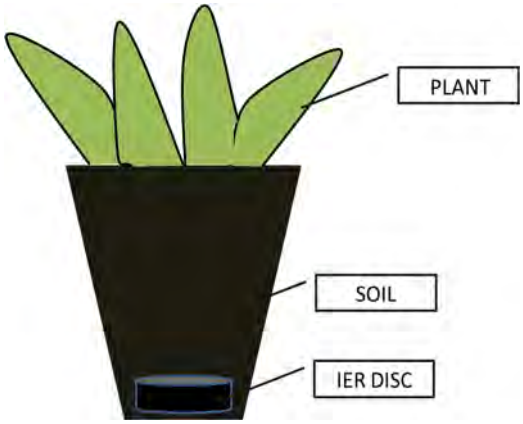
As experimental plant, salad *(Lactuca sativa* L.) was selected due to rapid growth of its biomass and sensitivity to changes in the substrate. To each container, 1000 g of soil was put and enriched with the addition of fertilizer. The control variant consisting of puresoil was prepared. Characteristics of the variant is given in Table 1. Experiment was founded on the 3rd January 2016 and ended on the 2nd March 2016. Subsequently, production biomass and level of root colonization by arbuscular mycorrhizal fungi (AMF) were measured.

Biochar

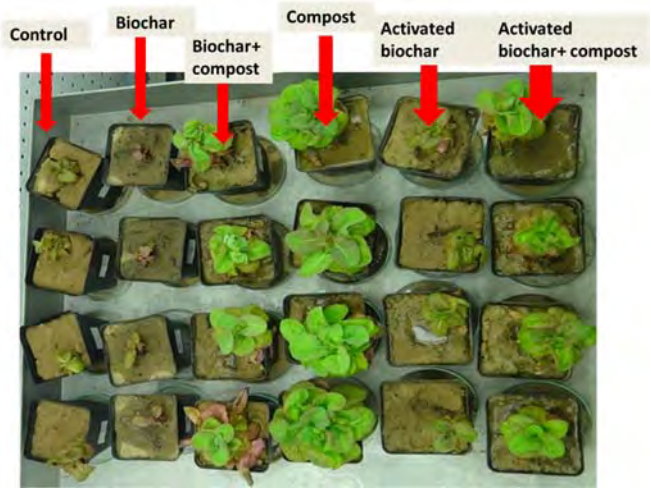
Biochar is certified in complying with the conditions of European Biochar Certificate and UK Biochar Quality Mandate. The principle of dry carbonisation is used by PYREG® system. At the very beginning, the biomass is heated up to 500–600 °C in the PYREG® reactor where biomass is not incinerated but carbonised to biochar. The second level encompasses complete burning of the syngas produced in the reactor. The temperature in the combustion chamber is about 1.250 °C. Information was obtained from PYREG GmbH (more information: www.pyreg.de).

Figure1 Experiment design (a) Pictures of differences between variants (b)

a) Design of experimental pot



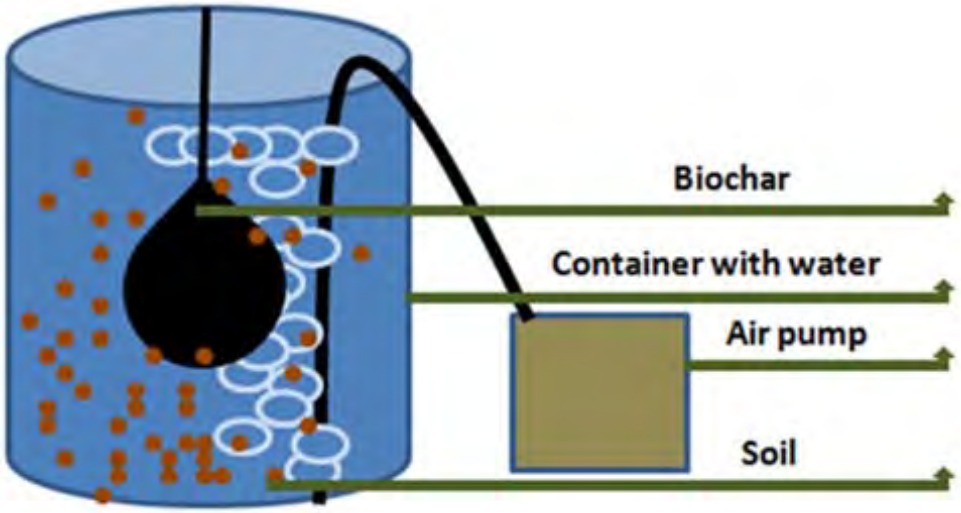
b) Differences between individual variants



Biochar biological activation process

Biochar was placed into the fabric and attached to a plastic container which was filled with distilled water (20 l), 50 g of soil from the area of Březová nad Svitavou was add in the container and stirred. The vessel aeration was carried out for 14 days. Afterwards, the activated biochar was removed, kept 2 hours under laboratory conditions, and used in the same manner as the inactivated biochar.

Figure 2 Schema of activating biochar



Root colonization by arbuscular mycorrhiza

Lactuca sativa was chosen as the experimental plant. Colonization of the roots of the plants was carried out according to the methodology of Koske and Gemma (1989) and it took place in several steps. From the underground biomass, each test vessel was trimmed off 5 g roots. The roots were projected and stored in FAA (50% ethanol, acetic acid, formaldehyde) solution during 24 hours.

After the roots were stained, solution of 10% KOH was used in order to brighten the roots. It was followed by immersion in 1% HCl for the acidification and finally the roots were stained with trypan blue in lactophenol. Stained roots were cut into 1 cm fragments, and observed under a microscope using magnification of 200X, Giovanetti and Moss (1980).

RESULTS AND DISCUSSION

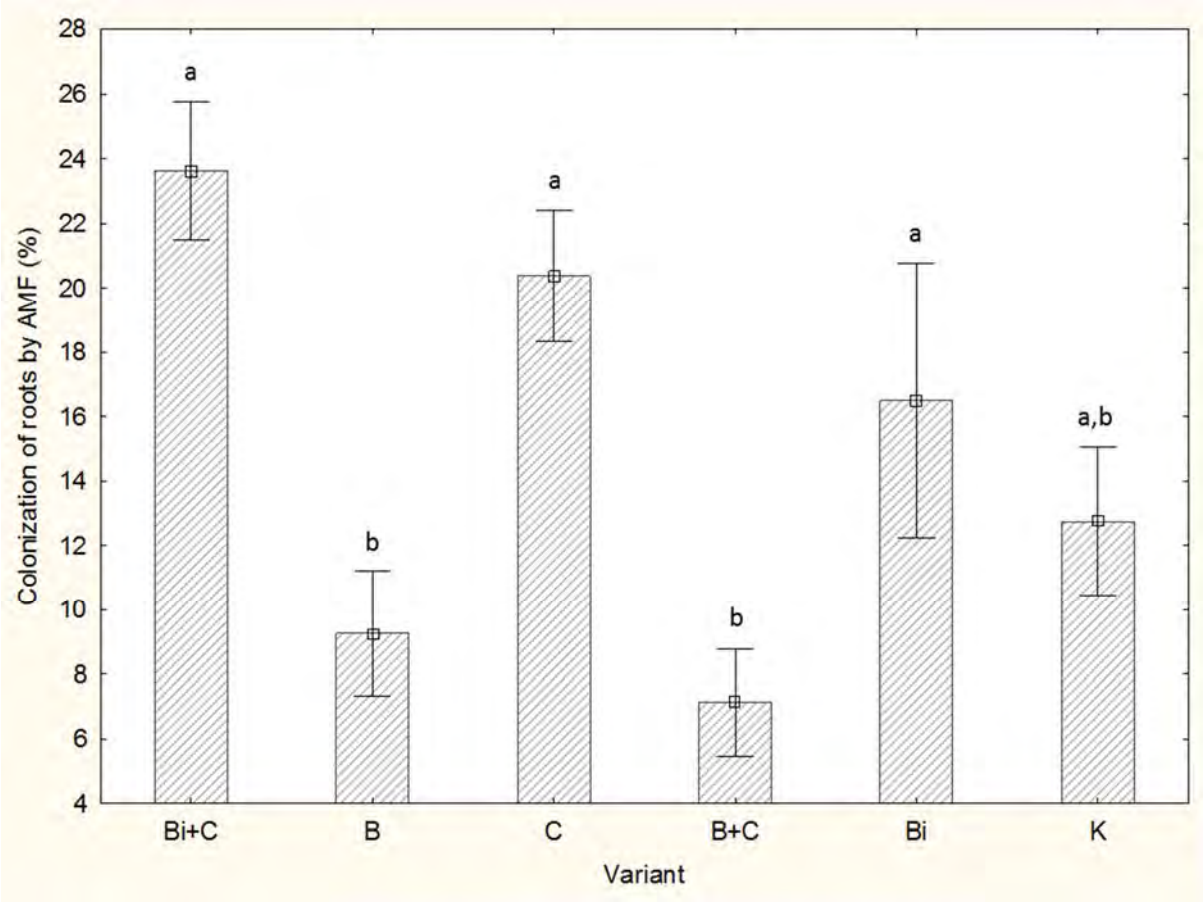
Colonization arbuscular mycorrhiza

Colonization of arbuscular mycorrhiza was measured in all variants at the end of the experiment and was in the range of 7–25% As the results show, the highest root colonization of arbuscular mycorrhiza was found in variants Bi + C (combination of compost and activated biochar) in comparison with the control, followed by a variant of Bi (activated biochar). However, these differences were not significant. Significant differences were only detected between variants B; B + C and other variants (except control). These data indicated that soil microbes were motivated to create cooperation with plant in variants B + C, C; Bi and K. Davies et al. (1992), Tobar et al. (1994), Miransari et al. (2008) state that increased colonization of root by arbuscular fungi (AMF) is result of stress. It may be a lack of moisture, nutrients or the presence of contaminants.

Above all, Smith and Read (2008) reported that there is a connection between the level of AM and the state of the microbial communities in rhizosphere. Therefore results of AM can show potential effect of the above soil amendments on microbial communities in rhizosphere zone.

In both variants, where activated biochar was used, increasing of colonization was measured, compared to conventional biochar. The explanation can be twofold. 1) Activated biochar did not contain a high concentration of substance which inhibited the growth of mycorrhiza, compared to the conventional biochar. 2) Conversely, activated biochar contains substances that make the plant not forced to invest in symbiosis with fungi. The substances in the biochar could be a slightly degraded due to its activation.

Figure 4 Colonization of salad roots by arbuscular fungi



Legend: Bi + C – Compost (50 kg/ha) Activated biochar (50 t/ha) 1:1, B – biochar (50 t/ha), C – Compost (50 kg/ha), B + C – Compost (50 kg/ha) Biochar (50 t/ha) 1:1, Bi – Activated biochar (50 t/ha), K – Control. Mean values ± SE from four repetition (n = 4) are presented, different letters indicate significant differences – ANOVA with Tukey test, P < 0.05.

As part of the experiment, biomass production was measured (see Table 1) *Lactuca sativa* L. (salad) was used as indicator plant due to its ability to react to small changes in soil properties (D´antuona and Neri (2001). There were found big differences between individual variants. The Figure 1 – part b presents important information about effects of biochar application on plant biomass production. These results, together with data from Table 2, clearly show positive effect of compost application on plant biomass production (Diaz and Bertoldi 2007) and decrease in phytotoxicity of biochar (Sparks 2011). The significantly higher biomass production was found in variant C where compost was applied. The second highest biomass production was detected in variant where mixture of compost and biochar was applied – significant in comparison with variant where only biochar was applied. Significant differences between individual kinds of biochar were not detected. On the other hand, the variant, where activated biochar was applied, show higher production of plant biomass in comparison with conventional biochar. This data can indicate that activated biochar was less (phyto)toxic than conventional biochar. For example, Borchard et al. (2012) found potential use of activated biochar – reduction in leaching of N a P from soil and decrease of phytotoxicity of conventional biochar.

Table 2 Biomass production

	Total biomass biomass production	Standard deviation	HSD (P < 0.05)
Bi + C	2.67	0.32	a
B	0.15	0.01	b
C	4.27	0.54	c
B + C	2.19	0.08	a
Bi	0.95	0.02	b
K	0.69	0.17	b

Option B (conventional biochar) and Option B + C (a combination of compost and convention biochar) demonstrated a reduction in root colonization compared to control. Toxic aromatics in a conventional biochar is likely to reduce colonization of the root mycorrhiza. Total biomass production = sum of aboveground and underground biomass production.

CONCLUSION

Presence of Arbuscular mycorrhiza represents very important soil property (Wright 1996). More than 52 million ha of land in countries of the European Union is threatened by desertification due to climate change (COM 2002). Arbuscular mycorrhiza increases root surface and thus enables the plant distant moisture. Applications of activated biochar supported the colonization of roots, in comparison with conventional biochar. Placing biochar in aerated water environment can reduce the toxicity of biochar to the limit, but it was not sufficient for the development of microbiological organisms in the soil. For deep understanding effect of activated biochar, it is necessary to analyse activated biochar and determine the concentration of aromatic compounds.

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INCREASING THE RESISTANCE OF MICROORGANISMS TO STRESS BY DROUGHT

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Abstract: Desertification is becoming a global problem and use of soil amendments is one of the options to decrease the danger. Many authors indicate that biochar can affect soil hydro-limits in a positive way. Unfortunately biochar also affects biological properties of soil. Predictions of the effect of biochar application are very difficult and must be based on aromatic substance concentration and on the dosage of biochar. In our experiment we prepared pots for establishing the effect on different substrates. Each container contained two separate parts with two different substrates. We measured the concentration of base groups of microorganisms on *Lactuca sativa* roots and the decomposition of cellulose. According to our results biochar can mitigate the impact of stress caused by drought on soil microorganisms. These amendments did not affect cellulose decomposition.

Key Words: biochar, winter wheat, activated carbon, CFU, desertification

INTRODUCTION

Climate changes cause loss of fertile soil all around the world. According to COM, more than 52 million ha in European Union are threatened by desertification and this number is growing. Hueso-Gonzalez et al. (2013) say using soil amendments can mitigate the impacts of climate changes that are soil erosion or desertification.

Biochar is a carbonized organic matter which can improve carbon sequestration, the stability of aggregates and soil retention as well (Besley et al. 2010). As Hardie et al. (2014) said the addition of biochar as fertilizer leads to increased water content at the permanent wilting point. On the other hand biochar is the product of pyrolysis. High temperature, high pressure and an atmosphere with low concentration of oxygen or oxygen–free are typical for this process. Unfortunately pyrolysis also used to produce toxic substances such as naphthalene or pyrene. According to Novak et al. (2009) studies these substances decrease biological activities during decades and centuries after biochar application, but eventually they decompose into non-toxic form as carboxyl acid.

The aim of our study was determinate effect of biochar or activated carbon on soil microbiota during dry season.

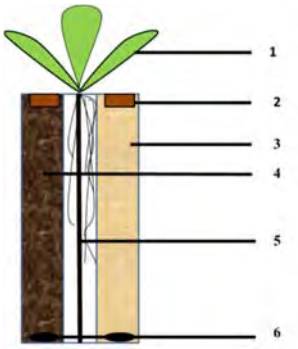
MATERIAL AND METHODS

Design of experiment

Experimental soil was collected in June 2015 from the area of Březová nad Svitavou, according to ČSN ISO 10 381-6 (ČSN – Czech Technical Standard). Soil samples were sieved through a 2 mm mesh. Experimental pots with two different chambers for substrates were used. These two chambers were separated by plastic iris. Between the substrate and the plastic iris was a polyamide mesh. As experimental plant *Lactuca sativa* was used. The plant was precultivated during a period of two weeks. After this period its roots were divided into two equal parts and put in the pot between the polyamide mesh and the plastic iris, according to figure 1. This means that in each container there was one plant, its root system divided into two equal parts and each part was placed in a different substrate. One part of the pot always included a control soil. Each container was filled with 4.2 kg of soil and two types of

amendments were used (Table 1). Different water regime was maintained during the experiment. Thirty percent available water capacity was maintained in containers with dry condition, and 70% available water capacity was in containers with wet condition.

Figure 1 Sectional view of experimental pots



Legend: 1 – Experimental plant (Lactuca Sativa), 2 – Mash bag with cellulose, 3 – Control substrate (Bare soil), 4 – Substrate with amendment, 5 – Plastic iris, 6 – IER discs

Table 1 Characteristic of variants

Variants	Characteristics	Repetitions
Ks	Control – Dry condition	4
Km	Control – Wet conditions	4
Am	Active carbon (50t/ha) – Wet conditions	4
Am k	Active carbon – Wet conditions –Control	4
As	Active carbon (50t/ha) – Dry condition	4
As k	Active carbon – Dry condition – Control	4
Bm	Biochar (50t/ha) – Wet conditions	4
Bm k	Biochar – Wet conditions –Control	4
Bs	Biochar (50t/ha) – Dry condition	4
Bs k	Biochar – Dry condition – Control	4

Microbiological analysis

For estimation of the microbiological activity in roots the dilution plate method was used. Four groups of microorganisms were analysed: the total amount of microorganisms, Actinomycetes, Nitrogen-fixing bacteria and Fungi. The determination was carried out according to CSN EN ISO 6887–1. The same methodology was used for choice and preparation of agars. One millilitre of the samples was watered by agar and left in constant temperature to grow as seen in Table 2.

Table 2 Summarization of methodology for estimate basic soil microorganisms group

Physiological groups of microorganisms	Agar	Temperature	Time
Total amount of microorganisms	MPA nonselective medium	30 °C	72 h
Actinomycetes	Ammonia agar	30 °C	120 h
Nitrogen-fixing bacteria	Ashby agar	25 °C	120 h
Fungi	Czapek Dox	30 °C	120 h

Litter mesh bag

Known amount of cellulose was used for establishing cellulose activity during the whole experiment. Cellulose was put into plastic bag (10 cm × 5 cm) and the bag was applied in the depth of 0–10 cm below surface. After the end of experiment the mesh bag was removed and the weight was

measured. The loss of cellulose was established by annealing. The analysis was performed based on Bocock and Gilbert (1957).

RESULTS AND DISCUSSION

Microbiological analysis

Dilution plate method was evaluated. Out results can be seen in Table 3 and Figures 2 to 6. We wanted to compare the results among different variants and results within the individual container (the two separate parts).

Table 3 Results of microbial analysis among different variants

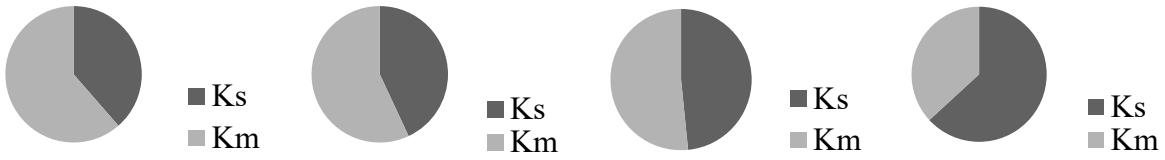
	TNM – Total number of microorganism (CFU)	Fungi (CFU)	Nitrogen – fixing bacteris (CFU)	Actinomycetes (CFU)
Km	670000	28200	1012	1400
Ks	421000	21300	950	1350
Am	676700	28400	12650	2800
As	509000	22050	10200	2195
Bm	422800	28850	1720	2270
Bs	1199600	51900	17450	4290

As Table 3 shows variant which was maintained in ideal water conditions has high colonization, on the other hand the variants which were stressed by drought resulted in decreased colonization. The only exception was found in the variant with biochar. Water is necessary for microorganism growth. According to many studies (Karhu et al. 2011, Laird et al. 2010, Ahmad et al. 2010) biochar can modify soil hydrolimits and because of that the stress by drought could not have effect on TNM. The highest colonization was found in the Bs variant, lowest in the Ks.

For Fungi, Nitrogen-fixing bacteria and Aktimomycetes the same trend was observed. The highest production was indicated in Bs variant, the lowest in Ks variant.

Figure 2 Results in Control variants

A) TNM *B) Fungi* *C) Nitrogen-fixing bacteria* *D) Actinomycetes*



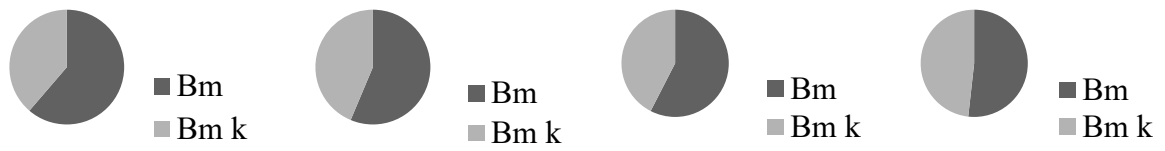
Legend: Ks – Control (Dry condition), Km – Control (Wet conditions). Different colours means different part of containers.

For control variant, higher colonization was measured in containers with moisture treatment than in containers stressed by drought. The only exception were the Actinomycetes (Figure 2).

This group has got a different survival strategy, Actinomycetes can use water and nutrients from larger environment then the other groups.

Figure 3 Results in Variant with biochar and wet condition

A) TNM *B) Fungi* *C) Nitrogen-fixing bacteria* *D) Actinomycetes*

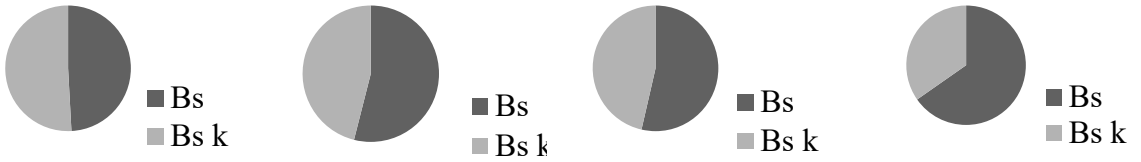


Legend: Bm – Biochar (Wet conditions), Bm k – Biochar (Wet conditions – Control). Different colours mean different part of containers.

If we compare colonization in the part of container where biochar was applied and where there was maintained the ideal moisture, with the control in the same container, we can see the higher number in biochar treatment for all groups of microorganisms (Figure 3).

Figure 4 results in Variant with biochar in condition stress by drought

A) TNM *B) Fungi* *C) Nitrogen-fixing bacteria* *D) Actinomycetes*

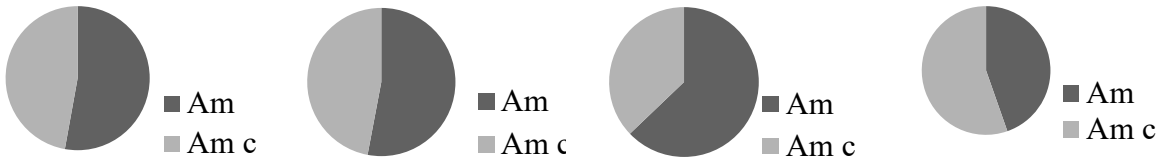


Legend: Bs – Biochar (Dry condition), Bs k – Biochar (Dry condition – Control). Different colours mean different part of containers.

In variants stressed by drought where one part was treated with biochar and the other was control, our results show lower colonization for biochar in TNO and higher for Fungi, Nitrogen-fixing bacteria and for actinomycetes as well (Figure 4).

Figure 5 results in Variant with active carbon and wet condition

A) TNM *B) Fungi* *C) Nitrogen-fixing bacteria* *D) Actinomycetes*



Legend: Am – Active carbon (Wet conditions), Am k – Active carbon (Wet conditions – Control). Different colours mean different part of containers.

This set of samples was in ideal moisture and consists of control and the part with active carbon. The highest colonization was measured for active carbon in all groups except Actinomycetes (Figure 5).

Figure 6 results in Variant with active carbon in condition stress by drought

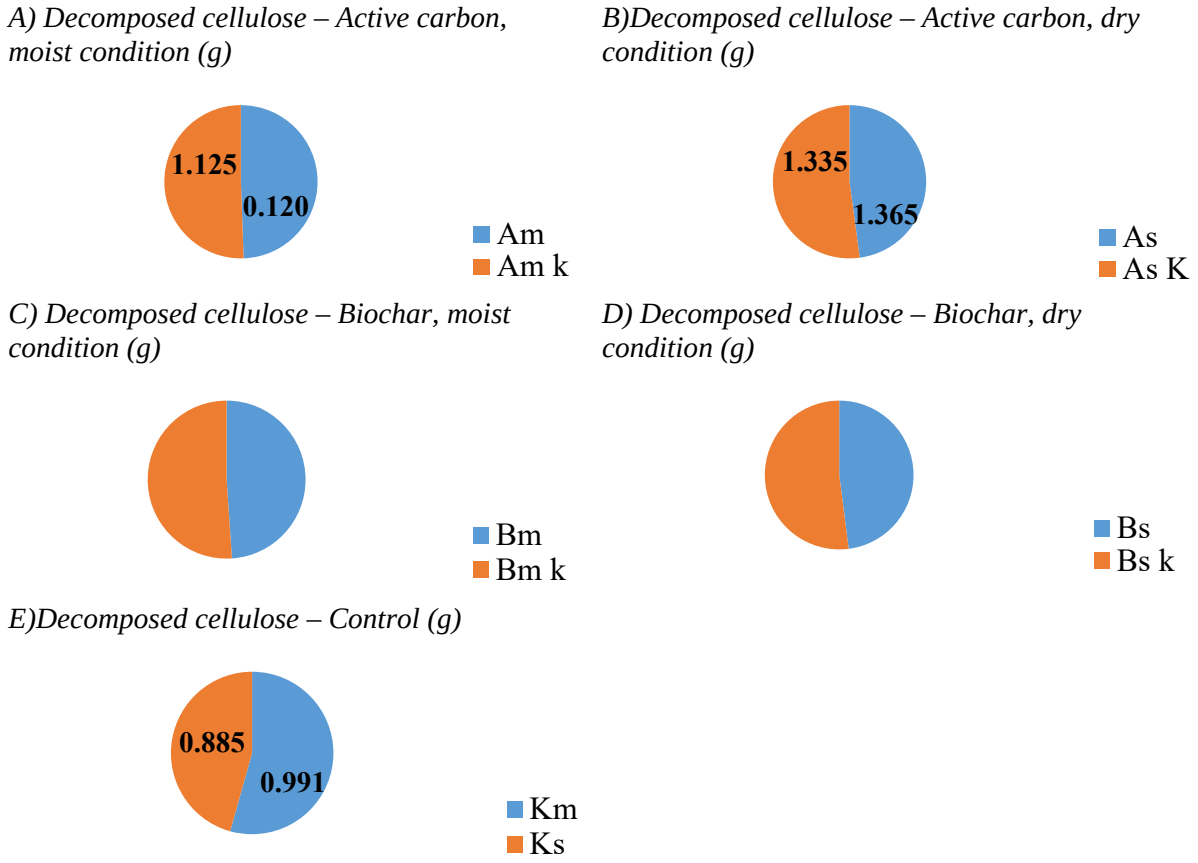
A) TNM *B) Fungi* *C) Nitrogen-fixing bacteria* *D) Actinomycetes*

Legend: As – Active carbon (Dry condition), As k – Active carbon (Dry condition – Control). Different colours mean different part of containers.

In containers stressed by drought, which contained active carbon, the same trend was observed as for the variant not stressed by drought (Figure 6).

Litter mesh bag

Figure 7 Decomposition of cellulose-Litter mesh bag results



Legend: Am – Active carbon (Wet conditions), Am k – Active carbon (Wet conditions – Control), Active carbon (Dry condition), As k – Active carbon (Dry condition – Control), Bm – Biochar (Wet conditions), Bm k – Biochar (Wet conditions – Control), Bs – Biochar (Dry condition), Bs k – Biochar (Dry condition – Control), Ks – Control (Dry condition), Km – Control (Wet conditions). Different colours mean different part of containers.

Figure 7 shows, stress by drought did not significantly affect cellulose decomposition. The difference in decomposition was measured only in control variants. In the containers with ideal moisture faster decomposition was observed.

According to our results biochar or active carbon can mitigate the effect of stress by drought for most group of microorganisms. We also indicated that these amendments could affect the ability to maintain the decomposition of soil even in times of drought. Many studies found biochar or active carbon can keep hydroclimates in soil (Kammann et al. 2011). But as O'Neill and Nicholson-Cole (2009), Liang et al. (2010) indicates especially biochar is very diverse and for each experiment it is very important to know as much as possible about the character of using feedstock and pyrolysis process. Many works show that biochar should only be applied in really small amounts (1–10 t) and in this case biochar does not affect soil microbiota in a bad way (Hossain et al. 2010). In our work we used higher concentration of biochar and our results show highest colonization, especially in variants with biochar stressed by drought.

CONCLUSION

Appropriate application of biochar depends on many factors such as climate, soil character, feedstock for the biochar etc. According to our result additives such as biochar or activated carbon can promote the development of microbial communities in the period of drought. Repeating the experiment under field conditions is necessary for supporting our hypothesis.

ACKNOWLEDGEMENTS

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BIOLOGICAL PARAMETERS OF SOIL QUALITY IN HAPLIC CAMBISOL

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Abstract: During two-years experiments (2014–2015) we evaluated intensity of basal soil respiration and microbial biomass content. Object of study was Haplic Cambisol (Vatín, Czech Republic) under two different management systems: ploughing and permanent grassland. Soil basal respiration was determined using GS-chromatography. Amount of microbial biomass was measured by fumigation-extraction method. Results showed there is a tendency of increasing amount of microbial biomass and intensity of basal soil respiration after conversion of arable soil in permanent grassland. After two years of experiments no statistically significant differences were found. Close correlation was determined between microbial biomass and intensity of basal soil respiration (r = 0.856).

Key Words: basal soil respiration, microbial carbon, Haplic Cambisol

INTRODUCTION

Soil's ability to act as a part of ecosystem in given by appropriate land use management. Maintain the biological productivity and promote plant and animal health is the main soil function (Doran and Parkin 1994). Chemical, physical and biological soil properties are therefore the main characteristics affecting soil quality/health. Chemical indicators include: soil reaction, exchangeable sorption capacity, and soil organic matter content and quality. Physical properties include: texture, structure, porosity, pedocompaction and others. Biological soil parameters include: microbial biomass content, microbial activity, soil basal respiration and substrate-induced respiration (Pospíšilová and Vlček 2015).

The presence of plants, animals and micro-organisms leads to the formation and accumulation of soil organic matter, causing biochemical weathering, and mixing soil substances. Moreover living organisms significantly accelerate cycling of elements and flows of energy, leading to the formation and stabilization of aggregates of soil particles (Šimek 2003). Biological activity is evaluated by various parameters and microbiological methods. Intensity of soil respiration, indicates the amount of CO_2 released from a certain amount of soil sample per unit time. Activity of heterotrophic soil organisms is evaluated by measuring of respiration intensity, which is highly dependent on the physiological conditions of microorganisms and other factors such as water content, temperature, pH, soil compaction and nutrient supply (Eisentraeger et al. 2000). Intensity of soil respiration inform us about degradation process, bioavailability of organic substances and intensity of mineralization. Under normal circumstances, there is an ecological balance between soil organisms and their activity. If the balance is broken (e.g. by addition of degradable organic compound), there is a significant increase in growth of microorganisms and their mineralization activity, resulting in a change in soil respiration. Soil respiration reflects also the speed of biodegradation of various materials and pollutants presented in soil, which is directly connected with microbial biomass diversity and content (Anderson and Domsch 1978a, b).

The aim of present contribution is to determine amount of microbial biomass and basal soil respiration during two years experiments (2014–2015). Experiments were carried out on Haplic Cambisol (Vatín, Czech Republic). Two types of land use were studies – arable soil and grassland.

MATERIAL AND METHODS

Haplic Cambisol was sampling in spring (2014 and 2015) from the top soil (0–5 cm) – see Figure 1. Two methods of farming were studied: ploughing (P) and permanent grassland (G). Soil was defined in terms of physical, chemical, and biological properties – see Table 1, 2. Area is situated in the climatic region slightly warm, slightly humid highland (Culek 1996). Total annual rainfall 550–700 mm, the long-term average rainfall is 594.4 mm (Quitt 1977). The average annual temperature is between 6–7 °C. Standard analytical method for basic soil properties were used. Soil reaction was determined by potentiometric method. Texture by pipette method. Cation exchange capacity (CEC) according to Kappen method. Conductivity by determination electric conductivity is soil. Carbonates were determined by volumetric method (Zbíral et al. 2010). Total organic carbon (TOC) content was measured by oxidimetric titration method (Nelson and Sommers 1982). Fractional composition of humus was determined by short fractionation method (Kononová and Bělčíková 1963). Soil respiration was determined by GS-chromatography (Vránová et al. 2009). Amount of microbial biomass (C_{mic}) was determined by fumigation-extraction method (Vance 1987). One-way ANOVA analysis and correlation analysis were applied for statistical data evaluation.

Figure 1 Locality Vatín (Czech Republic)

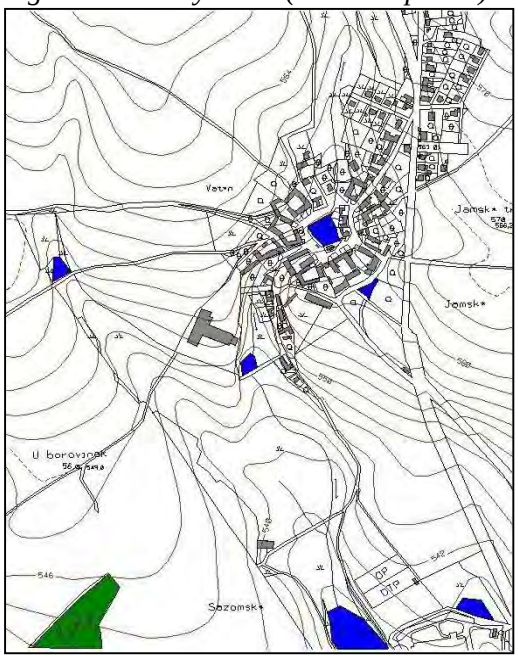


Table 1 Basic soil properties of Haplic Cambisol

Soil type	pH/H_2O	pH/KCl	Clay particles content (%)	Conductivity (mS/cm)
Haplic Cambisol (P)	5.1	4.7	22	0.2
Haplic Cambisol (G)	4.8	4	22	0.2

Table 2 Basic soil properties Haplic Cambisol

Soil type	CEC (cmol/kg)	TOC (%)	Humus (%)	∑ HS (mg/kg)	HA/FA
Haplic Cambisol (P)	14.2	1.43	2.47	4.50	0.80
Haplic Cambisol (G)	14		3.45	5.50	0.83

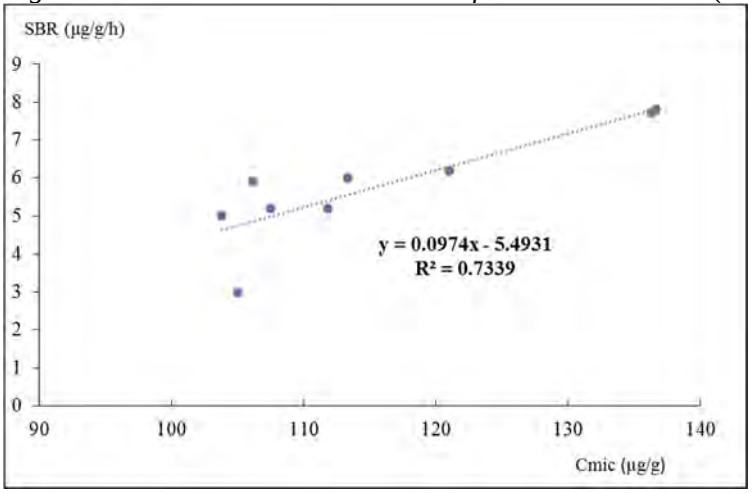
RESULTS AND DISCUSSION

Studied Haplic Cambisol had acid active (pH/H_2O) and exchangeable soil reaction (pH/KCl), very low conductivity (0.2 mS/cm) and was sandy loam textured (22%) – see Table 1. Cation exchange capacity was about 14%, which can be evaluated as low value. Total organic carbon content varied from 1.43% to 2% depending on land use. Higher humus content was determined under permanent grassland. Generally humus amount in soil was low. Comparison with literature data showed that Cambisols are usually low in humus and humic substances quality (Němeček et al. 2011). Average humic substances (HS) content and does not reach 6 mg/kg. Ratio humic acid and fulvo acid (HA/FA) was less than one, that means lows humic substances quality on both variants of experiment. Obtained results also indicated higher microbial biomass content under permanent grassland (G) to compare with arable soil (P). This can be explain by higher humic substances content, plant debris and microbial diversity under grassland. However statistically significant differences were not found. Similar results were published by Tesařová et al. (2006). Intensity of basal soil respiration was also higher under permanent grassland (G) to compare with arable soil (P). No statistically significant differences between intensity of basal soil respiration were found (see Table 3). On the other hand close correlation between an amount of microbial biomass and intensity of basal respiration was found (r = 0.856) – see Figure 2. We can conclude that soils, which were converted into permanent grassland showed tendency of higher humus accumulation. This is a main factor influencing increasing of microbial biomass and intensity of basal soil respiration. Two years experiments will continue and it is supposed that differences between two different types of land will increase. Literature data also confirmed that amount of soil microorganisms and microbial activity are directly influenced by agricultural systems, agro- and prato-technical measures applied on soils (Zaujec et al. 2009, Hauptaman 2009).

Table 3 One-way ANOVA analysis

Source	Number	Sum	Average	Variance		
Cmic (P)	7	807.41	115.344	188.919		
Cmic (G)	7	922.45	131.779	1201.6		
Source of variability	*SS*	*Difference*	*MS*	*F*	*P*	F_{crit}
Between sources	945.300114	1	945.3	1.35964	0.26625	4.74723
All sources	8343.11506	12	695.26			
Total	9288.41517	13				
Source	Number	Sum	Average	Variance		
Soil basal respiration (P)	6	33.77	5.62833	0.32802		
Soil basal respiration (G)	6	29.46	4.91	11.6225		
Source of variability	*SS*	*Difference*	*MS*	*F*	*P*	F_{crit}
Between sources	1.54800833	1	1.54801	0.25907	0.6218	4.9646
All sources	59.7524833	10	5.97525			
Total	61.3004917	11				

Figure 2 Correlation between amount of microbial biomass (Cmic) and soil basal respiration (SBR)



CONCLUSION

Amount of microbial biomass and intensity of basal soil respiration in Cambisols were directly influenced by land use. Close correlation between both studied parameters was determined (r = 0.856). Studied soil had low humus content and quality but differences between two types of soil management were not statistically different after two years of experiment. It is supposed that differences after conversion of arable soil into permanent grassland are going to increase.

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RESPONSE OF MICROBIAL ASSOCIATIONS TO FERTILIZERS APPLICATION

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Abstract: The calcium ammonium nitrate with single superphosphate and muriate of potash; digestate and farmyard manure were applied into the soil with aim to study their effect on biological soil parameters. Field experiments started in 2014 on the experimental plots of Mendel University in Brno (Vatín, Czech Republic). We evaluated intensity of basal soil respiration, soil organic matter stability and N/B, G/N ratios. Measurements were carried out using Vaisala GMT 222 device. Results indicated lower basal soil respiration after digestate application. However statistically differences in time of collecting were not found.

Key Words: basal soil respiration, farmyard manure, digestate, mineral fertilizer

INTRODUCTION

Soil organisms act irreplaceably in all pedogenic processes determining soil fertility and quality. Biological activity evaluated by measuring of soil respiration activity (for example, CO_2 production or O_2 loss in a closed system) is generally regarded as one of the basic soil properties, which can be used as an indicator of soil quality and health (Šimek 2004). Basal soil respiration measured as the amount of produced CO_2 help us to define the activity of soil microorganisms. From one point of view, higher intensity of respiration is a positive factor indicating the ability of soil microorganisms make available nutrients bounded to soil organic matter. On the other hand, rapid respiration leads to depletion of organic matter, increasing of mineralization intensity and to faster nutrients loss. A high respiration rate may also indicate microorganisms stress (Novak and Apfelthaler 1964, Anderson and Domsch 1978). Potential respiration is an indicator of the response of the microbial associations to the easily available organic substrate, and therefore is referred as a substrate-induced respiration (SIR). Measurements are necessary to do immediately after the addition of easily degradable substrate (e.g. glucose, ammonium sulfate) as quoted Anderson and Domsch (1978) and Foukalová (2011).

MATERIAL AND METHODS

Characterization of locality

Field experiment was located at Vatín (Žďár nad Sázavou county, the Czech Republic) on the experimental plots of MENDELU. Each experimental plot had 10 m^2. Soil was classified according to Němeček et al. (2011) as haplic cambisol, eutric variety: (KAme'), sandy-loam textured soil on weathered gneiss. Average annual rainfall is 594 mm; average annual temperature is about 6.1 °C. The experiment was established in 2014 and we evaluated experimental data from 2015: 14 days (April) and 30 days (June) after fertilizers application in two different depth (5 and 15 cm).

Experimental design:

Three different fertilizers on monoculture *Zea mays* for corn were applied - *calcium ammonium nitrate* (CAN), *farmyard* manure (F) and *digestate* (D). The amount of used fertilizer was calculated in accordance with nitrogen content. Applied value of nitrogen was 150 kg/ha. All fertilizers were applied in two doses during the vegetative season (spring – 60% of total delivered N and summer – 40% of total delivered N). The mineral fertilizer CAN with single superphosphate and muriate of potash was applied. Digestate were made from farmyard manure, cereals silage, corn silage, green grass, grass silage and

was reached from biogas plant station in Pikárec (Czech Republic). The harvest residues were left on the soil surface and were incorporated into the soil during the cultivation by disking to the depth 0.16 m. First dose of digestate and mineral fertilizer was applied during the spring on cultivated parcel before the maize sowing. Digestate was applied into the furrow and mineral fertilizer on the parcel surface with subsequent covering by soil. Second dose was applied between the row spacing on the surface.

Sampling and analysis

Soil sampling should follows instruction for microbiological analysis, which are directly depending on precise handling, storage and time. Samples are quickly sieved through a 5 mm sieve, removing the greater part of soil skeleton fraction, impurities and residues of vegetable or animal material; as soon as possible after sampling they are stored in a fridge. Samples are re-moistured to the same moisture value within one experiment. Water is added to eliminate the potential difference before the addition of glucose or ammonium sulphate solution (measuring of substrate induced respiration). A detailed description of the methodology is given by Foukalová (2011). Measurements were carried out using Vaisala GMT 222 device. The results were evaluated according to Střalková et al. (2011).

RESULTS AND DISCUSSION

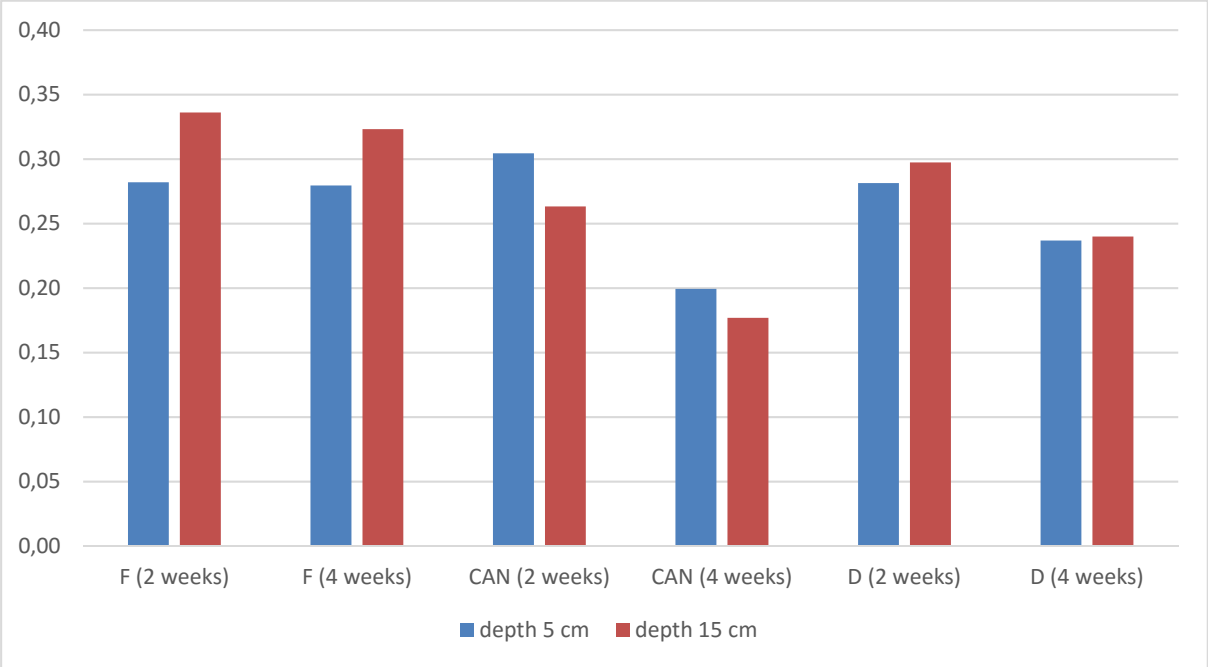
Basal respiration (B) (see Figure 1) for farmyard manure (F) was low at the depth of 5 cm under the surface in two weeks after application (0.28 ± 0.01 mg CO_2/h in 100g of soil); one month after the application remained the same (0.28 ± 0.01 mg CO_2/h in 100g of soil); at the depth of 15 cm the values were slightly higher, but still low (0.34 ± 0.002 mg CO_2/h in 100g of soil after 14 days from the application or 0.32 ± 0.01 mg CO_2/h in 100g of soil after one month from the application). Basal respiration for mineral fertilizers (CAN) was at the depth of 5 cm under the surface, two weeks after application, it was low (0.30 ± 0.01 mg CO_2/h in 100g of soil); one month after application stayed at this depth almost unchanged (0.28 ± 0.01 mg CO_2/h in 100g of soil); at the depth of 15 cm the values were somewhat lower (0.26 ± 0.01 mg CO_2/h in 100g of soil after 14 days from the application – still assessed as low basal respiration; or 0.32 ± 0.02 mg CO_2/h in 100g of soil after one month from the application). Similar (but long term) trends in results showed Pavan Fernandesa et al. (2005). Basal respiration in digestate (D) was low (depth 5 cm) in two weeks after application (0.28 ± 0.01 mg CO_2/h in 100g of soil); one month after application decreased to 0.24 ± 0.01 mg CO_2/h in 100g of soil); at the depth of 15 cm were the values initially slightly higher, but still low (0.30 ± 0.02 mg CO_2/h in 100g of soil 14 days after application); after one month they were falling to levels similar to those observed in the upper layer of 0.24 ± 0.01 mg CO_2/h in 100g of soil. Němeček et al. (1990) showed for cambisol basal respiration from 0.21 (in oligobasic cambisol) to 0.50 (in eutric cambisol) mg CO_2/h in 100g of soil. Sáňka et al. (2000) showed average basal respiration for arable land 0.29 mg CO_2/h in 100g of soil. Our results corresponded more with oligobasic cambisol according Němeček et al. (1990) or with average soil according Sáňka et al. (2000).

The differences in time of collecting were not statistically significant (Table 1).

Table 1 Analysis of variance between groups with different time of collecting.

Source of variation	SS	df	MS	F	P-Value	F crit
Between groups	0.008	1	0.008	4.614	0.057	4.965
Within groups	0.017	10	0.002			
Total	0.025	11				

Figure 1 Basal respiration in different depth, values are in mg CO_2/h in 100g of soil.



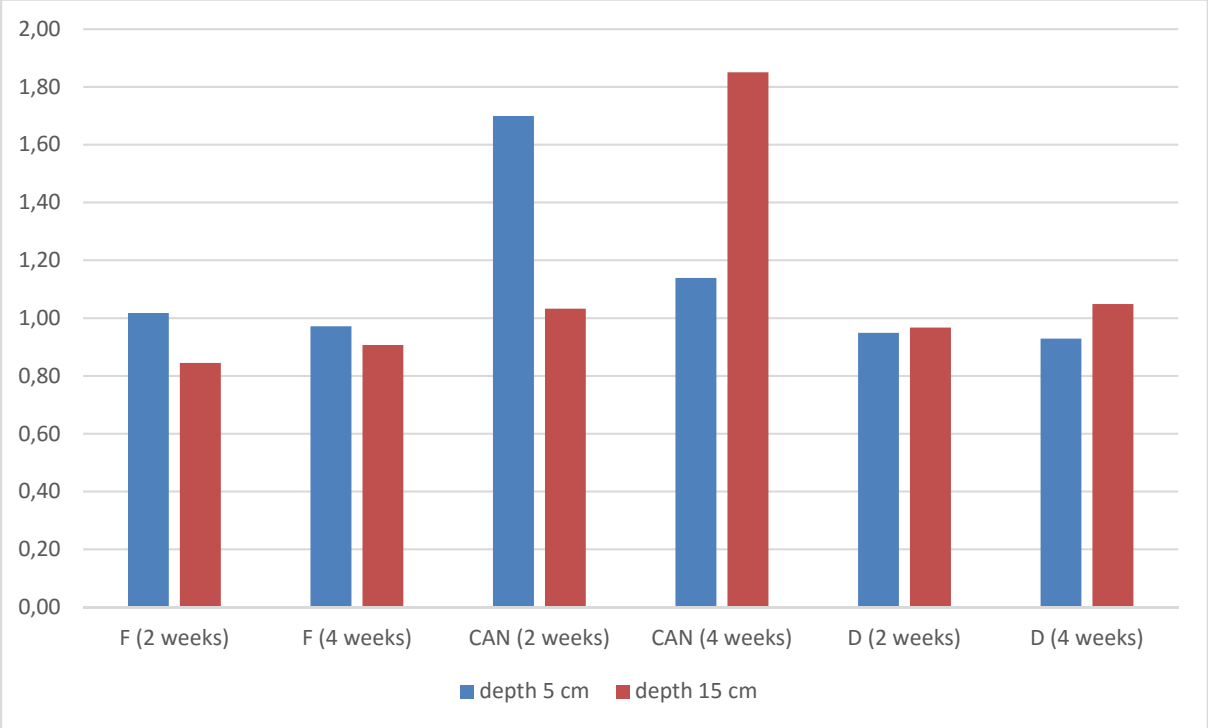
Ratio N/B (respiration of the samples with nitrogen/basal respiration) indicates physiological utilization of soil nitrogen. High N/B ratio values show lower nitrogen physiological availability for microorganisms (see Figure 2). If there is enough nitrogen, the addition of nitrogen does not increase the respiration rate and N/B ratio is close to value one. Ratio N/B for farmyard manure (F) was good (ratio 1.02) at a depth of 5 cm under the surface two weeks after application; one month after the application it slightly decreased to low (ratio 0.97); at a depth of 15 cm, the values were slightly higher, but still low (ratio 0.84) after 14 days from the application and after one month of application (ratio 0.91). The results showed that in the soil there was enough of usable nitrogen, addition of nitrogen was not increasing the respiration. Ratio N/B for mineral fertilizers (CAN) was high (ratio 1.70) at a depth of 5 cm under the surface, two weeks after application; one month after the application it went to good (ratio 1.14); at a depth of 15 cm an opposite effect occurred and the physiological availability of nitrogen went from good (ratio 1.03 in 14 days after application) to high (ratio 1.85) after one month from application. The results showed that in the soil, the lack of available nitrogen for the microorganisms in the upper parts of the soil profile decreased but in the depth increased in time. Ratio N/B for digestate (D) was at the depth of 5 cm under the surface virtually identical - low (ratio of 0.95 after 14 days and 0.93 after one month); at the depth of 15 cm the values were similar, from low usability became good (ratio 0.97 after 14 days and 1.05 after one month from the application). From the results it was apparent, like with the manure, that there was sufficient amount of physiologically available nitrogen for microorganisms in the soil and additional nitrogen did not increase the respiration. Similar results showed Foukalova et al. (2008) N/B ratio 0.97 (autumn 2007) and 1.06 (spring 2008) or Němeček et al. (1990) for cambisol (N/B ratio 0.85–0.95).

The differences in time of collecting were not statistically significant (Table 2).

Table 2 Analysis of variance between groups with different time of collecting.

Source of variation	*SS*	*df*	*MS*	*F*	*P-Value*	*F crit*
Between groups	0.009	1	0.009	0.084	0.778	4.965
Within groups	1.116	10	0.112			
Total	1.125	11				

Figure 2 Ratio N/B (indicates physiological utilization of soil nitrogen)



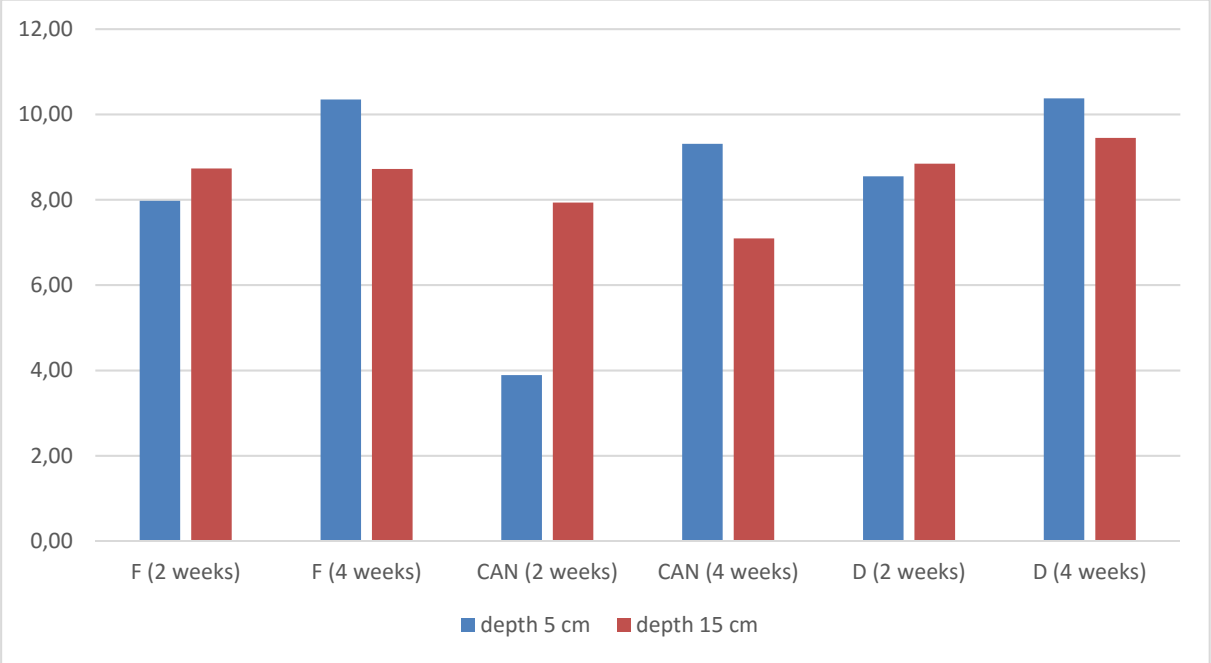
Ratio G/N (respiration of the samples with glucose/ respiration of the samples with nitrogen) (Figure 3) characterize connection between physiologically available carbon and nitrogen content in soil. In spite of the fact that the carbon is always used in greater amount by soil microorganisms than the nitrogen, the balanced physiological ratio of these two elements is approximately equal to value five (5). Values higher than 5 indicate that soil microorganisms are relatively better supplied with organic substances (carbon) than with nitrogen. Lower values than 5 showed the opposite (soil microorganisms are better supplied by nitrogen). Ratio G/N for farmyard manure (F) was at the depth of 5 cm under the surface, two weeks after application it was very high (ratio 7.98); one month after the application it was still increasing (ratio 10.35), at the depth of 15 cm were the values virtually identical in both dates of sampling - very high (ratio 8.73 and 8.72). Ratio G/N for mineral fertilizers (CAN) was two weeks after application at the depth of 5 cm low (ratio 3.89); one month after the application it grew rapidly at the rate in similar levels as in manure (ratio 9.31, very high), at the depth of 15 cm, although this ratio in both dates was very high, decreased in time (ratio 7.93 after 14 days of application and 7.09 after one month from application). Ratio G/N for digestate (D) was very high too at the first depth (ratio 8.55 after 14 days and 10.38 after one month); at the depth of 15 cm the values were very high again, but did not increase so significantly (ratio 8.85 after 14 days and 9.45 after one month from the application).

All studied variants showed that soil microorganisms were better supplied with nitrogen than with the organic substances. Remarkably it was evident on variant with CAN application, where significant over-fertilization with nitrogen it found in the depth of 5 cm. The differences in time of collecting were not statistically significant (Table 3).

Table 3 Analysis of variance between groups with different time of collecting.

Source of variation	SS	df	MS	F	P-Value	F crit
Between groups	7.321	1	7.321	2.905	0.119	4.965
Within groups	25.198	10	2.520			
Total	32.520	11				

Figure 3 Ratio G/N (describe physiologically available carbon and nitrogen)



Stability of organic matter was evaluated by ratio NG/B (Figure 4)**.** Higher values indicate a higher stability, in general. Stability of organic matter was in the case of farmyard manure (F): in the depth of 5 cm below the surface, in both periods, it was very high (two weeks after the application is the ratio 12.30, one month after the application it was further increased to 14.42); at the depth of 15 cm, the situation was similar, the values were also very high in both periods, 10.97 ratio after 14 days from the application and ratio of 12.48 one month after application. Stability of organic matter was for mineral fertilizers (CAN): at the depth of 5 cm under the surface two weeks after application it was very high (ratio 12/11); but one month after the application it risen fast to the ratio of 18.37. By nitrogen launched microbial activity was not accompanied with an adequate application of the carbonaceous material for the microorganisms. At the depth of 15 cm the same phenomenon occurred when the physiological availability of nitrogen changed from 10.32 ratio (14 days after application) to (one month after application) the 18.77 ratio. In both cases the organic substances begun to be inaccessible to microbial decomposition, more significantly in deeper parts of soil profile. And in variant with digestate (D) was the situation at both depths very similar - the stability was very high in each case after 14 days from the application, with the ratio of 10.14 (5 cm depth) and 10.64 (15 cm depth), and changed one month after application to the ratio of 15.03 (the depth of 5 cm) and 15.11 (depth 15cm).

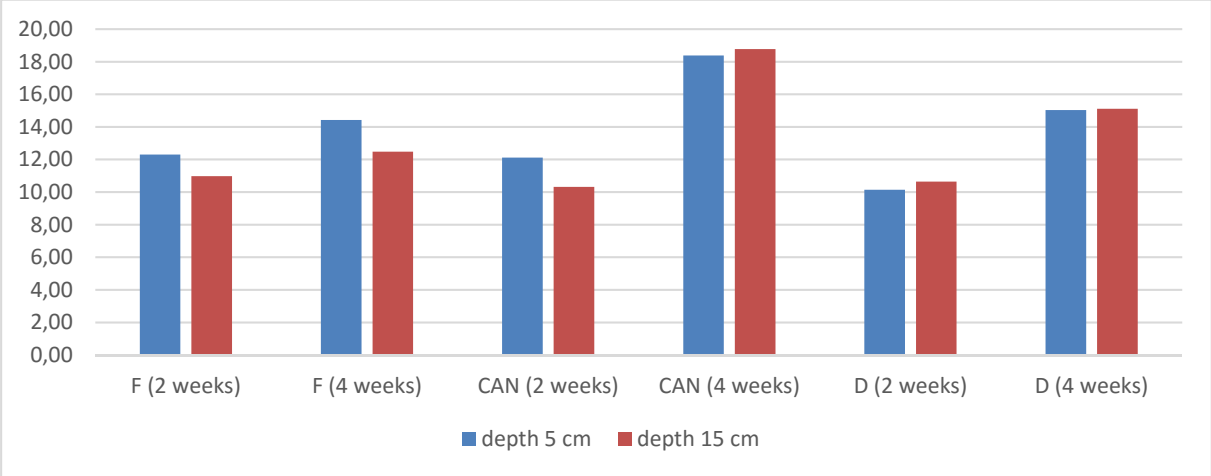
Foukalova et al. (2008) showed in Vatin lower ratio NG/B 9.08 (autumn 2007) and 8.48 (spring 2008). Němeček et al. (1990) showed the lowest ratio for common cambisol from 2.21 (oligobasic cambisol) to 5.87 (eutric cambisol). In all our studied variants the organic substances started to become inaccessible to microbial degradation, but with manure less strongly.

The differences in time of collecting were statistically significant (Table 4).

Table 4 Analysis of variance between groups with different time of collecting.

Source of variation	SS	df	MS	F	P-Value	F crit
Between groups	63.985	1	63.985	19.029	0.0014	4.965
Within groups	33.625	10	3.362			
Total	97.610	11				

Figure 4 Stability of organic matter by ratio NG/B. Higher values indicate a higher stability



CONCLUSION

Basal respiration in studied soil was low. No influence of the type of applied fertilizer or depth was found. Intensity of basal soil respiration was the lowest after digestate application and the highest after farmyard manure application. In all variants there was sufficient amount of physiologically available nitrogen for microorganisms in the soil and additional nitrogen did not increase the respiration (ratio N/B was ± 1). All studied variants showed that soil microorganisms were better supplied with nitrogen than with the organic substances (with CAN application was significant over-fertilization with nitrogen in the depth of 5 cm). In all our studied variants the organic substances started to become inaccessible to microbial degradation, but with manure less strongly. However, the differences were not mainly statistically significant.

ACKNOWLEDGEMENTS

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DEVELOPMENT OF-LAND USE CHANGES IN SELECTED VILLAGES IN THE MIDDLE-HRON RIVER REGION

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Abstract: In this paper we focus on the development of land-use changes in two villages – Tekovské Nemce and Hronský Beňadik. These villages have old vineyards and a fruit growing tradition and are situated in the Middle-Hron river region (Slovakia). We evaluated and compared changes of secondary landscape structure (SLS) in two time periods (1949–1986 and 1986–2016). The result is identification of landscape development trends, which are an important basis for planning sustainable future development of the municipality and biocultural diversity.

Key Words: vineyard landscape, Hronský Beňadik, Tekovské Nemce, land-use changes

INTRODUCTION

The cultural landscape reflects all political, social, economic and technological changes and developments of the society which produces it (Lipský et al. 1999). There is a long history of studies of the vineyard landscape as part of the broader cultural landscape. The first written reference to vineyards in Slovakia is from the village Hronský Beňadik in 1075 (Hronský and Pintér 2009). The historical structures of the agricultural landscape (HSAL) consist of a mosaic of small-scale arable fields and permanent agricultural cultivations such as grasslands, vineyards and high trunk orchards (Dobrovodská and Štefunková 1996). The current trend is that area of HSAL is constantly decreasing.

Changes of landscape features are significantly influenced by the socio-economic activities of the people who determine the future character of landscape. In this paper we focus on the development of changes in the landscape over the last 70 years. We have evaluated land-use changes in two time periods. For the analysis of historical landscape structure (HLS) we chose the years 1949 and 1986, and for the current landscape structure (CLS) the year 2016. The aim of this paper is to evaluate and compare the development of the secondary landscape structure (SLS) in the socialist era (for the years 1949 and 1986) and in the era of post-socialist agricultural transformation and restructuring of agriculture (for the years 1986 and 2016), and identify the main trends in SLS development for these two time periods.

MATERIAL AND METHODS

The first step of our study was field research in the selected area, which was necessary for creating maps of the current landscape structure. Processing of graphic layers and visualisation were done in computer program for geographic information systems ArcGIS 10.1. We used two-code marking for mapping landscape features according to the adjusted map legend of Petrovič et al. (2009), which divides landscape features into six groups. This map legend was chosen because it is applicable for interpretation of the historical and current landscape structure as well. Also, it is usable for the whole territory of Slovakia, from the local to the national level.

For analyzes of historical landscape structures we used panchromatic aerial photos from 1949 and 1986. For analyzes of current landscape structure we used ortophoto images from 2007, which we updated with data from the web site http://www.mapy.cz and field research.

To simplify the data presentation we transformed the original four-number code classification of SLS into a two-number code. By grouping the various types of landscape elements into broader classes we ended up with 16 types of landscape elements at the second level of SLS classification (Table 1).

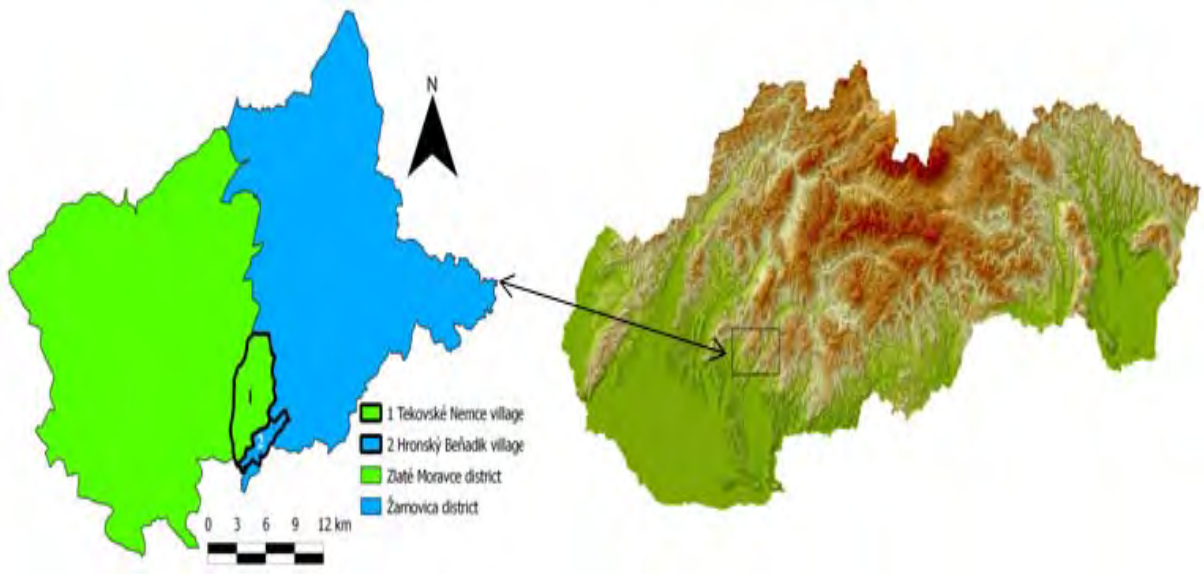
Table 1 Legend of secondary landscape structure (SLS)

SLS 1	Name 1	SLS 2	Name 2
1	Tree and scrubland vegetation	11	Forest
		12	Non-forest woody vegetation
2	Grasslands	21	Meadows and pastures
3	Agricultural crops	31	Large-block fields
		32	Small-block fields
		33	Gardens
		34	Large-block vineyards
		35	Small-block vineyards
		36	Orchards
4	Mining areas and raw soils	41	Quarries, rocky hills, ridges and walls
5	Surface water and wetlands	51	Lakes, ponds and water-courses
6	Houses and built-up areas	61	Housing and amenities
		62	Residence and technical vegetation
		63	Sports, cultural, recreational objects and grounds
		64	Production, technical objects and grounds
		65	Transport objects and grounds

Localization Of The Study Area

The study areas consist of the cadastral units of two vineyard villages, Hronský Beňadik and Tekovské Nemce, both situated in the Middle-Hron river region (Figure 1). The area of Hronský Beňadik (Žarnovica district) is 725.63 ha, and that of Tekovské Nemce (Zlaté Moravce district) is 2831.62 ha. These areas represent the northern boundary of winegrowing in the Nitra wine region.

Figure 1 Study area location in Slovakia



RESULTS AND DISCUSSION

The changes in secondary landscape structure were evaluated separately for each period and village. We focused on the most significant changes.

Historical landscape structure of Hronský Beňadik in 1949

In 1949, groups of small-block fields (33.71%) and meadows with pastures (32.15%) took up 2/3 of the area. Fragmented land ownership and a variety of farming technologies and products had created an outstanding example of a traditional rural landscape. In total, agricultural crops took up 66% of the territory, while forests and non-forest woody vegetation accounted for only 12%. Small-block vineyards, which for a long time had represented the traditional land use of the village, covered 14.13 ha (1.95%).

Historical landscape structure of Hronský Beňadik in 1986

Significant changes in land use had occurred by 1986, especially in agriculture (Table 2). The intensification and collectivisation of agriculture, which began in the 1950s, involved merging small-block fields into large-block fields. As a result, in 1986 large-block fields covered 43% of the study area, while small-block fields had almost completely disappeared, falling to 0.01%. Also, there was a significant loss of meadows and pastures, which had fallen by more than a half. The amount of small-block vineyards had also decreased, to 1.63% of the study area. Conversely, the area of forests had slightly increased, and in the southern part of cadastral area there had arisen the new feature of large-block vineyards, covering an area of 64.91 ha (8.95%). A slight increase in gardens was related to the construction of new houses.

Current landscape structure of Hronský Beňadik in 2016

Nowadays, large-block fields are the biggest group of features, representing an area of 329.15 ha (45.36%). Relative to 1986, the forest area was significantly increased, to about 53.32 ha. This was caused by the fact that people had stopped breeding and grazing cattle. The area of both small- and large-block vineyards had decreased as well. Some of them had been changed to agricultural fields, while others had undergone plant succession.

Table 2 Comparison of area of SLS features in Hronský Beňadik 1949–2016

Year	1949		1986		2016	
SLS	**Area (ha)**	**Area (%)**	**Area (ha)**	**Area (%)**	**Area (ha)**	**Area (%)**
11	42.93	5.92	64.1	8.83	117.42	16.18
12	44.08	6.07	47.12	6.49	73.37	10.11
21	233.26	32.15	93.16	12.84	43.25	5.96
31	64.14	8.84	312.05	43.00	329.15	45.36
32	244.58	33.71	0.07	0.01	0.24	0.03
33	23.58	3.25	34.64	4.77	27.94	3.85
34	-	-	64.91	8.95	15.51	2.14
35	14.13	1.95	11.81	1.63	5.63	0.78
36	0.16	0.02	0.72	0.10	1.17	0.16
41	2.76	0.38	2.15	0.30	1.45	0.20
51	26.3	3.62	27.2	3.75	23.85	3.29
61	12.78	1.76	13.42	1.85	16.59	2.29
62	4.51	0.62	12.33	1.70	22.43	3.09
63	0.74	0.10	1.27	0.18	1.15	0.16
64	0.79	0.11	24.23	3.34	25.35	3.49
65	10.89	1.50	16.45	2.27	21.13	2.91
Total	**725.63**	**100**	**725.63**	**100**	**725.63**	**100**

Historical landscape structure of Tekovské Nemce in 1949

In 1949, the forests were the most widespread landscape element (Table 3). They occupied 53.61% of the study area. Non-forest woody vegetation covered 205.97 ha (7.27%). Meadows and

pastures (13.83%) and small block fields (13.42%) occupied almost the same area. In this year, large-block fields represented an area of 255.42 ha (9.02%). This indicates that relatively advanced and intensive agriculture had already occurred in the study area before the period of agricultural collectivization under socialism. Small-block vineyards occupied only 2.08 ha (0.07%) and housing and amenities 9.99 ha (0.35%).

Historical landscape structure of Tekovské Nemce in 1986

By 1986, the forests had increased by 149.66 ha. The area of non-forest woody vegetation had decreased to 2.42%. The main reason was succession of forest. Small-block fields represented an area no bigger than 0.18 ha (0.01%). This was caused by combining small-block fields into the large-block fields, which covered 626.13 ha (22.11%). We noted two new landscape elements: water courses and technical objects had been built here. It is interesting that in Tekovské Nemce socialist collectivisation did not result in large block vineyards. In fact, small-block vineyards had increased by 2.15 ha. The area of orchards had decreased by 8.31 ha.

Current landscape structure of Tekovské Nemce in 2016

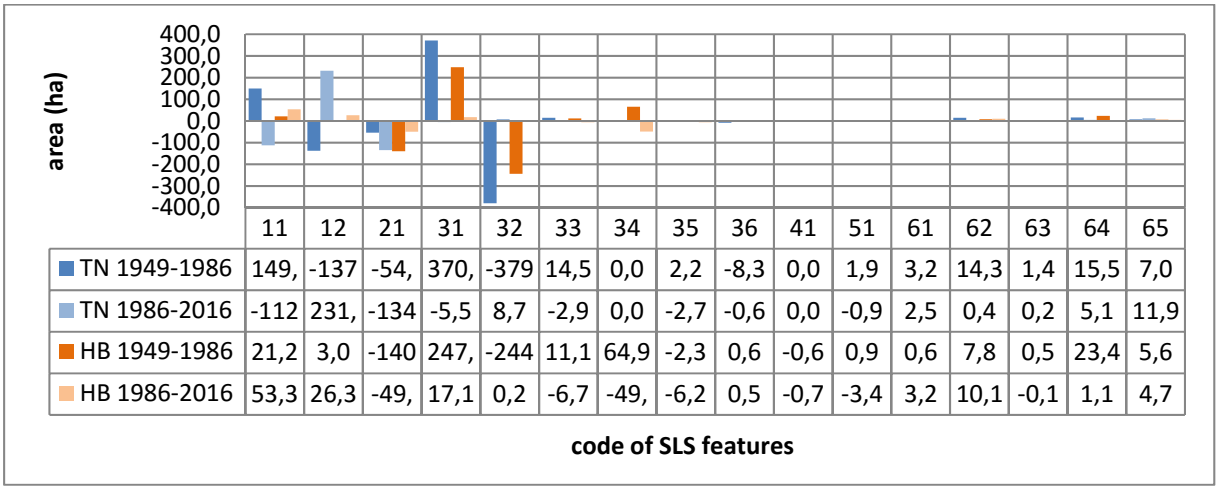
Forests still occupy the largest area, but compared to 1986, they have decreased by 112.86 ha. On the other hand, non-forest woody vegetation has increased to 299.87 ha (10.59%). We note the increase of small-block fields to 8.86 ha (0.31%). Small-block vineyards decreased to 1.5 ha. Compared to 1986, the areas of transport objects and of production and technical objects are around 50% larger. This is caused by construction of the R1 highway.

Table 3 Comparison of area of SLS features in Tekovské Nemce 1949–2016

Year	1949		1986		2016	
SLS	**Area (ha)**	**Area (%)**	**Area (ha)**	**Area (%)**	**Area (ha)**	**Area (%)**
11	1517.97	53.61	1667.63	58.89	1554.77	54.91
12	205.97	7.27	68.4	2.42	299.87	10.59
21	391.59	13.83	337.11	11.91	202.35	7.15
31	255.42	9.02	626.13	22.11	620.61	21.92
32	380.01	13.42	0.18	0.01	8.86	0.31
33	50.39	1.78	64.89	2.29	61.96	2.19
34	-	-	-	-	-	-
35	2.08	0.07	4.23	0.15	1.5	0.05
36	11.19	0.40	2.88	0.10	2.33	0.08
41	-	-	-	-	0.01	0.00
51	-	-	1.85	0.07	0.97	0.03
61	9.99	0.35	13.18	0.47	15.72	0.56
62	0.19	0.01	14.48	0.51	14.88	0.53
63	0.13	0.00	1.48	0.05	1.65	0.06
64	-	-	15.47	0.55	20.53	0.73
65	6.69	0.24	13.71	0.48	25.61	0.90
Total	**2831.62**	**100**	**2831.62**	**100**	**2831.62**	**100**

After comparison of changes in area of SLS features we have identified the main development trends in both villages (Figure 2). Using the classification scheme of Feranec et al. (2002) we have identified 9 principal trends in the land-use changes.

Figure 2 Changes of area of SLS features in Tekovské Nemce (TN) and Hronský Baňadik (HB) between 1949–1986 and 1986–2016



	11	12	21	31	32	33	34	35	36	41	51	61	62	63	64	65
TN 1949-1986	149,	-137	-54,	370,	-379	14,5	0,0	2,2	-8,3	0,0	1,9	3,2	14,3	1,4	15,5	7,0
TN 1986-2016	-112	231,	-134	-5,5	8,7	-2,9	0,0	-2,7	-0,6	0,0	-0,9	2,5	0,4	0,2	5,1	11,9
HB 1949-1986	21,2	3,0	-140	247,	-244	11,1	64,9	-2,3	0,6	-0,6	0,9	0,6	7,8	0,5	23,4	5,6
HB 1986-2016	53,3	26,3	-49,	17,1	0,2	-6,7	-49,	-6,2	0,5	-0,7	-3,4	3,2	10,1	-0,1	1,1	4,7

In the period of 1949–1986, the trend of intensification of agriculture was the most significant in both villages. In Tekovské Nemce, the trend of reforestation was significant as well. This is probably a continuous trend from the previous time period because we observe a change of non-forest woody vegetation to forest. Over this period, trends of urbanization were important in both villages. Compared to the previous period, areas of urban vegetation significantly increased. Areas of manufacturing and technical facilities increased too.

During the 1986–2016, the appearance of the landscape was influenced by the process of restructuring and transformation of agriculture. The significant trend of afforestation is especially apparent in Tekovské Nemce by reduction of the acreage of grassland. Abandonment of grasslands and they overgrowing by woody vegetation over the period 1986–2016 was probably related to the production restructuring of the agricultural cooperative in Tekovské Nemce. Simultaneously, a pronounced deforestation trend is manifested here, caused by harvesting. The continuing trends of urbanization are particularly evident in Hronský Beňadik.

Both villages are very well situated in terms of transport accessibility. Their distance between them and the three district towns – Zlaté Moravce, Levice and Žarnovica – is under 25 km. This fact leads us to the conclusion that the trend of urbanization will continue. Construction of houses and apartments will increase due to cheap building land. This leads to an increase of garden area. We also expect an increase of production and technical objects, which has arisen mainly as a result of the construction of highway R1 in 2011. R1 is going through both villages and connects the towns Ružomberok and Trnava. In Trnava, R1 connects to the D1 highway and from here it heads to the capital city Bratislava.

According to available historical sources, the traditional land use in both villages was viticulture and fruit growing. However, we have found that at the beginning of 1949, traditional vineyards and orchards did not significantly contribute to land use. At the end of the study period, the proportion of orchards and vineyards (whether traditional or large-block) does not exceed 3% of the area in either village.

Changes in land use resulted in a reduction in the landscape functions. During the analysed period, the landscape types had changed in both villages. In Hronský Beňadik, the original meadow-pasture-arable landscape was transformed into a forest-arable landscape. In Tekovské Nemce the change from the original meadow-pasture-arable-forest landscape to arable-forest landscape was marked. The proportion of grasslands decreased below 10%. Grasslands and traditional agricultural mosaics are disappearing from both villages.

CONCLUSION

Our aim was to evaluate and compare the development of vineyard villages in two time periods and determine the main development trends. We can say that the landscape in both villages has changed

significantly in last 70 years. The biggest changes we can observe are in agriculture, related to the change of political system after 1948. Socialism in Slovakia caused a relatively swift transition from extensive to intensive agriculture. The results of these processes were a gradual fading of traditional rural features and support of urbanization and industrialization. Traditional agricultural landscape is now in decline in both villages. Therefore, it is necessary to monitor these areas and prevent the extinction of these areas. The most important task for local communities and stakeholders is to lead and support younger generation to ensure sustainable development of biocultural values in both study areas. Otherwise, we are risking a loss of and negative impact on biological and cultural diversity. Despite the changes that have affected both villages, they have been able to retain a typical rural character.

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LONG-TERM DEVELOPMENT ANALYSIS OF ECOLOGICAL STABILITY AND LAND USE AROUND JEVÍČKO

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Abstract: This contribution deals with development evaluation of land use and analysis of long-term ecological stability development around Jevíčko. The area of interest is formed by Basic territorial units Bělá u Jevíčka. From the Lucc Czechia database and data from Czech Office for Surveying, Mapping and Cadastre were selected and compiled the required data with individual acreages of cultures for interest Basic territorial unit. From these data was calculated Coefficient of ecological stability according to Míchal (1985) and according to Miklós (1986). Generally, can be stated the largest acreage on the area of interest are occupied by forests and arable land. Arable land had the highest acreage in 1948 and since 1990 is evident decrease of arable land and rise of the other areas. Ecological stability quantified by Coefficient of ecological stability according to Míchal (1985) is the lowest in 1948 and highest in 2016. It should be noted step increase in value of Coefficient of ecological stability in 1990, but since 1990 is value stagnant. Reduction of the coefficient in 1948 is due to increase of arable land acreage. Increasing of the coefficient since 1990 is due to reduction of arable land and increasing quantities of permanent grassland, forest areas, water areas and permanent cultures. According to Míchal (1985) methodology area remains classified as heavily used area with weakened self-regulatory processes. According to Miklós (1986) methodology values of Coefficient of ecological stability ranges from 0.48 to 0.51. The lowest value was also achieved in 1948 and the highest in 2016. It may be noted stagnating of values after 1990 again.

Key Words: ecosystem, biodiversity, landscape structure, coefficient of ecological stability, database

INTRODUCTION

The landscape is a set of enclaves forming the landscape matrix and between the enclaves is possible to distinguish between stable and unstable constituents. Cultural landscapes are the result of consecutive reorganizations of the land in order to adapt its use and spatial structure better to changing societal demands. Landscape changes are seen as a threat, a negative evolution, because the current changes are characterized by the loss of diversity, coherence and identity of the existing landscapes (Antrop 2005). Ecological stability and biodiversity of Czech cultural landscape has been very negatively affected by development in the period of socialist agriculture from the 50s to the 80s of the 20th century but since 1990 previous ecologically highly unfavourable development of the rural landscape stopped (Lipský 2000). Knowledge of the landscape history is essential for understanding the spatial and chronological context of current and future landscape (Skånes 1996).

It is possible to quantify the ecological stability of the landscape using the coefficient of ecological stability (CES). CES can be calculated using several methods, for example by Miklós (1986), Míchal (1985) and Agroprojekt (1987) (Lipský 2000). Ecological stability is the ability of the ecosystem to balance changes caused by external factors and to maintain its natural properties and functions (§ 4 of Act number 17/1992 Sb.).

An important term is also Land use. Land use is characterised by the arrangements, activities and inputs people undertake in a certain land cover type to produce, change or maintain it (FAO/UNEP 2016). Land use integrates the landscape environmental and socio-economic component. Current and historical data are analysed from which are deduced changes in land use. Land-use changes reflect

different phases of socio-economic development (i.e. social metabolism) and political climates, as well as environmental changes (Bičík et al. 2001).

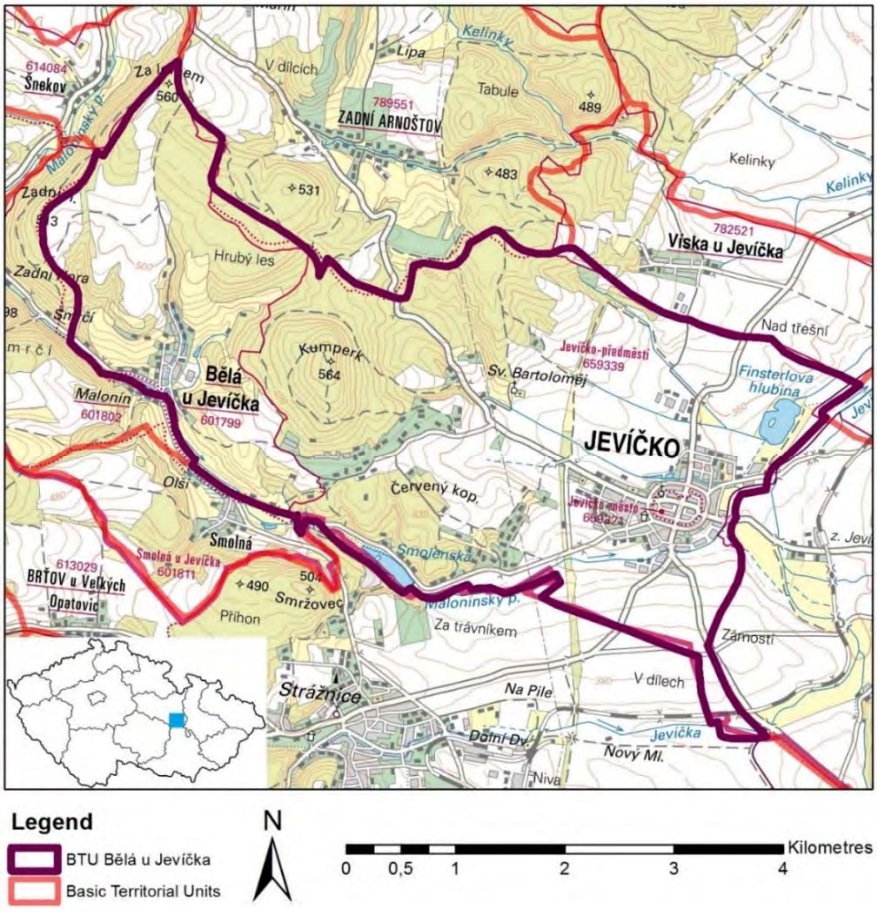
The data of evolution of the landscape can serve as a model for assessing development of the territory and it can be considered as important source material for landscape planning (Tress 2005).

MATERIAL AND METHODS

Definition and description of the interest area

The interest area is located in the south-eastern part of the Pardubice region, 39 km west from Olomouc and 49 km north of Brno. It is formed by Basic territorial unit (BTU) Bělá u Jevíčka. The BTU is composed of cadastral areas Bělá u Jevíčka, Jevíčko předměstí and Jevíčko město. Map of BTU is shown in Figure 1.

Figure 1 BTU Bělá u Jevíčka on the base of the Basic map ČR



Data Source: Database Lucc Czechia. Basic map: Czech Office for Surveying, Mapping and Cadastre. Modified by author.

The area lies geomorphologically in the Boskovická furrow. It is a rift valley with permo-carboniferous filling (Česká geologická služba – ČGS 2016).

The area around Jevíčko is geologically formed by consolidated or unpaved sedimentary rocks in the form of loess and loess loam, claystone, siltstone, sandstone, greywacke and conglomerate. In Bělá u Jevíčka are occurred stony almost loamy-stony sediments, sandy marl claystone almost spongilitic claystone, locally silicified (argillite), calcareous sandstone-clay, glauconitic, sometimes with cornea. Overall, the region of Boskovice furrow is homogeneous rock (ČGS: Geologie 2016).

Hydrogeologically the eastern part of the territory belongs to Rajon Boskovická furrow-northern part in permocarboniferous sediments and in the eastern part belongs to Velkoopatovická cretaceous in the upper cretaceous sediments. The main basins of both Rajons is the Dunaj. Sub-basin of Boskovická furrow is Dyje and has an acreage 323.27 km^2. Velkoopatovická cretaceous belongs to the Morava river basin and has an acreage 49.59 km^2 (ČGS: Hydrogeologie 2016).

From the viewpoint of hydrological characteristics in the area of interest flows Malonínský stream and stream Jevíčka There are water reservoirs of which the largest is Finsterlova deep and Smolenská reservoir.

Pedologically is a valley around Jevíčko formed predominantly by black soil luvic and subsoil of water streams by fluvisol glay. Closer and around to Bělá u Jevíčka are dominated cambisols, especially modal dystric, mesobazic. In agriculturally used part of the area is located brown soil modal (ČGS: Půdy 2016).

Within forestry characteristic are in the area of interest dominated forests composed by spruce, with a minor admixture of pine, larch, beech, maple and other deciduous trees. Coniferous are prevalent type of vegetation (ÚHUL 2016).

The area of interest belongs to a slightly warm climate region MT9, which can be characterized by a long summer that is warm and slightly dry. Spring and autumn are slightly warm. Winters are shorter and slightly warmer. The duration of snow cover is a medium short (Quitt 1971).

From the perspective of habitation are on the area of interest two municipalities. Jevíčko with 2,827 inhabitants and Bělá u Jevíčka with 351 inhabitants. Both municipalities were founded in the 13th century AD. They are agricultural villages since their foundation (risy.cz 2016).

Methodics

Firstly, a characteristic of area of interest was elaborated. From the data of Lucc Czechia database for the years 1845, 1948, 1990 and 2000 and from Czech Office for Surveying, Mapping and Cadastre (COSMC) data for 2016 have been selected and compiled the required data with individual acreages of cultures for interest BTU. From these data was subsequently calculated the CES according to Míchal and according to Miklos. Method of calculation is provided below. Representation of cultures development was evaluated and ecological stability of the area was evaluated.

Calculation of CES according to Miklós (1986 in Lipský 2000)

Methodology according to Miklós distinguishes ecological significance areas by introducing numerical coefficients. The calculation of the value is in the range of 0–1. The closer to 1, the more stable territory is.

Calculation formula: $CES = (\sum p_{ni} \times \sum k_{pn\,i}) / p$

$p_{n\,i}$: acreage of individual cultures

$k_{pn\,i}$: ecological significance of cultures coefficient (k_{pn} for each category of land use: arable land 0.14; meadows 0.62; pastures 0.68; gardens 0.50; orchards 0.30; forested areas a voda 1.00; others 0.10)

p: acreage of the cadastral area

Calculation of CES according to Míchal (1985 in Lipský 2000)

Methodology according to Míchal presents a proportion number and specifies the area ratio between stable and unstable landscape forming features in the interest area. The higher the value of CES comes out, the more stable and quality a landscape is.

Calculation formula: $CES = (FA + WA + PG + Pa + We + Or + Vi) / (AL + HA + Ho)$

$CES = \text{stable ecosystems} / \text{unstable ecosystems}$

Stable ecosystems: FA: Forested areas, WA: Water areas, PG: Permanent grassland, Pa: Pastures, We: Wetlands, Or: orchards, Vi: vineyards (+ gardens)

Unstable ecosystems: AL: Arable land, HA: Human areas, Ho: hopgardens

RESULTS AND DISCUSSION

Development of land use evaluating

For years 1845, 1948, 1990 and 2000 was used data from the Database Lucc Czechia. For the year 2016 was used actual data which provides COSMC. COSMC currently not distinguish meadows and pastures and values are listed as summed permanent grassland.

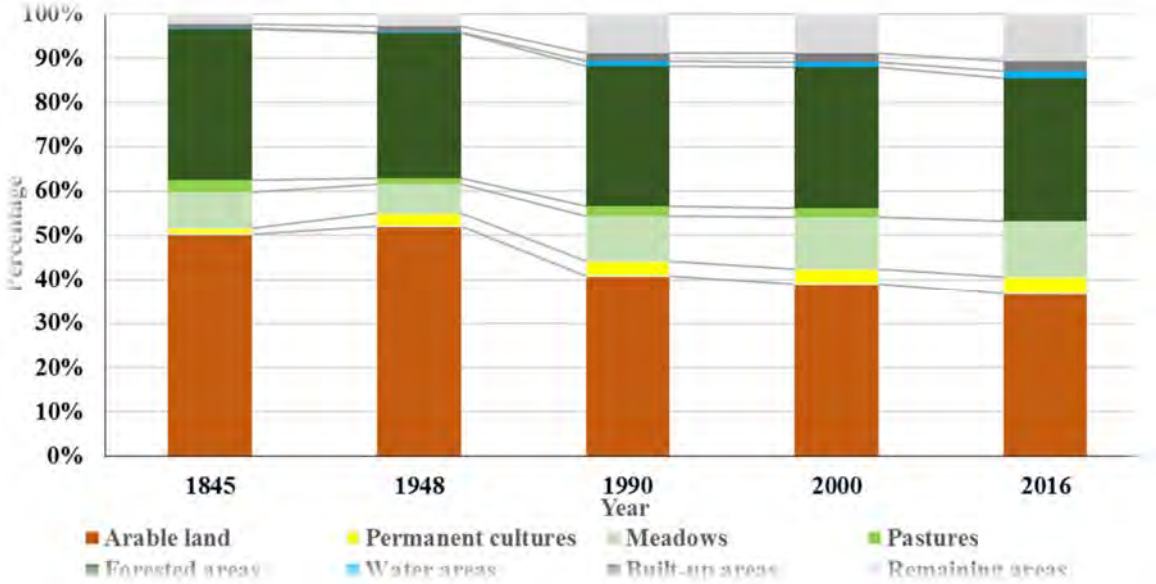
Acreage development of individual cultures in hectares is shown in Table 1. Percentage representation of individual cultures over the years is shown in Figure 2.

Table 1 Development of acreage of cultures in ha in BTU Bělá u Jevíčka

Bělá u Jevíčka	**Year**				
Culture name	**1845**	**1948**	**1990**	**2000**	**2016**
Arable land	924.1	954.1	745.9	713.8	673.1
Permanent cultures	21.3	53.8	63.8	62.4	68.4
Meadows	151.2	122.4	186.7	215.1	231.7
Pastures	50.9	25.3	39.2	37.0	
Agricultural land	***1 147.50***	***1 155.60***	***1 035.60***	***1 028.30***	***973.2***
Forested areas	***623.5***	***601.1***	***582.4***	***583.7***	***593.4***
Water areas	3.5	3.5	20.4	21.1	30.2
Built-up areas	16.9	25.5	32.2	36,6	38.6
Remaining areas	43.2	49.4	162.2	164.3	196.8
Other land	***63.6***	***78.4***	***214.8***	***222.0***	***265.7***
Total [ha]	**1 834.60**	**1 835.10**	**1 832.80**	**1 834.00**	**1 832.23**

Data source: Database Lucc Czechia: 1845, 1948, 1990 and 2000; COSMC: 2016

Figure 2 Comparison of Land Use in % for years 1845, 1948, 1990, 2000 and 2016, own calculation



Highest acreage in BTU has arable land. The acreage of arable land was highest in 1948, which occupied 52% of the total acreage. Lowest acreage of arable land was occupied in 2016 and reached 37% of the total acreage. From 1990 to the present acreage of arable land is decreasing in favor of mostly remaining areas, built-up areas and forest areas. Forest area over the years occupied the second highest acreage of the whole. In 1845, forest area occupied most of the total area, namely 34%. After the reduction between 1948 and 1990 the acreage of forest area has grown since 1990 to the present. Permanent grassland (meadows and pastures) had the lowest acreage in 1948, because some part was probably converted to arable land. Permanent grassland currently occupy 12.7% of the total acreage. Built-up areas and remaining areas has increasing tendency. Mainly remaining areas between 1948 and 1990 increased by 6.2%, from 2.7 to 8.9% of the total acreage. Permanent cultures recorded the highest increase in acreage between 1845 and 1948, and the acreage continues to grow after a small decline in 2000 until today, when occupying 3.7% of the total acreage. Water areas grew the most between 1948 and 1990, namely from 0.2 to 1.1% of the total acreage. Other growth of water areas was recorded in 2016, namely 1.7% of the total acreage.

Generally, can be stated the largest acreage on the area of interest are occupied by forests and arable land, that is currently 69.1%. After 1990 is apparent loss of arable land and an increase in other

areas, slow increase of the forest and water areas that are important for improving ecological stability of the area.

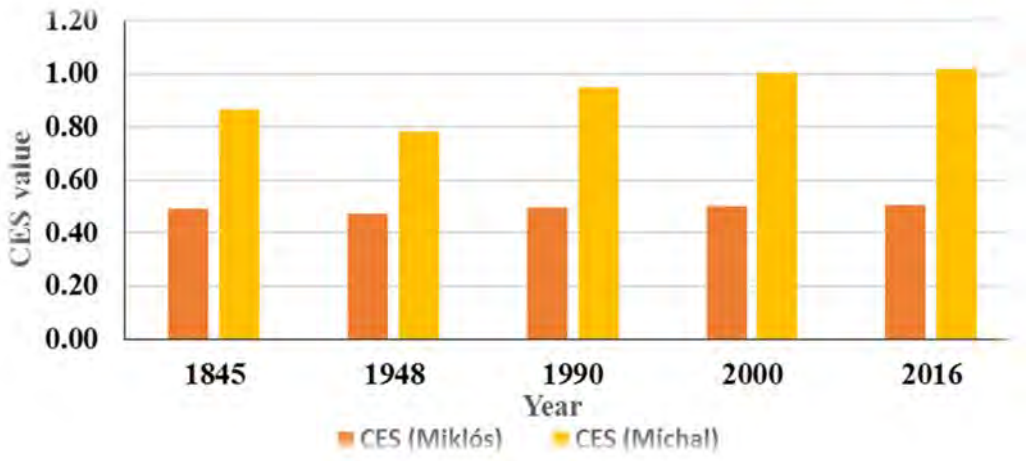
Evaluation of the ecological stability

The value of CES according to Míchal methodology is between 0.78–1.02. The lowest value was in 1948 and the highest in 2016. It should be noted step increase in value of CES in 1990, and increase of only 0.01 between 2000 and 2016. Increasing of environmental stability after 1990 is therefore stagnating. Reducing of CES in 1948 is due to an increase in acreage of arable land. Increasing of the coefficient since 1990 is due to reduction of arable land and increasing quantities of permanent grassland, forest areas, water areas and permanent cultures.

For evaluation of CES according Míchal the area of interest can be classified as follows: 0.30<CES<1.00: intensively used area, especially with agricultural mass production, weakening of self-regulation processes in their agroecosystems causes significant ecological lability and requires high energy supplementary deposits; little stable area: intensive use of cultural landscape (agricultural landscape), (Lipský 2000).

The value of CES by Miklós methodology is between 0.48–0.51. The lowest value was reached in 1948 and the highest in 2016. It may be noted stagnating values since 1990 again. In comparison with Míchal method are different values achieved in relation to another method of calculation. The value of CES after 1990 isn't increasing probably because of increasing acreages of built-up areas and remaining areas. Development of CES for years is shown in Figure 3.

Figure 3 Development of the CES for the period, own calculation



CONCLUSION

This contribution deals with development evaluation of land use and analysis of long-term ecological stability development around Jevíčko. The interest area is located in the south-eastern part of the Pardubice region and is formed by BTU Bělá u Jevíčka. From the Lucc Czechia database and data from COSMC were selected and compiled the required data with individual acreages of cultures for interest BTU. From these data was calculated CES according to Míchal and according to Miklós.

On the interest area since 1990 is evident decrease of arable land and rise of the other areas. It is also evident a slow increase in forest and water areas after 1990, which are important for improving ecological stability of the area. Ecological stability quantified by CES was increased in 1990, but since 1990 is value of CES stagnant.

The most important period of evolution of Czech Republic landscape was between years 1948 and 1989. The processes of nationalisation and so-called socialist industrialisation led to an enormous increase in the exploitation of natural resources. The impacts on land-use structure were enormous (Bičík et al. 2001). The overall coarse-grained structure of our rural landscape was not significantly changed since 1990 because it is corresponding with technology used in Europe and overall European trend of increasing cultivated lands (Lipský 2000).

Land use changes are the result of a complex interplay of drivers and processes operating at different spatial and temporal levels. Landowners play a crucial role in land use changes and are the target of many policy interventions and instruments (Kristensen 2016).

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DISPERSED SETTLEMENT IN THE VILLAGE TERCHOVÁ

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Abstract: Dispersed settlement is a unique settlement type which appears in Slovakia. Dispersed settlement was created by one of the newest waves of settlement in 15th and 16th century in marginal and houseless parts of our country. This settlement was just seasonal at first, but it became permanent later. This kind of settlement appears in the village Terchová which belongs to area called "Javornícko-beskydská kopaničiarska oblasť" and Žilina subarea.We studied the development of dispersed settlement in Terchová from 1949 up to the present time. We studied current possibilities and tried to propose another development oportunities for dispersed settlement. The use of dispersed settlement transformed several times since its creation. It was mainly functional or physiognomic changes. Dispersed settlement was used for living and husbandry at first. Later the residents started to migrate from hamlets to village centre and some hamlets became abandoned. In past several decades these abandoned hamlets have begun to be used by cottagers. Nowadays hamlets in our area of interest are used either for living or for recreation. During our research we were curious about how will dispersed settlement develop in the area that is a centre of tourism in Malá Fatra National Park and how increased number of visitors will effect it.

Key Words: hamlet, Terchová, landscape potential, land use, "Javornícko-beskydská kopaničiarska oblasť"

INTRODUCTION

Dispersed settlement is according Sitar (1967) a settlement type, which is spatially limited and fixed on husbandry. Its beginning is linked to a period of social need, which led to reclaiming unused mountain land. According Veresik (1974) dispersed settlement consists of individual settlement units or groups of houses which include 2–10 or more houses. Dispersed settlement according Horvath (1980) consists of dispersed settlement units which were originated outside the village centres and have unique names.

Dispersed settlement at Slovakia is divided into 5 areas and each area has several subareas (Huba 1990). Terchová belongs to area called "Javornícko-beskydská kopaničiarska oblasť" and Žilina subarea. In Slovak republic there are used several different regional names for dispersed settlement, for example: "rále, role, štále, kopanice, lazy, pľace or osady". In Terchová there is used name hamlet.

Terchová can be found in the north of Slovak republic, in Žilina county and Žilina district (see Figure 1). This village belongs to micro region "Terchovská dolina" valley and there are the most hamlets of all villages which are included in these micro region. There are 74 hamlets in Terchová (see Figure 2). Beside the fact that Terchová is the village with dispersed settlement, it is also the centre of tourism of international interest (Mariot 2002). Terchová is the start point for hiking in Malá Fatra National Park. House fund in some hamlets is used as accommodation for tourists. Accommodation facilities are situated by main roads near to the village centre. These accommodation facilities are in form of lodgings and pensions. Characteristic traits, mainly authentic architecture, are substituted by unoriginal elements.

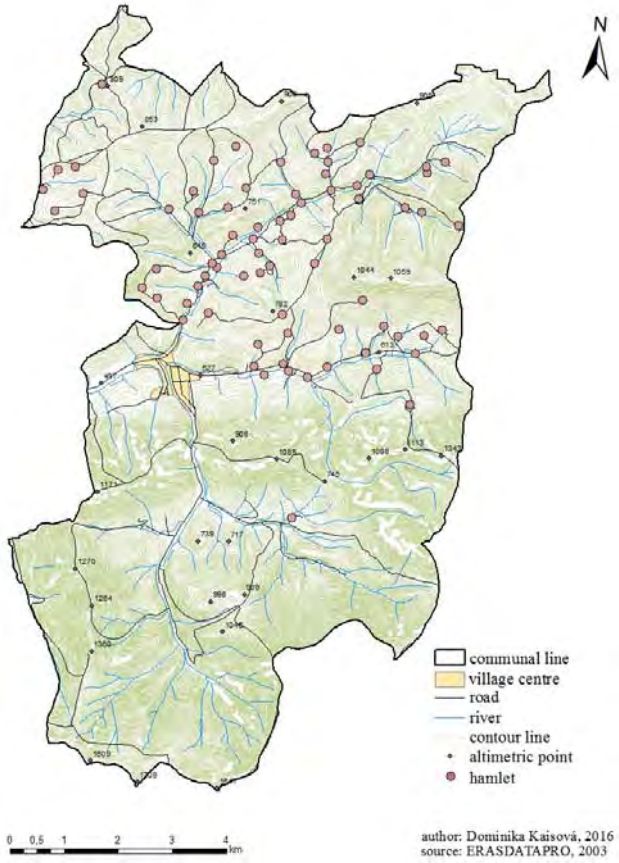
The main aim of this research was to evaluate present state of dispersed settlement in Terchová and to suggest arrangements in the field of recreation, agriculture and house fund development. These

suggestion was made on the ground of acquired information about our area which should lead to preservation of valuable elements in the country and prevent them from destruction.

We decided to study development and changes in Terchová because we think that land with dispersed settlement deserves preservation. The results of these study could be used by creating new spatial plan of Terchová and as information source for National Park Malá Fatra.

Figure 1 Placement of the village Terchová within Slovak republic

Figure 2 Dispersed settlement in the village Terchová in 2016



MATERIAL AND METHODS

Field research (identification of hamlets in the selected area, road types that lead to the hamlet, civic amenities, hamlet function, land use)

Main part of our study was field research, by which we identified 74 hamlets and noted down their co-ordinates by using GPS. We used co-ordinates in coordinate system WGS 84, which we transformed into coordinate system S-JTSK Ferro/Krovak in programme QGIS 2.2.0 later.

During our research we evaluated road types that lead to the hamlets and we divided it into two categories – metalled road (asphalt or concrete surface) and earth road (gravel or clay surface). This indicator is important because of availability aspect.

Another indicator, which was evaluated, was civic amenities. We used Sitar's (1967) methodology. In his study he selects several objects which should be in the hamlets that are important crossroads or they are creating certain centre. These objects are school, policlinic, groceries and bus stop.

We determined hamlet's function on the ground of field research and according information about houses and inhabitants which we obtained at municipal office in Terchová. We divided hamlets with housing function, housing and recreational function and recreational function. In the hamlets with housing function there were houses used by village inhabitants and not by recreants. In the hamlets with housing and recreational function there were houses tenanted by residents and by recreants. In the hamlets with recreational function there were houses used only by recreants and there were no residents. In few cases there lived less than 5 residents and they lived there for their whole life.

When we were evaluating land use, we focused on husbandry and we evaluated how residents and recreants are taking care of their properties (presence of orchards, meadows, pasture land, arable land and succession). We used information about house fund development plans form spatial plan of Terchová. There were also information about landscape, civic amenities, demographic information, infrastructure and development information, but we focused mainly on house fund development information.

The hamlets distance from the village centre

We evaluated hamlets in terms of their distance from the village centre. We measured these distance from the village centre to each hamlet in kilometres measured on road. We used Nahalka et al. (1966) methodology who selected 4 distance categories: 0–2 km, 2.1–5 km, 5.1–10 km and more than 10 km.

House fund and the hamlet's residents

We evaluated house fund by Nahalka et al. (1966) methodology. We were working only with lived-in houses, so we had to change the first category on 1–5 houses (initial category was 2–5 houses) the second category was 6–9 houses, the third category was 10–20 houses, the fourth category was 20 and more houses and we added the last category – without lived-in houses.

There were 5 categories while we were evaluated residents. There were hamlets with 1–10, 11–50, 51–100, 100 and more residents and hamlets without residents.

Area changes of the hamlets

Based on historical ortophotographs from the year 1949 and current ortophotographs from the year 2015 we created in QGIS 2.2.0 two polygon shapefiles which we used for calculating of real area by script Realna_rozloha. Sh (Sevcik and Jakab, 2015). It is a tool that counts area on the grounds of slope which helps us to integrate digital elevation model and not only its 2D representation. According calculated values we could compare build-up area changes. These values tell us which hamlets have tendency for development and which will perish.

Central hamlets

Following information about civic amenities, road type, hamlet's distance from village centre, number of residents and houses we suggested several hamlets that could create centre and in which should concentrate civic amenities and which should constitute initial point for surrounding hamlets.

RESULTS AND DISCUSSION

Road type and the hamlet's distance from the village centre

During our field research, we discovered that in 55.4% there are metalled roads mainly with asphalt surface. There were 44.6% hamlets with earth roads and these hamlets were mainly more distant from village centre than hamlets with metalled roads. Earth roads are one of the reasons why these hamlets are used mainly by recreants.

While we were measuring hamlet's distance we recognized that 8.1% of hamlets are 0–2 km distant from the village centre, 55.4% of hamlets are 2.1–5 km distant and 36.5% of hamlets were distant 5.1–10 km. In the last category – 10 and more km there were no hamlets. The furthest hamlet was 9.7 km distant from the village centre. The nearest hamlet was only 0.9 km distant from the village centre.

The distance influences hamlets function and influences presence of the residents or cottagers in the hamlets.

The house fund and hamlet's residents

We evaluated only lived-in houses during our research and we found out that there are 45.9% of hamlets with 1–5 houses. In these category, there are the most hamlets and we can see that the majority of hamlets has quite small lived-in house fund. In the category 6–9 houses there were 10.8% of hamlets, in the category 10–19 houses there were 8.1% of hamlets, in the category 20 and more houses there were 16.2% of hamlets and without lived-in houses there were 18.9% of hamlets.

There were 18.9% of hamlets without residents in Terchová. The majority of hamlets (36.5%) was in the first category (1–10 residents). In the second category (11–50 residents) there were 21.6% of hamlets, in the third category (51–100 residents) there were 8.1% of hamlets and in the fourth category (more than 100 residents) there were 4% of hamlets. In 10.8% of hamlets there was no information about houses at municipal office in Terchová.

House fund and number of residents in hamlets showed us hamlets which are used most and which are used least by residents. Thereafter we could establish those hamlets that have potential to grow and hamlets that are on their way to stagnation or destruction.

Civic amenities

Bus stop occurs at 20.3 % of hamlets. Bus transportation connects hamlets with the village centre, another villages and the town Žilina. Scholl occurs in one hamlet and the groceries is in two hamlets. Policlinic is in no hamlet. Current amenities are deficient and that’s why we suggested placing smaller shop in few hamlets with strategic location, in hamlets on crossroad or surrounded by other hamlets so they are creating certain centre. Deficient civic amenities could be the reason of depopulation of hamlets.

Hamlet's function

27% of hamlets had housing function and housing and recreational function. The most of the hamlets (46.6%) had recreational function. These hamlets could have also few houses with residents, but if there were residents, there were mostly only 1 or 2 of them in post productive age. We could claim that in several years these hamlets will be with no residents.

The area progress

We recognized area growth by 86.5% of hamlets while we were comparing area from the year 1949 and the year 2015. The most area growth there was in one hamlet–its area in 1949 was 0.33 ha and in 2015 is was 8.49 ha. There is a school, bus stop, its distance from the village centre is only 2.7 km and there are no natural limits to stop its development. Area reduction was in 5.4% of hamlets. There was no hamlet with area reduction more than 1 ha. Without area changes was 8.1% of hamlets.

Central hamlets

We selected 11 hamlets of 74 hamlets which should be the central hamlets. These hamlets are creating the start point for surrounding hamlets and that is why there should be more civic amenities that could satisfy needs of residents of the central hamlet and surrounding hamlets. If there are insufficient civic amenities we suggested its completion. There are 52 hamlets that belonged to

11 central hamlets. There are 11 hamlets without central hamlets. These hamlets are too distant or they are using civic amenities of another villages.

On the base on required information we suggested concrete arrangements for hamlets in the area of house fund that involve proposal of new build-up and reconstruction of old wooden houses and farm buildings. We partly used information from ground plan of the village Terchová. In our proposal, we also used information about area changes of hamlets, number of residents and live-in houses. We suggested new build-up by 35.2% of hamlets that are near to the village centre and their infrastructure is developed and so they are more attractive for residents. Reconstructions were proposed at 31.1% of hamlets that are used mainly by cottagers and their architecture is quite well-preserved. We suggested to keep in natural state 33.7% of the hamlets. Development of these hamlets is limited by conditions of the land. Dispersed settlement is a kind of settlement where is quite massive depopulation (Huba 1989). Negative conditions in combination with great distance are causing downfall of dispersed settlement (Huba 1990). Today there are the most distant hamlets without residents. But if there are favourable conditions like good roads, valley position and civic amenities, not even great distance should be a barrier to another development. If the hamlet is not efficiently connected with village centre, it is probable that this hamlet will perish.

Next field that involved suggestions was tourist traffic. We don't recommend to channel massive tourist traffic into the area with dispersed settlement because of preservation characteristic traits of the landscape that will with high probability be destroyed or changed as it happened in several hamlets nearby the village centre which house fund is used as accommodation for tourists. There are new recreational centres in these hamlets that are constructed in a new architectural style and they don't fit into the current landscape. The future of using some hamlets is agrotourism. This way the visitors could learn about dispersed settlement history and about authentic agricultural use of the land. It could help to preserve characteristic traits of the land with dispersed settlement. There are 58.1% of hamlets that cross cyclorouts, tourist routes or cross-country skiing routes. In hamlets that are positioned in valleys there are restaurants and pubs, but there are no such facilities in higher areas. The solution could be that there will be 2 or 3 small pubs or restaurants that will be open only during weekends. These facilities should be on the most frequented routs and they will serve local meals and drinks. The last option for recreational use of hamlets is their using by cottagers, mainly during weekends. The last option is used the most frequently.

Husbandry in Terchová is limited by lower soil quality and climate conditions. There are typical sheep and beef raising in Terchová. Arable soil was in the past obtained by deforestation and in the present days these areas are used as meadows and pasture land. Almost in all hamlets we can find old and newly founded plum orchards. It is necessary to keep agriculture in Terchová alive and to produce local products and sell them to tourists. Barnes and Robinson (1940) and Stone (1991) were focusing on agriculture, but in their areas were farms with high soil quality.

Omasta (2011) sees the future of dispersed settlement in intersection of current functions and he claims that by use of local sources, cultural traditions and handicraft we can fully utilize potential that is not economic and social, but it is cultural, historical, aesthetic and environmental and thus ensure its sustainable development. We think that Terchová has potential for sustainable development, but nowadays the village Terchová prefers the massive tourist traffic instead of protecting historical value of the land with dispersed settlement. The only hamlets that are protected are those that are distant enough from the village centre. The hamlets pass through functional and physiognomic transformations (Omasta 2011) and in our research area it is visible mainly by building material choices and vegetation choices. Hansen (1964) saw high potential of dispersed settlement in Scandinavia in their historical values.

Norling (1960), Petrovic (2006) and Petrovic and Muchova (2013) sees the preservation of dispersed settlement in its using for recreation. We agree with these claims only partly, because not only in Terchová there are hamlets that are nearby the village centre, and they are used especially for living not recreation. Hamlets used by cottagers and for agro tourism are more distant from the village centre.

CONCLUSION

We can see the landscape changes in the village Terchová where fields, meadows, orchards and woods with dispersed settlement are changing into land with dominating recreational centres with unfitting vegetation, that are decreasing landscape value. In terms of tourist traffic, it would be advisable to focus on renewal of handicraft, traditions and husbandry that are related to dispersed settlement. Parish council shouldn't focus only on propagation of famous Juraj Jánošík and Malá Fatra National park, although is important part of the history and nature beauties of the village.

The hamlets in the village Terchová are heterogeneous and it's visible that the hamlets in the valleys differ from the hamlets on the hills and that's why we couldn't set a certain tending of all hamlets.

The valley hamlets have better conditions to keep their housing function. It happens at the expense of destroying characteristic land lines. The hamlets on the top of the hills are mostly used by cottagers and they are fit for recreation. New construction shouldn't be realized in here. These hamlets are more preserved because cottagers are trying to preserve house fund and characteristic architecture.

It is necessary to consider characteristic elements in the landscape during projecting spatial plan. Obtained information is valuable for designing spatial plan and for Malá Fatra National Park in which protective zone the hamlets are situated.

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USE OF PHYTOTOXKIT™ TEST IN ASSESSMENT OF TOXICITY OF TWO TYPES OF SEWAGE SLUDGE

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Abstract: The investigations aimed to determine a possible application of Phytotoxkit™ biotest for the assessment of soils amended with sewage sludge. The experiment was performed in a laboratory on samples of sewage sludge. The test bases on estimation of germination and early growth inhibition of sweet sorghum (*Sorghum saccharatum* L.). Two kinds of sewage sludge: dewatered and anaerobically stabilized sludge with dry matter content of about 24%, and dewatered sludge with dry matter content of about 92%. The results indicate that the tested samples of sewage sludge are toxic. Growth inhibition (%) at the studied samples ranged from 94.97% to 100%. Phytotoxkit is a good method to evaluate the toxicity of sludge's, and can be a valuable addition to the physico–chemical methods.

Key Words: sewage sludge, sewage treatment plant, phytotoxicity, sweet sorghum (*Sorghum saccharatum* L.), land application

INTRODUCTION

Sewage sludge generated from wastewater treatment process is a menace to environment. The cost for disposal of excess sludge accounts for 25–60% of the total operating cost of wastewater treatment plant (Zhang et al. 2009). Sewage sludge pose a threat to the environment and the problem of their utilization. Alongside the undeniable fertilizing properties, the sludge characteristics include the presence of pollutants, mainly heavy metals, pathogens and harmful organic compounds (Oleszczuk 2008), which are associated with a potential danger to the environment (Rosińska and Karwowska 2016).

Studies carried out (Smith et al. 2001, Oleszczuk 2006a) showed that sewage sludge in the conditions of its land application can be a significant source of a lot of undesirable substances in the soil and plants. Heavy metals and organic compounds are among the "most popular" pollutants present in the sewage sludge (Stevens et al. 2003, Oleszczuk 2006b). The sewage sludge's application can pose an indirect risk to human health; due to the possibility of pollutants migration to groundwater, or their accumulation in plants (Adamcova et al. 2016). On the other hand, however, high contents of organic matter and nutrients make sewage sludge a perfect material for fertilization and recultivation of degraded soils (Albiach et al. 2001, Selivanovskaya et al. 2003, Oleszczuk 2008).

There has been a series of research studies carried out in recent years (Stevens et al. 2003, Oleszczuk 2006b) that has been aimed at the evaluation of the organic and inorganic pollutant contents in sewage sludge and composts. Every year new pollutants that may have, or actually have a negative influence on the living organisms are recognized. Biological tests are a method which determines their negative influence and possible interactions among them (Oleszczuk 2008, Adamcova et al. 2016).

Plants are essential primary producers in the terrestrial ecosystem. In addition, the crop yield and quality are important success criteria in agriculture. Therefore, it is important to identify potential phytotoxins and understand the magnitude of their impact on different terrestrial ecosystems (Schowanek et al. 2004, Oleszczuk 2008, Adamcova et al. 2016). Reports have considered phytotoxicity test to be useful in assessing environmental (soils, sediments) and anthropogenic (compost, sewage sludge) matrix toxicity (Czerniawska–Kusza et al. 2006, Oleszczuk 2008). Many of authors confirmed

that the Phytotoxkit microbiotest is effective in identifying toxic samples contaminated with heavy metals, PAHs, pesticides (Czerniawska–Kusza et al. 2006, Wadhia and Thompson 2007, Mankiewicz–Boczek et al. 2008, Oleszczuk 2008, Sekutowski and Sadowski 2009).

The research aimed at assessing the toxicity of sewage sludge by means of Phytotoxkit™ test. The general aim of the present work was: (1) to characterize the sewage sludge's, (2) to assess the phytotoxicity of two types of sewage sludge's and (3) to investigate the effect of stabilization strategy used on sludge phytotoxicity. The effects on seed germination and root growth were determined in *Sorghum saccharatum* L. Such bioassays are simple and rapid methods to indicate phytotoxicity (Wong et al. 2001, Adamcova et al. 2016).

MATERIAL AND METHODS

Characteristic of sludge's

Sewage sludge samples were collected from the sewage treatment plant (mechanical–biological treatment system) in Czech Republic. The wastewater treatment plant serves around 374.000 inhabitants with an influent flow rate of about 4.22 m^3/s. The treatment plant consists of a conventional extended aeration activated sludge process (Adamcova et al. 2016).

The experiment was carried out on two sludge's. The samples (1 kg) were collected in triples (sample A, B and C) at the end point of the sewage sludge digestion process. Sewage sludge's were typical aerobically digested. The two types of sludge's had been stabilized in different ways as follows: II – dewatered and anaerobically stabilized sludge with dry matter content of about 24%, I – dewatered sludge with dry matter content of about 92%.

Chemical characteristic of the sewage sludge's is presented in Table 1. The collected samples were stored in glass bottles and immediately transported to the laboratory. All sewage sludge samples were air–dried and crushed to obtain representative samples. Sewage sludge's were crushed in a mortar and then sieved through a 2 mm sieve for chemical and ecotoxicological analysis (Adamcova et al. 2016).

Table 1 Chemical characteristic of the sewage sludge's (Adamcova et al. 2016)

I – Dewater sludge "Palikal" (92% DM)	**Hg (mg/kg)**	**Cd (mg/kg)**	**Ni (mg/kg)**	**Cr (mg/kg)**	**Cu (mg/kg)**	**Zn (mg/kg)**	**Pb (mg/kg)**
Sample A	1.60	0.640	31.6	68.6	200	964	30.4
Sample B	2.31	0.450	29.9	61.7	204	872	30.2
Sample C	1.86	0.570	28.4	65.5	213	907	28.4
II – Stabilized sludge (24% DM)	**Hg (mg/kg)**	**Cd (mg/kg)**	**Ni (mg/kg)**	**Cr (mg/kg)**	**Cu (mg(kg)**	**Zn (mg/kg)**	**Pb (mg/kg)**
Sample A	1.92	0.840	35.5	79.6	184	765	27.7
Sample B	1.69	0.880	32.8	71.2	199	895	24.8
Sample C	2.10	0.590	29.4	71.2	210	906	26.6

Phytotoxicity test

The toxicity of sewage sludge was assessed with a commercial toxicity bioassay–Phytotoxkit™ Test (Microbiotests, Nazareth, Belgium) (Phytotoxkit™ 2004). The test was based on measurement of germination and growth of the plant roots after three days of exposure to the soil/sewage sludge in comparison with germination and growth of these plants in the soil. The test was conducted following the procedure recommended by the manufacturer (Phytotoxkit™ 2004). The phytotoxkit makes use of flat and shallow transparent test plates composed of two compartments, the lower one which contains

soil saturated to the water holding capacity (Adamcova et al. 2016). In the experiment *Sorghum saccharatum* L. was chosen.

The phytotoxkit measures the decrease (or the absence) of seed germination and of the growth of young roots after 3 days of the exposure of selected seeds of higher plants to a contaminated matrix, in comparison to the controls in a reference soil. Water saturation is calculated according to the user's manual. The distilled water was spread over the entire surface of the soil in the test plate. Ten seeds of *Sorghum saccharatum* L. were positioned at equal distances near the middle ridge of the test plate on a filter paper placed on the top of the hydrated soil/sewage sludge mixture.

After closing, the test plates were placed vertically in a holder and incubated at 25 °C for 3 days. At the end of the incubation period a digital picture was taken of the test plates with the germinated plants. The analyses and the length measurements were performed using the Image Too l3.0 for Windows (UTHSCSA, San Antonio, USA). The bioassays were performed in three replicates. The percent inhibition of seed germination (SG) and root growth inhibition (RI) were calculated with the formula (1):

$$SG / RI = A - B / A \times 100 \quad (1),$$

where A means seed germination and root length in the control; B means seed germination and root length in the test (Adamcova et al. 2016).

RESULTS AND DISCUSSION

To evaluate the toxicity tests with the test plants *Sorghum saccharatum* L. the parameters shown in Table 2 (the basic characteristic of the growth inhibition and the degree of toxicity) were used.

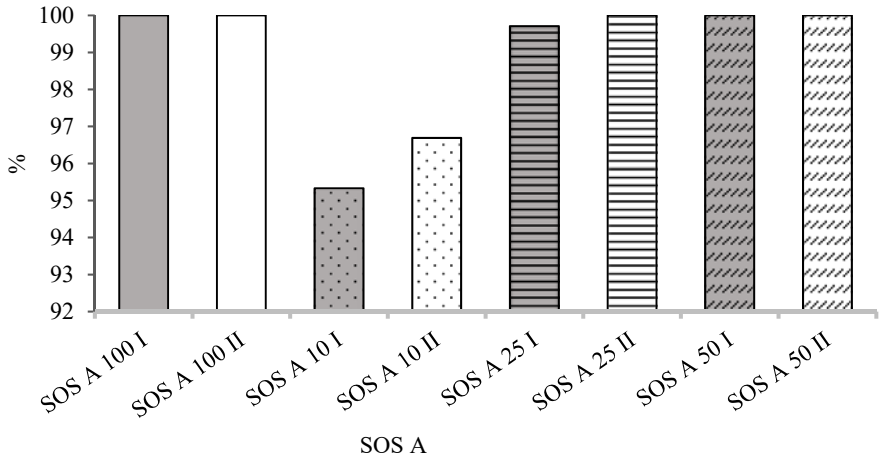
Table 2 The degree of toxicity (www.um.prf.jcu.cz, adjust 2016)

Inhibition (%)	The degrese of toxicity	Evaluation
I* < 10	1	Non – toxic or slightly toxic
10 < I < 50	2	Toxic
50 < I	3	Strongly toxic

** I – growth inhibition (%)*

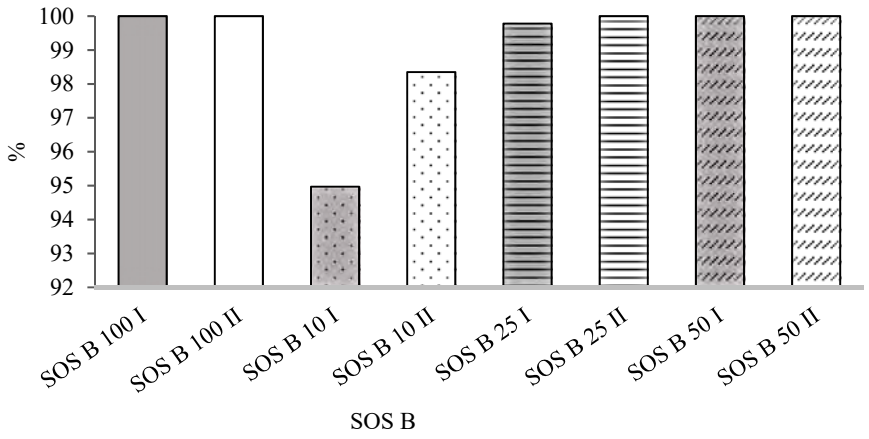
Figure 1–3 presents the effect of the sewage sludge (concentration 100%, 10%, 25% and 50%) on the inhibition of seed germination and root growth as related to the test plants *Sorghum saccharatum* L. (SOS), Samples A, B and C.

Figure 1 SOS Sample A



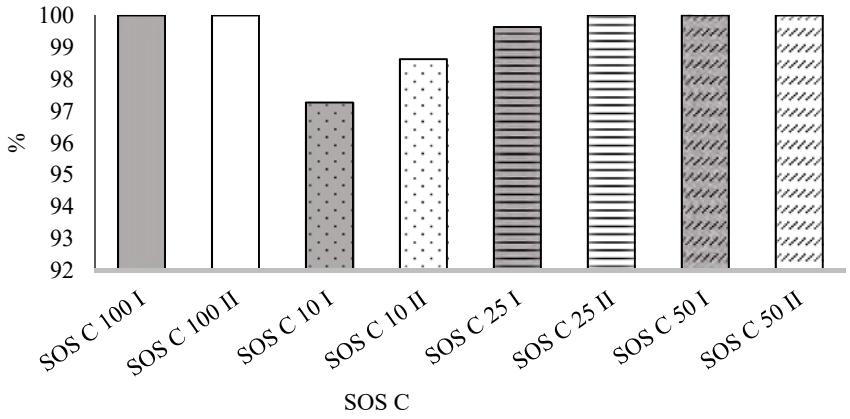
The growth inhibition (%) of *Sorghum saccharatum* L. for dewatered and anaerobically stabilized sludge with dry matter content of about 24%, and dewatered sludge with dry matter content of about 92%, Sample A was in the range of 95.33–100%. These samples are strongly toxic, the degree of toxicity 3, 50 < I.

Figure 2 SOS Sample B



The growth inhibition (%) of *Sorghum saccharatum* L. for dewatered and anaerobically stabilized sludge with dry matter content of about 24%, and dewater sludge with dry matter content of about 92%, Sample B was in the range of 94.97−100%. These samples are strongly toxic, the degree of toxicity 3, 50 < I.

Figure 3 SOS Sample C



The growth inhibition (%) of *Sorghum saccharatum* L. for dewatered and anaerobically stabilized sludge with dry matter content of about 24%, and dewatered sludge with dry matter content of about 92%, Sample C was in the range of 97.27−100%. These samples are strongly toxic, the degree of toxicity 3, 50 < I. The image of the *Sorghum saccharatum* L. control Sample and Sample A.

Sewage sludge is the by–product from the wastewater treatment plant. It is a complex mixture of organic and inorganic materials and contains a wide variety of substances and microorganisms in suspended or dissolved form (Werther and Ogada 1999, Niu et al. 2016).

Due to mixed contamination of sewage sludge with potentially hundreds of different substances, environmental quality assessment of sewage sludge or composted sewage sludge is challenging and cannot be achieved with chemical analysis alone. Ecotoxicity assessment provides valuable information on the environmental fate of these materials. Biotest could be used as an indicator for potential risk for example when sewage sludge–based products are targeted to agricultural or landscaping applications (Kapanen et al. 2013).

The problem of the effect of sewage sludge on seed germination and plant growth has been addressed by numerous researchers (Fjällborg and Dave 2004, Fuentes et al. 2006, Hu and Yuan 2012, Oleszczuk 2008, Ramirez et al. 2008, Oleszczuk et. al. 2012, Adamcova et al. 2016). Among different toxicity indices based on germination and seedling growth of various higher plants, the growth inhibition seemed to be a good method for the evaluation of the toxicity of sewage sludge (Czerniawska–Kusza 2006, Adamcova et al. 2016). In the present study, the plant species of the Phytotoxkit microbiotest

responded differently to the degree of contamination of the sewage sludge samples. In general, growth inhibition values clearly revealed the inhibitory effects of sewage sludge contaminants on seed germination and root elongation of *Sorghum saccharatum* L.

CONCLUSIONS

In the present study, two types of sewage sludge: dewatered and anaerobically stabilized sludge with dry matter content of about 24%, and dewatered sludge with dry matter content of about 92% caused phytotoxic effects on the tested plant species, manifesting as root and shoot growth reduction or total inhibition. The results indicate that the tested samples are toxic. Growth inhibition (%) at the studied samples ranged from 94.97% to 100%. In the control, the average root length of *Sorghum saccharatum* L. reached 33.47 mm. In conclusion, the data of this study revealed that the Phytotoxkit microbiotest was effective in identifying toxic sample. In this context, further studies should be performed on sewage sludge characteristics for a better understanding of the biological/ecotoxicological response to the contaminants present.

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ANALYSIS OF SOIL AGGREGATE DEGRADATION IN HEAVY SOILS SITUATED IN LOCALITIES AT RISK OF WIND EROSION

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Abstract: We present the results of our study of wind erosion in heavy soils. The areas that are at highest risk of wind erosion usually display light soils; however, in some localities with the occurrence of heavy soils, wind erosion may also be observed in particular meteorological conditions. The meteorological conditions in winter seasons induce degradation of soil aggregates. This degradation in mainly effected by the action of water and subsequent freezing/thawing of the soil surface. Due to the pressure of freezing water in the soil and its subsequent resolution, the soil aggregates are degraded to erodible fractions, with a possible erosion event in the case of erosion-effective wind. The quality of agricultural land fund in the Czech Republic is assessed via a valuation system based on the ecological-productive land evaluation. This system was established in the 1960–1980s after a complex survey of agricultural land. It provided integral information on the agricultural land quality and on the price of agricultural land parcels derived from their productive capacity. Starting from the 1990s, evidence in the database of Evaluated Soil-Ecological Units (ESEU) has been regularly updated. To analyse the susceptibility of heavy soils to wind erosion we used the database of ESEU and selected soil types according to the main soil unit (further referred to as MSU) – 06, 07, 20, 61, 63, and 57. The studied area is situated in the surroundings region of South Moravia (Czech Republic), where all the above-mentioned MSU can be found. In all cases, soil samples were collected at the beginning and end of winter. After collection, the soil samples were analysed for aggregates and the proportion of erodible and non-erodible fractions was determined. Climatic data on the temperature of soil surface and condition of soil moisture were obtained from the nearest professional station of the Czech Hydrometeorological Institute (CHMI).

Key Words: wind erosion, heavy soil, soil structure, erodible particles

INTRODUCTION

Erosion is a natural process in which the action of water, wind and other factors cause disruption of the soil surface and subsequent transportation of soil particles. Wind erosion thus represents a natural process of erosion consisting in disruption of the soil surface by the mechanical force of wind (abrasion), transportation of the soil particles by wind (deflation) and their deposition at another place (accumulation). Wind erosion is a physical phenomenon and is directly influenced by the physical soil properties (Janeček 2008). Localities susceptible to wind erosion are characterized by low and fluctuating precipitation, fluctuating and high wind velocity, frequent occurrence of droughts, and extreme changes in temperature and high evaporation (Pasák 1984). The periods with highest risk of wind erosion usually occur in spring and autumn, when the soil is not protected by the vegetation cover. Wind erosion is mostly observed in localities with soils of low clay particle content (light soils). A particular effect is found in soils with higher content of clay particles (heavy soils), which under specific climatic conditions become better erodible. During winter seasons, the meteorological conditions induce degradation of soil aggregates. This degradation is mainly brought about by the action of water and its freezing and thawing. The effects of these two factors are manifested by the growth of erodible fraction, with a possible erosion event in the case of erosion-effective wind. One of the basic soil factors involved in the loss of soil particles by wind is the granularity and aggregate composition of the soil. The key role is played by the size of the soil particles, while differences in the particle shape have only little impact (Pasák 1970, Dufková 2008). Non-erodible particles in the soil are detected by

aggregate analysis by means of sieving the soil sample collected from the soil surface and dried in air through a 0.8 mm sieve; however, in case of heavy soils the threshold value for non-erodible soil particles must be shifted to 2 mm (Švehlík 2002, Holý 1994). The phenomenon of wind erosion in heavy soils is most significantly manifested in the Czech Republic in southeast Moravia in the White Carpathian Mountains foothills (Švehlík 1987, 2002).

MATERIAL AND METHODS

Study area

To analyse the erodibility of heavy soils by wind erosion we used the database of valued Evaluated Soil-Ecological Units (ESEU), the soils were selected according to MSU (06, 07, 20, 61, 63, and 57). The studied localities were situated in the surroundings of city Strážnice in the Hodonín district, where all the MSU of interest can be found. Figure 1 shows the localities of sample collection in green. Collection of soil samples was done in winter seasons 2013/2014, 2014/2015 and 2015/2016. Specifically, sampling was done in cadastral areas of Strážnice, Tvarožná Lhota, Kněždub, Rohatec, Veselí nad Moravou and Blatnička. From the geological point of view, the study area belongs to the external West Carpathian Mountains. Their geological structure includes part of the Flysch Belt and the Vienna Basin. The main part of the study area belongs to the flysch zone that is part of the Magura nappes. Maximum precipitation occurs in the summer, namely in July, minimum precipitation in winter. At the lowest altitudes, the maximum annual precipitation is close to 600 mm; in contrast, at the peak areas of the mountains the annual precipitation exceeds 920 mm. The wind velocity and direction depend on the local terrain morphology and altitude. The peaks of the White Carpathian Mountains are characterized by southeast currents that prevail in the annual average, with their frequency increasing during the summer. The southeast to south currents in southeast Moravia may display a föhn effect carried out by the downward current fraction. These dry and warm falling winds cause strong wind erosion, i.e., loss of fine soil and rock particles and their subsequent accumulation in the form of drifts of even tens of centimetres. From the pedological aspect, the prevailing soil types are cambisols. The southwest part of the White Carpathian Mountains is characterized by mesotrophic cambisols on limestone subsoil; the peak parts of the Carpathian ridge and northeast region are typical by oligotrophic cambisols on acidic substrates (Nature Conservation Agency of the Czech Republic (AOPKČR) 2010, Hrdoušek 2007).

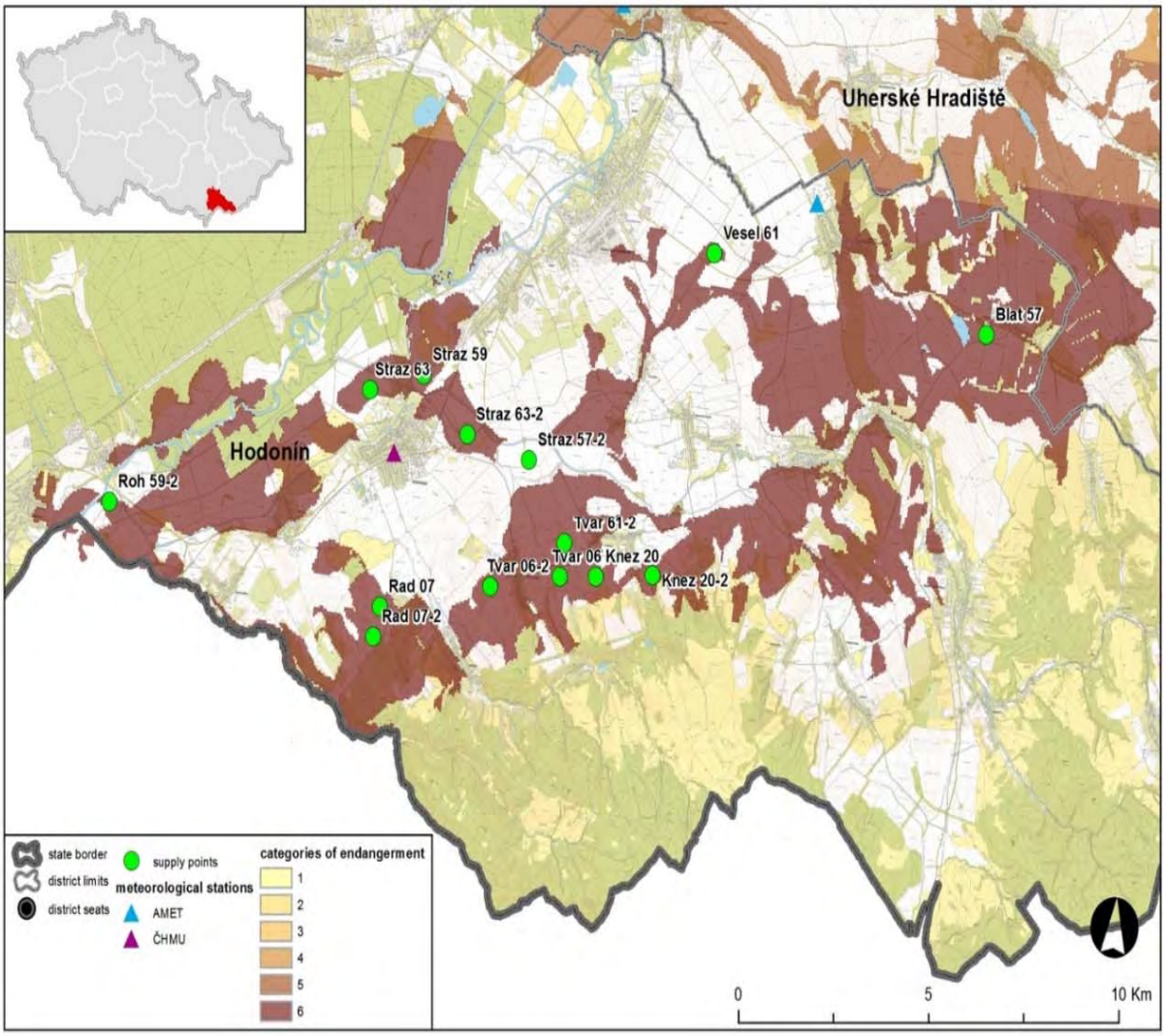
Pedological analyses of selected soil samples from the studied localities

Soil samples were collected at the beginning and end of the winter season. After collection, the samples were subjected to aggregate analysis, defining the erodible and non-erodible fractions. To determine the erodible or non-erodible fraction we used a 2 mm sieve. The soil samples were collected from a straight, smooth soil surface (to max. 2.5 cm). Samples from two localities were collected for each MSU. Selection of these localities was based on the certified Map of Potential Risk of Wind Erosion in Heavy Soils Based on Meteorological Conditions during the Winter (Podhrázská 2014). This map document was created from a specific database of relevant data (cycles of thawing and subsequent freezing of the soil surface; periods of specific degree of soil moisture) from selected meteorological stations and their following regionalization. The studied area belongs to the region at potential risk category 6 (the highest risk). The highest risk for heavy soil means that these localities experience the most frequent events of freezing/thawing in combination with frequent soil humidification.

Analysis of meteorological conditions

We analysed the climatic conditions for the winter seasons 2013/2014, 2014/2015 and 2015/2016. For these seasons, we obtained hourly data from the Czech Hydrometeorological Institute (CHMI) on the soil surface temperature and soil humidification. The climatic data were obtained from the nearest professional CHMI station in Strážnice. Figure 1 shows the main climatic station for the studied locality. The additional climatic station selected for calibration of the climatic data was stations in Association Litschmann & Suchý (AMET) in Blatnice pod svatým Antonínkem.

Figure 1 Map showing localities for collection of samples at risk category 6



RESULTS AND DISCUSSION

Pedological analyses of selected soil samples from the studied localities

Table 1 shows the results of aggregate analysis for individual MSU. In the first season 2013/2014, only soil samples of MSU 57, 59, 93, and 61 were collected. These MSU were selected based on the samples analysed in previous reports (Dufková 2007). In the following seasons, the sampling localities were extended by MSU 06, 07, and 20. The presented results show that all selected MSU display a growing tendency in erodible fraction during the winter season. The highest growth of erodible fraction was recorded in the 2013/2014 season for MSU 57 and 63. In season 2014/2015, the highest growth of erodible fraction was again observed in MSU 57 and 63. The highest growth of erodible fraction during the last season was recorded in MSU 59 and again in MSU 63.

Table 1 Results of aggregate analysis (percentage of erodible fraction) for individual winter seasons and analysed MSU

Sampling month / MSU	November	March	Difference in erodible fraction [%]
2013–2014	25.52	74.63	49.11
57	31.01	93.55	62.53
59	25.20	51.17	25.97
61	26.97	80.09	53.12
63	18.91	73.69	54.77
2014–2015	17.55	59.03	41.48
6	18.34	56.65	38.31
7	13.69	47.16	33.47
20	16.27	53.79	37.52
57	17.06	75.42	58.36
59	20.74	49.13	28.39
61	21.73	58.11	36.38
63	15.45	83.76	68.30
2015–2016	32.12	77.82	45.70
6	53.78	73.70	19.92
7	33.96	77.22	43.26
20	35.69	83.43	47.74
57	21.05	73.60	52.54
59	19.30	80.79	61.49
61	27.55	69.60	42.05
63	27.05	84.62	57.57
Total mean	24.92	69.29	44.37

Table 2 shows analysis of soil aggregate degradation in relation to the surface conditions during soil sample collection. We defined two types of soil conditions – covered by vegetation and coverless. The table shows that in samples collected on the coverless surface, the growth of erodible fraction was not so pronounced as in the covered surface. The greatest difference between the surface conditions was observed in season 2014/2015. The difference in erodible fraction was 32.19%.

Table 2 Results of aggregate analysis (percentage of erodible fraction) for individual winter seasons for covered and coverless surface

Surface conditions	Coverless			Covered by vegetation		
Sampling month	November	March	Difference in erodible fraction [%]	November	March	Difference in erodible fraction [%]
2013–2014	22.94	76.89	53.95	28.11	72.36	44.25
2014–2015	17.02	79.19	62.17	17.85	47.83	29.98
2015–2016	26.92	83.28	56.36	36.02	73.73	37.71
Total mean	22.50	81.42	58.92	26.58	61.88	35.30

Analysis of meteorological conditions

To analyse the climatic conditions we utilized data from CHMI (professional station in Strážnice). For the analysed winter seasons we obtained hourly recordings of temperature measured directly above the soil surface. Based on these data we established the number of periods of freezing/thawing in individual months and seasons. We analysed two types of periods. The first period was defined by the initial value of -5 °C (number of (-5 °C) events) and the second by initial value -2 °C (number of (-2 °C)

events). Each period was defined by its duration in hours. The analysed periods were terminated when the temperature exceeded 0 °C. The temperature of -5 °C was defined as the mean temperature at which water in solution freezes (Šantrůčková 2014). The second type of period was characterized by temperature -2 °C. To get more detailed analysis of the freezing/thawing periods we also performed analysis at -2 °C. For a start period is determined by the temperature below -5 °C or -2 °C. Each table contains data on the number of soil condition types enhancing degradation of the aggregates. These condition types are: wet soil surface (soaked – water standing in smaller or larger puddles) snow or melting snow (with or without ice) covering less than half of the soil; snow or melting snow (with or without ice) covering more than half of the soil but not covering the soil completely. The highest number of evaluated soil condition types (26) occurred in season 2013/2014, then in season 2014/2015 with 22 evaluated condition types. In the last season, we evaluated 18 condition types. The number of events with the threshold value of -5 °C was highest in the season 2015/2016, with 34 events and total duration of 446 hours. The threshold value of -2 °C recorded most events in season 2013/2014. This season recorded 62 events with total duration of 673 hours.

Table 3 Analysis of the number of periods based on surface data including soil conditions – CHMI station (Strážnice)

Period	Number of -5 °C events	Duration of -5 °C events [hrs]	Number of -2 °C events	Duration of -2 °C events [hrs]	Number of evaluated soil condition types
2013/2014	27	327	62	673	26
January	6	116	16	233	8
February	8	82	20	166	5
March	3	22	3	33	0
November	3	31	8	68	12
December	7	76	15	173	1
2014/2015	19	284	48	632	22
January	6	98	15	179	3
February	8	79	21	241	17
March	0	0	1	16	0
November	0	0	1	8	2
December	5	107	10	188	0
2015/2016	34	446	50	660	18
January	14	255	18	362	11
February	6	58	6	68	4
March	5	36	8	68	3
November	2	19	2	22	0
December	7	78	16	140	0
Sum total	80	1 057	160	1 965	66

When comparing the results of aggregate analysis with the analysis of climatic data, the best correlation was observed between the results of aggregate analysis of the soil covered by vegetation and the results of climatic analysis for the threshold value of -5 °C. In season 2013/2014, the difference in the erodible fraction content was the highest of all evaluated seasons and represented 44.25%. In this season, 27 periods with total duration of 327 hours were analysed and 26 evaluated soil condition types were noted. In season 2015/2016, the difference in erodible fraction was 37.71% with 34 periods with total duration of 446 hours and 18 evaluated soil condition types assessed. Comparison of results of both seasons shows that the decisive role in the growth of erodible fraction is played by the high number of periods and the high number of soil condition types enhancing the aggregate resolution. In season 2014/2015, the growth of erodible fraction was the lowest and represented 29.98%. This season recorded

the lowest number of periods (19) with 22 evaluated soil condition types. However, correlation of the results of aggregate analysis with climatic data is not unambiguous. When the land parcel is not protected by vegetation cover, the degradation of aggregates in most probably also caused by other factors such as the effects of drying winds. Other climatic factors have not been taken into account.

CONCLUSION

The presented results show that during the winter season, the soil aggregates degrade, resulting in the growth of erodible fraction. Considering the soil surface conditions during the soil sample collection (covered or coverless), it is evident that samples collected on the coverless surface do not display such pronounced growth of the erodible fraction as on the surface covered by vegetation. The highest difference in the soil surface conditions was recorded in the season 2014/2015. The difference in erodible fraction was 32.19%. Comparing the results of aggregate analysis and analysis of climatic data, the best correlation was observed between the results of aggregate analysis in surfaces covered by vegetation and the results of climatic analysis for the threshold value of -5 °C. For this contribution have not been processed correlation between number of cycles and % of erodible fraction.

ACKNOWLEDGEMENTS

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PILOT STUDY ON SILVER BIRCH (*BETULA PENDULA* ROTH) OCCURRENCE IN MORAVIA-SILESIAN REGION

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Abstract: Silver birch (*Betula pendula* Roth) is common tree in forest stands and potentially important source of wood and non-wood forest products. It seems that for future reforestation of forest stands which were destructed by bark beetles in Moravia-Silesian Region, birch will be promising tree species with multiple utilisation. The aim of our study is identification of influence of forest type on occurrence of silver birch in Moravia-Silesian Region, the Czech Republic, Central Europe. Study was done on data from National forest inventory I. Results of our research shows positive influence of rich forest sites on higher occurrence of silver birch. Although occurrence of silver birch is partially influenced by forest management we can recommend to continue in research on encouraging of silver birch occurrence in Moravia-Silesian Region.

Key Words: forest type, forest vegetation grade, silver birch, occurrence

INTRODUCTION

Silver birch (*Betula pendula* Roth) is common tree species in Czech forests. It is very well growing tree which very fast occupy forest land after final felling or windthrow damage (Vodde et al. 2010) and not utilised agricultural soil (Zasada et al. 2014). Silver birch is valuable source of timber which is used in many fields of wood processing industry (Gobakken 2000). Birch is important source of non-wood forest products like bark, leaves, birch sap or twigs.

It is important to know where the best conditions for grow of birch tree are in forest. Hypothesis of authors was to find relations between birch occurrence in forest stand and forest types classification (Plíva 1987), which is used for classification of forest stands in the Czech Republic.

MATERIAL AND METHODS

The data source was measurement, which took place under the National forest inventory I (NFI). The measurements were carried out according to the methodology in the areas of deployment of regular network throughout the Czech Republic. Database provided by the Institute of Forest Management, which is the guarantor of NFI data contained NFI for all surfaces in the observed region. Within each area were, inter alia, monitoring and data (events) that have significance for non-wood forest products.

Table 1 List of events contained in the database

Name of phenomenon	Units	Name of phenomenon	Units
No. area	Unique number	Herb, shrub	The word
Elevation	m	Abundances	Scale
Sub-plot number	Number	Age level	Number
Exposure	Letters	Occurrences	%
Note	The word	Canopy	%
Region	The word		

Region was firstly evaluated as a whole, and then was subjected to evaluation occurrence birch. For statistical assessment of data outside the classical methods of exploratory analysis utilized methods

of multivariate data analysis using mainly principal components, factor analysis and cluster analysis. Finally, the table or the key to creating allocation table, or at least show the potential of the species bearing non-wood forest products. Table 1 shows a list of events contained in the database that has been given.

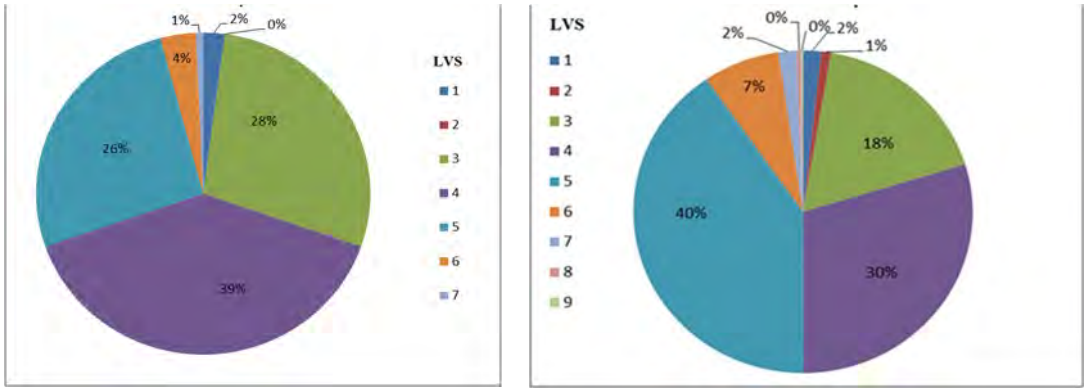
RESULTS AND DISCUSSION

Occurrence of birch recorded under forest vegetation grades (FVG) large differences in all major FVG (see Figure 1). Larger differences can be seen at 3 and 4 FVG, when an increase of more than 9%. At 5 FVG then there was a drop of 14%. 6 FVG is then noticeable drop. Relative to the altitude birch occurs in areas from 240 to 1100 m a.s.l. In the region, this is basically in its entirety. The average elevation is 476 m a.s.l.

For group of forest types (GFT) this rise is situated just to the GFT 3D, 3O and then to 4B and 4S (see Figure 2) where occurrence of silver birch is higher compare to region occurrence of group of forest types. Other GFT show a large increase. Rather, it is seen that a large number of GFT birch ever have or its incidence is so infrequent that GFT is below the frequency of occurrence of 1%. This result shows birch bound to certain habitats within the Region. Results of this study are the first step in identification of optimal forest sites for silver birch grow and production. There are just studies about grow of birch in polluted and mining areas (Podrazsky et al. 2010, Frouz et al. 2015).

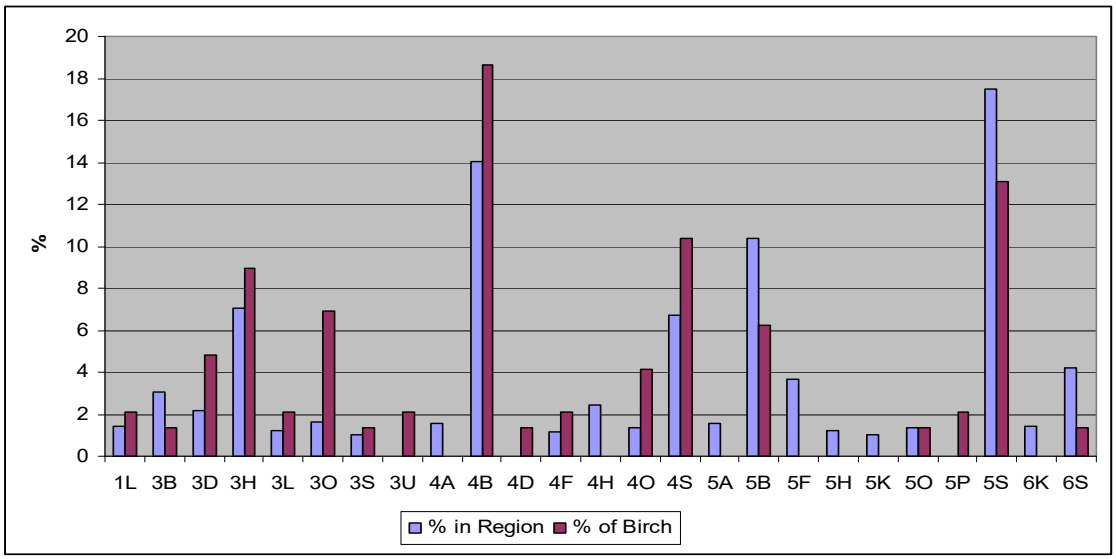
Figure 1 Percentages of the forest vegetation grades (in %)

A) Birch in grades *B) Grades in region*



Legend: LVS = forest vegetation grades

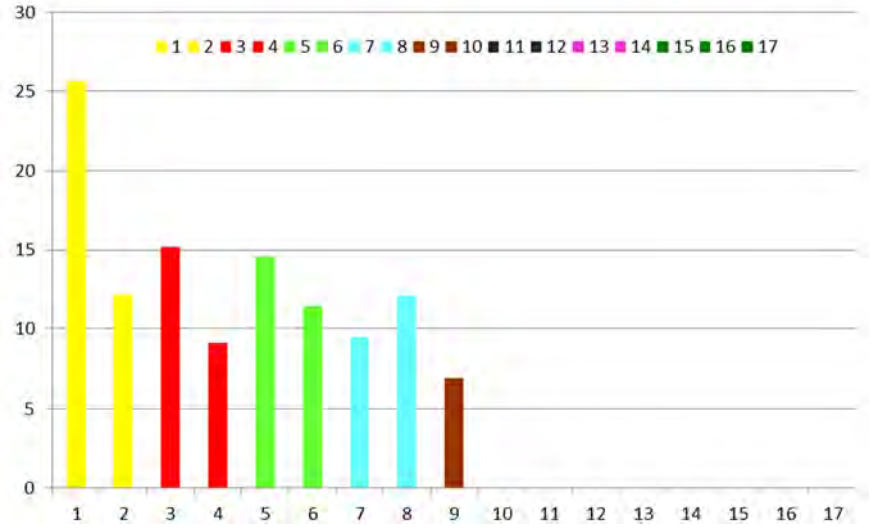
Figure 2 Percentages of the group of forest types (in %)



Legend: L – the floodplain, B – normal, D – deluvial, H – loam, O – moderately rich, S – fresh, U– valley, A – stony, F – slopes, K – acidic, P – pseudogley

The results show important information about higher occurrence in the younger age levels (see Figure 3). This in turn corresponds to a certain formula management, when the birch was removed from younger forest stands. Absence at higher ages (older stands) is again given the durability of birch. Birch in older age rather suffers various pathogens and naturally leaves from forest stands.

Figure 3 Birch occurrence in age grades (in %)



Legend: x axes - Age grades; 1 age grade = 10 years

CONCLUSION

Results of this pilot study are important for future planning of utilisation of wood and non-wood forest products from birch. It is important to know on which group of forest sites we can encourage occurrence of birch forest stands. Although birch is not popular species in forestry practice, it seems that for future reforestation will be very important tree species, especially it will be very helpful species in spruce forest stands which are destroyed by bark beetles.

It can be recommended to continue in research on encouraging of portion of birch forest stands in study area and utilisation of all products which could be obtained from birch tree.

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LOCALIZATION OF COMMERCIAL SUBURBANIZATION SUBJECTS IN MLYNÁRCE DISTRICT

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Abstract: Areas of commercial suburbanization can be found mainly along major communication lines on the periphery of cities. These, used to have primarily residential or agricultural function. Due to a boom of commercial activities, process of commercial suburbanization can be observed also in Nitra city intensively. The aim of this paper is to evaluate the progress of localization of commercial areas in Mlynárce district between 1998 and 2016. Mlynárce district (Nitra city) recorded an increase in commercial subjects from 18 in 1998 to 156 in 2016. Comparison of aerial photographs combined with field research lead to an assessment of the intensity of growth of commercial activities. In addition, it enabled recording of real number of commercial activities in the observed area. Moreover, basic localization conditions of commercial suburbanization were identified.

Key Words: commercial suburbanization, localization conditions, Nitra city, Mlynárce district

INTRODUCTION

Urban planning in the 1990s is considered to be liberal (central government) as well as highly individualized by ad hoc decisions made by local politicians in the vast majority of post-communist countries (Suditu et al. 2014). Significant transformational changes affecting political, social and economic life gradually spread across Slovakia in this period. One of the most significant economic changes in this period was the increased interest of foreign investors in entrepreneurial activities in Slovakia. As a result, substantial growth of foreign capital as well as boom of commercial subjects established by international companies can be observed (Midler and Dubcová 2014). Today, there is a strong increase in business activities by multinational companies in the vast majority of larger towns in Slovakia. As a result, former rural character of suburban areas of major towns was transformed by those companies. These areas became suitable zones for localization of new commercial facilities for retail and wholesale units, various logistic and production halls, storehouses as well as administrative buildings. The described process is called "commercial suburbanization" in the specialized geographical literature. Commercial suburbanization can be identified by its significant dynamic character. The development dynamics of commercial suburbanization depend on several factors such as appropriate geographical location of the city, its availability and accessibility (mainly by road), qualified workforce, scientific base as well as industrial potential of the surrounding region.

The aim of this paper is to point out the development of commercial suburbanization and its characteristics in Mlynárce district (Nitra city) between 1998 and 2016.

REVIEW OF LITERATURE RELATED TO THE SCOPE OF THE RESEARCH

Commercial suburbanization is generally associated with the decentralisation of industrial, commercial, retail and administrative as well as high-tech sector (Champion 2001). This process leads to the formation of commercial areas consisting of hypermarkets, shopping centres and galleries, industrial zones with production halls, storehouses and distribution centres in the peripheral parts of cities. According to Maštálka and Valíková (2014) supermarkets, hypermarkets, extensive areas occupied by warehouses or logistic centres belong to commercial areas. Commercial suburbanization compared to the residential suburbanization is strongly bound to the morphological process of ribbon

development. Commercial areas are usually located on territories with very good transport possibilities along highways, motorways and important transport hubs (Sýkora 2002, Matlovič 2004, Masný and Dubcová 2010). In addition to the mentioned factors, commercial suburbanization is influenced by e.g. sufficiency of free areas allowing new construction activities or existing areas suitable for revitalization or construction of necessary large parking spaces for clients and employees.

The commercial suburbanization in Slovakia is studied mainly on the example of major Slovak cities representing regional centres. Aspects of commercial suburbanization focused on the area of city of Prešov was studied by Sedláková (Sedláková 2005). Danielová (2008) conducted a research focused on the residential changes caused by commercial suburbanization in the city of Trenčín since 1989. The processes related to suburbanization in the Nitra city were assessed by Repaská (2012) and by Repaská and Masárová (2013). The mentioned suburbanization processes influenced also changes in the Radvaň and Kremnička districts in Banská Bystrica, Slovakia. These processes were evaluated by Masný and Dubcová (2010). Development of commercial suburbanization in Bratislava was explored by Šveda and Križan (2012). Among Czech geographers, aspects of commercial suburbanization were studied by Sýkora (2002), Konečný (2010) on the example of the city of Brno, Štefánek (2010) on the example of the municipality of Rudany pri Prahe, Nemeškal (2013) or Krejčová (2014) who focused on Prague, the capital of the Czech Republic. The growing importance of monitoring the aspects associated with commercial suburbanization was documented also by Chuman and Romportl (2011). They established a methodology for monitoring the extent of commercial suburbanization in the Czech Republic.

RESULTS AND DISCUSSION

Localization and forms of commercial suburbanization in Mlynárce district

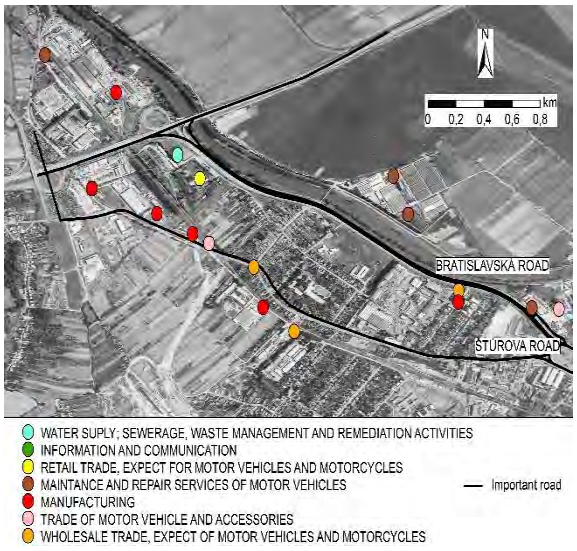
The city of Nitra is one of the examples of cities with clear impact of commercial suburbanization. The city belongs to 5 biggest cities of Slovakia and is divided into 13 districts. Commercial suburbanization can be mainly observed in 5 districts: Mlynárce, Horné Krškany, Dolné Krškany, Čermáň and Chrenová. Although Mlynárce focus of our study is the 4th smallest district with area of 379 ha, it is the most developed district. Mlynárce had a dominant residential function with few production facilities until the 1990s. Additionally, rural character of Mlynárce district is formed by construction of residential units (n = 605) and by low public facilities. The impact of commercial suburbanization on the target area is also demonstrated by decrease in the number of inhabitants (from 599 in 2001 to 555 in 2014).

Amount of commercial subjects increased from 18 to 156 between 1998 and 2016 (Figure 1).

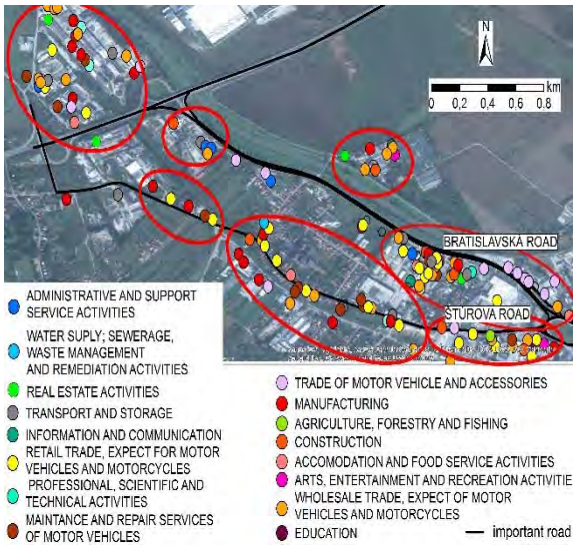
The most important factor influencing the development of commercial suburbanization of Mlynárce is good accessibility of important roads. The 1st class road 65 (Bratislavská road) is directly linked to the expressway R1. The expressway R1 is connecting Nitra city with the capital city Bratislava. Another important road is the 3rd class road 1674 (Štúrová street) connecting Nitra city with Hlohovec town. Commercial areas are located along these two main lines in area of almost 105 ha representing more than 27% of Mlynárce's territory.

Commercial suburbanization in Mlynárce has two forms based on the intensity and character of built-up area along Bratislavská road and Štúrová street. On the one hand, Bratislavská road is characterized by large areas of the free plots as well as functionless production areas. Underused but large enough plots for production halls (eg. the area of the former Zelokvet) provide ideal conditions for the location of new commercial entities. Formation of new commercial subjects on free plots leads to the development of clusters (e.g. Turancar Travel Agency, large areas of retail stores with extensive parking spots of OC Gallery Tesco, Kärcher Slovakia, Siko, Decodom).

Figure 1 Localization of commercial subjects in area of Mlynárce district in 1998 and 2016



Own elaboration with using ArcGIS 10.3



Own elaboration with using ArcGIS 10.3

Development projects and construction activities on former open space (Figure 2) cause an extensive non-renewable degradation of fertile land, elimination of greenery. In general, commercial activities affect the environment extensively (e.g. Turancar Travel agency, OC Gallery Tesco).

Figure 2 Example of the construction of subjects of commercial suburbanization at former open space

Area OC Gallery Tesco 1991

Own elaboration with using ArcGIS 10.3

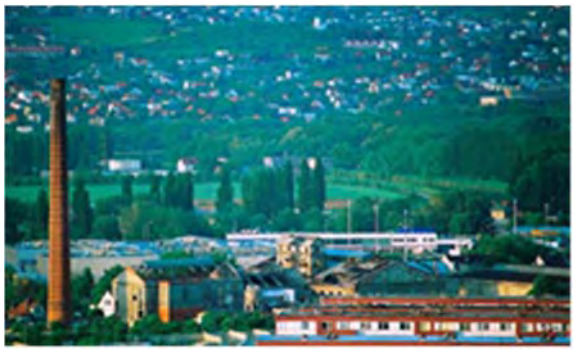
Area OC Gallery Tesco 2016

Own elaboration with using ArcGIS 10.3

On the other hand, Štúrova street is typical for higher density of family houses that do not offer much space for construction of new commercial areas. Hence, commercial activities were established into existing areas on both sides of the street. One of the significant consequences of use transformation of the area are changes from the original residential purpose into commercial function. As a result, residents move from peripheral parts of cities to other city districts. Eventually, they sell or rent their properties and move to nearby municipalities (e.g. stores Boel, installation and heating material Praktik, ironmongery Mikulášik or other commercial subjects). Commercial suburbanization process in the Štúrova street is characterized by transformation of area use of existing properties. Due to revitalization of abandoned and devastated areas, this transformation has a positive influence on the environment (Figure 3). Demolition of premises of former sugar factory and consequent construction of Merkury Market retail store represent a typical example for this process (Figure 3).

Figure 3 Example of revitalization area of sugar factory

The original use of the area



www.nitrafoto.estranky.cz

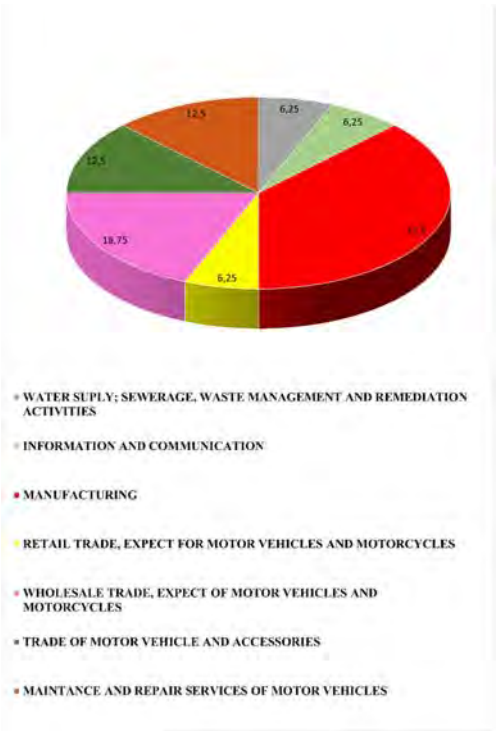
Current use of the area

Own elaboration

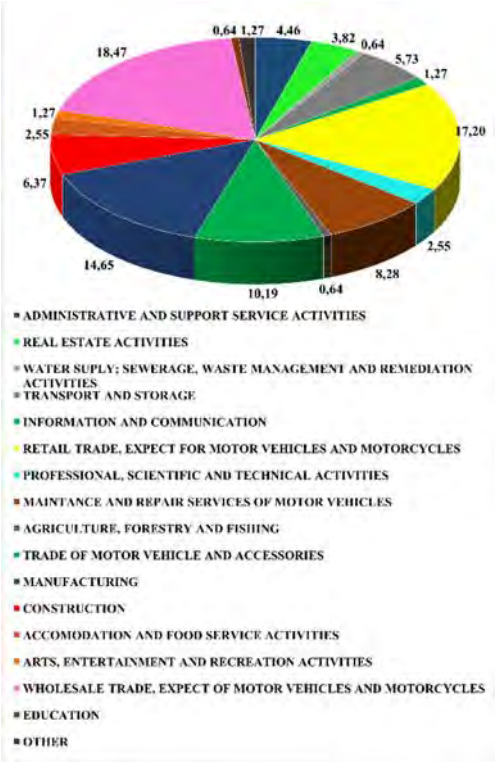
The development of commercial suburbanization in the peripheral parts of the city caused that the former function of the area was replaced by new commercial function. Mostly, logistics prevails. Areas of commercial suburbanization are e.g. areas of the former asbestos factory called Ferrenit, whose premises are currently used as logistic halls or halls for car repair services.

Based on the diachronic aspect, the prevalent sector in analysed district was wholesale and retail store along with repair of motor vehicles and motorcycles in 1998. They reached 56% of all commercial areas (Hagard: Hal, Kartel, AUTODRUKOS, s.r.o., Auto-Nitra-Peugeot, gas station, coal warehouses and two car services). Industrial production covered 33% of the commercial area represented by 6 commercial subjects: Ferrenit, Bramac, Nipek (today Penam), ICT Polysack, RST s.r.o., Služba VDI (Figure 4). Information services (SPS), communication services (6%) and water supply represent the least spread industries in the analysed district in 1998.

Figure 4 Proportion of particular economic sectors in 1998 and 2016



Year 1998



Year 2016

Development of commercial suburbanization was reflected in change of structure of the industry. The most dominant sector is wholesale and retail, repair of motor vehicles and motorcycles characterized by an increase in commercial subjects from 8 subjects in 1998 to 86 subjects in 2016. Additionally, there

is a change in the industrial production. Establishing of new commercial subjects leads toward restructuring and streamlining of production. Moreover, decrease in unemployment is observed. Generally, the industrial structure in the observed area changes as new commercial subjects are established.

Based on the character of particular commercial subjects, car industry is dominant represented by repair and service of motor vehicles (13 commercial areas), sale of cars and light motor vehicles together with car rentals (16 commercial areas), transport and warehousing (9 commercial areas). Free greenfield areas were occupied mainly by large-area retail stores e.g. OC Gallery Tesco, Merkury Market. In addtion, small retail stores and service providers are located in individual residential areas. Micro-enterprises were dominant in the structure of commercial subjects (50.6%) together with small enterprises (19.2%) in 2016. Medium-sized enterprises achieved just 9% of the total amount of commercial subjects. Large enterprises reached the 4.5% share of all commercial subjects within the studied district. 16.7% of commercial subject in Mlynárovce do not belong to any of the above mentioned classifications (Table 1).

Table 1 Structure of commercial areas in Mlynárce based on the number of employees in 2016

Number of employees	Commercial subjects	
	abs.	%
less than 10	79	50.64
10–49	30	19.23
50–250	14	8.97
250 and more	7	4.49
unidentified	26	16.67
Total	***156***	***100.00***

Sources: www.zisk.sk, own elaboration

CONCLUSION

Commercial suburbanization recorded a sharp increase of 138 areas in the Mlynárce district between the years 1998 and 2016. The increase in the number of commercial activities is the result of the very good geographical location, availability of infrastructure facilities as well as overall economic growth in the city of Nitra. The development of commercial areas in the studied area was affected also by undeveloped and inexpensive plots, sufficiency of workforce as well as availability of good infrastructure that connects Nitra with the capital city Bratislava. Commercial areas in the studied district are located along the 1st class road 65 (Bratislavská road) and 3rd class 1674 road (Štúrova street).

Commercial areas along Bratislavská road were localized in a number of mutually isolated commercial clusters. Commercial areas along the Štúrova street were localized in the linear row on both sides of the street. It was caused by different forms of built-up areas along these communications. On the one hand, extensive free plots as well as existing functionless industrial areas along Bratislavská road enable localization of several commercial subjects within the same plot resulting in building of clusters. On the other hand, Štúrova street is characterized by higher density of family houses that do not offer sufficient space for construction of new commercial areas. Therefore, there are commercial activities located into the existing areas on the both sides of the street. Revitalization of abandoned and devastated areas results into the functional change of area.

ACKNOWLEDGEMENTS

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IS IT ARANEOPHAGY A REASON FOR SPREADING OF DADDY LONG-LEGS SPIDER *PHOLCUS PHALANGIOIDES*?

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Abstract: The aim of this study was to find out new information about the predation and araneophagy of *Pholcus phalangioides*. The actual experiment was conducted in laboratory conditions and it had been used a total of 248 spiders there. Together with studied *Pholcus phalangioides* there were gradually tested five other species: *Hasarius adansoni*, *Psilochorus simoni*, *Parasteatoda tepidariorum*, *Tegenaria atrica* and *Tegenaria domestica.* All experiments were carried out in laboratory containers under the same laboratory conditions. The existing results have brought findings that juvenile individuals of these tested synantropic species are not able to defend against adults of *Pholcus phalangioides* and via this way could this species spread so quickly across Europe.

Key Words: Pholcus phalangioides, synantropic species, araneophfagy, predation, experiments

INTRODUCTION

Invasive and expansive species, among them *Pholcus phalangioides*, pose a serious threat to the nature not only in the Czech Republic, but also throughout the world. Together with the increasing use of natural resources, climate change and environmental pollution they are among the main negative factors which threaten existing biodiversity of native ecosystems (Agentura ochrany přírody a krajiny 2015). Potential hazard of *P. phalangioides* lies primarily in its interactions with other species of spiders (Bristow 1941). We can watch a similar phenomenon also with our alien spiders.

P. phalangioides has been recorded in the Czech Republic in 1959 for the first time. Since then it has successfully spread into almost all synanthropic habitats throughout our territory. Due to the rapid colonization of new environments and relatively quick adaptation to synanthropic habitats, it can be assumed that this species is evolutionarily very successful (Buchar and Kůrka 2001). However it seems that in the wildlife in our conditions the spider cannot survive winter (Česká arachnologická společnost 2016). With the expansion of *Pholcus phalangioides* there is a disappearance of our "native" synanthropic species (Hula pers. Comm).

MATERIAL AND METHODS

It was captured the total of 248 spiders for this experiment. The captured animals had been keeping separately in the breeding containers with the conical size – length: 14 cm, diameter of the bottom portion of 5.5 cm, diameter of the upper portion of the container 9.5 cm. Each breeding container was labeled with exclusive number. All containers were in the upper part provided with a perforated plastic wrap, which secured aeration within the container. To ensure moisture and liquids for the animals there was 1 cm below the upper edge of each container cut hole for a cotton ball which was moistened regularly at an interval of five calendar days. Into each container there was also placed a paper strip in the shape of U which was used to facilitate habituation of the captured spiders. Before being placed in the breeding containers the spiders were determined and it was also determined the sex and a stage of development of each individual. The spiders were fed at regular intervals of every 5 calendar days. Before the planned experiment (14 days) the feeding of the spiders was terminated.

For the experiment itself there was prepared 20 new experimental containers with the conical size – length: 14 cm, diameter of the bottom portion of 5.5 cm, diameter of the upper portion of the container 9.5 cm in which there were placed various old individuals by random selection. Into one new experimental container there was always placed one spider species *P. phalangioides* and into the second

new experimental container was placed a test spider of another species. There were used five kinds of other spiders (*Hasarius adansoni*, *Psilochorus simoni*, *Parasteatoda tepidariorum*, *Tegenaria atrica*, *Tegenaria domestica*) against *P. phalangioides*. All spiders were particularly numbered until end of the experiment. These containers were then connected temporarily so the spiders were not able to get together. It was ensured with the insertion of the paper square between both containers. Spiders were left to acclimatise for 20 min. Observation was carried out for a total of 3 days. On the first day the observation was carried out at two minute intervals for 120 minutes. Subsequently, the observation was recorded at 30 minute intervals for the next 180 minutes (5 hours). This has completed the first day of the observation. In the next two days there was done one more observation always at 8:30 am. After completion of the experiment the spiders were killed and subsequently stored in test tubes with alcohol and properly labeled with exclusive numbers. Subsequently, there was measured body size and evaluated the results.

RESULTS AND DISCUSSION

It was used a total of 248 spiders for this experiment. It consists of 124 spiders *Pholcus phalangioides* (Fuesslin, 1776), 19 spiders *Hasarius adansoni* (Audouin, 1826), 23 spiders *Tegenaria atrica* C. L. Koch, 1843, 25 spiders *Tegenaria domestica* (Clerck, 1757), 33 spiders *Parasteatoda tepidariorum* (C. L. Koch, 1841) and 24 spiders *Psilochorus simoni* (Berland, 1911). However these numbers are not final. The experiment will take place in the longer term so that these results are preliminary.

It was achieved of these preliminary results by testing:

Table 1 Pholcus phalangioides X Hasarius adansoni

Developmental stage	**Achievement in % /(number of individuals)**	**Achievement in % /(number of individuals)**	**Interaction without deaths in % (number of individuals)**
	Pholcus phalangioides	*Hasarius adansoni*	
Pholcus phalangioides juvenil X *Hasarius adansoni* juvenil	50% (2)	25% (1)	25% (1)
Pholcus phalangioides female X *Hasarius adansoni* juvenil	40% (2)	20% (1)	40% (2)
Pholcus phalangioides female X *Hasarius adansoni* female	60% (3)	40% (2)	0% (0)
Pholcus phalangioides juvenil X *Hasarius adansoni* female	40% (2)	60% (3)	0% (0)

In this experiment a total of 19 observations were done. A mutual duel of the spiders ended in 9 cases by consumption of the species *Hasarius adansoni* and in 7 cases of killing and consumtion of spiecies *Pholcus phalangioides*. In three cases there was no interaction which would lead to death or consumption of anyone of the spiders. The results also show that the adult female *Hasarius adansoni* are able to catch and kill not only the juveniles *Pholcus phalangioides* but it is not unrealistic for them to catch also a much larger adult females of this species. In one case even a juvenile individual *Hasarius adansoni* managed to kill an adult female *Pholcus phalangioides*. Among all the five species tested in this experiment, *Hasarius adansoni* seems to be the most competitive to *Pholcus phalangioides*.

Table 2 Pholcus phalangioides X Psilochorus simoni

Developmental stage	Achievement in % /(number of individuals)	Achievement in % /(number of individuals)	Interaction without deaths in % (number of individuals)
	Pholcus phalangioides	*Psilochorus simoni*	
Pholcus phalangioides juvenil X *Psilochorus simoni* juvenil	80% (8)	0% (0)	20% (2)
Pholcus phalangioides female X *Psilochorus simoni* juvenil	100% (5)	0% (0)	0% (0)
Pholcus phalangioides female X *Psilochorus simoni* female	50% (2)	0% (0)	50% (2)
Pholcus phalangioides juvenil X *Psilochorus simoni* female	80% (4)	0% (0)	20% (1)

In this case a total of 24 observations were done. *Pholcus phalangioides* was successful in 19 cases when it killed and consumed his opponent. In the remaining 5 cases there was no death of any spider after mutual interactions. It is interesting that *Psilochorus simoni* failed to kill his rival in every case. And it failed even in the case of an adult female of this species, where it stood against juvenile individual *Pholcus phalangioides*, therefore the individual of approximately the same size. In contrast the adult females *Pholcus phalangioides* were able to easily cope with all juvenile opponents. Similarly successfully, in 8 of 10 cases even juvenile *Pholcus phalangioides* were able to cope with juvenile *Psilochorus simoni*.

Table 3 Pholcus phalangioides X Parasteatoda tepidariorum

Developmental stage	Achievement in % /(number of individuals)	Achievement in % /(number of individuals)	Interaction without deaths in % (number of individuals)
	Pholcus phalangioides	*Parasteatoda tepidariorum*	
Pholcus phalangioides juvenil X *Parasteatoda tepidariorum* juvenil	69% (9)	0% (0)	31% (4)
Pholcus phalangioides female X *Parasteatoda tepidariorum* juvenil	57.1% (4)	14.3% (1)	28.6% (2)
Pholcus phalangioides female X *Parasteatoda tepidariorum* female	0% (0)	16.7% (1)	83.3% (5)
Pholcus phalangioides juvenil X *Parasteatoda tepidariorum* female	14.3% (1)	14.3% (1)	71.4% (5)

In this experiment were involved in total 33 individuals of *Parasteatoda tepidariorum.* In 14 cases there was killing of individuals *Parasteatoda tepidariorum*. Only in three cases the combat of these spiders was completed by killing and consumption of *Pholcus phalangioides* and in 16 cases there was no attack which would result in the death of one of the spiders. From the observation it is showed that juvenile *Pholcus phalangioides* can quite easily cope with juvenile stages of his opponent, specifically in 9 of 13 cases, in the rest cases there was no attack which would end with the death of spiders. Interestingly, however, is finding that the adult female *Pholcus phalangioides* killed the adult female *Parasteatoda tepidariorum* in no one of six experiments. In 5 out of 6 observations duel ended without deaths and in one case, moreover, with killing of adult female *Pholcus phalangioides*. Similar results are also showed in the combat of juvenile *Pholcus phalangioides* with adult females of *Parasteatoda*

tepidarourum. Thus it appears that the adult female *Parasteatoda tepidarourum* is able to resist the attacks of *Pholcus phalangioides* and vice versa they can also attack.

Table 4 Pholcus phalangioides X Tegenaria domestica

Developmental stage	**Achievement in % /(number of individuals)**	**Achievement in % /(number of individuals)**	**Interaction without deaths in % (number of individuals)**
	Pholcus phalangioides	*Tegenaria domestica*	
Pholcus phalangioides juvenil X *Tegenaria domestica* juvenil	50% (5)	0% (0)	50% (5)
Pholcus phalangioides female X *Tegenaria domestica* juvenil	66.7% (4)	0% (0)	33.3% (2)
Pholcus phalangioides female X *Tegenaria domestica* female	20% (1)	20% (1)	60% (3)
Pholcus phalangioides juvenil X *Tegenaria domestica* female	0% (0)	75% (3)	25% (1)

Overall, there were tested 25 individuals of *Tegenaria domestica.* The results showed that in the overall success rate in combats, the individuals of *Pholcus phalangioides* had predominance. The results also show that juveniles and adult females *Pholcus phalangioides* can quite easily cope with juvenile stages of spider *Tegenaria domestica*. Exactly the opposite is in the case when compared the adult females *Tegenaria domestica* with juvenile stages of *Pholcus phalangioides*. In these cases the adult female *Tegenaria domestica* has considerable predominance, moreover, it is also able to cope with the adult female *Pholcus phalangioides* as it was observed.

Table 5 Pholcus phalangioides X Tegenaria atrica

Developmental stage	**Achievement in % /(number of individuals)**	**Achievement in % /(number of individuals)**	**Interaction without deaths in % (number of individuals)**
	Pholcus phalangioides	*Tegenaria atrica*	
Pholcus phalangioides juvenil X *Tegenaria atrica* juvenil	66.7% (5)	0% (0)	33.3% (3)
Pholcus phalangioides female X *Tegenaria atrica* juvenil	66.7% (2)	0% (0)	33.3% (1)
Pholcus phalangioides female X *Tegenaria atrica* female	0% (0)	75% (3)	25% (1)
Pholcus phalangioides juvenil X *Tegenaria atricaa* female	0% (0)	37.5% (3)	62.5% (5)

In this case a total of 23 observations were done. From the observation it is showed that, similarly as in the case of the spider *Tegenaria domestica*, in the case of the species *Tegenaria atrica* the juveniles of this species are unable to cope with adult females, but also juvenile *Pholcus phalangioides*. Exactly the opposite is in the case when the adult females *Tegenaria atrica* face the juvenile individual or the female *Pholcus phalangioides*. The results are very clear for the female *Tegenaria atrica* in this interaction.

Human settlements in the central European countries provide appropriate conditions for the existence of our non-native species of spiders (Krumpálová 2001). Those synanthropic species in our countries inevitably collide together and it often leads to mutual interactions. In these situations may therefore apply versatile predatory behavior, which for instance *Pholcus phalangioides* hold as it has the prerequisites to prefer other types of spiders like hunted prey (Jackson et al. 1990). An important finding of this work is the confirmation that the juveniles of other synanthropic species especially adult individuals of *Pholcus phalangioides* can defend their attacks only very hard and are often a subject to their attacks. This is confirmed also in the work of Havlová (2013) who has observed similar behavior against the juvenile stages of other studied spiders in her observations.

Looking at the map of the occurrence of all 5 tested synanthropic spiders in the Czech Republic (Česká arachnologická společnost 2016) we can find out that for all of these spiders there are greatly predominate records of findings from the past over those that were made in the last 15 years. However, a similar situation can be seen in Germany (Arachnologische Gesellschaft e.V. 2016). The question is to what extent and whether this disparity can be caused also by araneophagous behavior of *P. phalangioides* towards other synanthropic species.

CONCLUSION

The aim of this study was to find out new information about predation and araneophagy of the spider *Pholcus phalangioides*. Preliminary results suggest that this spider can affect populations of our native species in a negative way. There is the fact that the adult individuals of this species are able to kill and consume juveniles of other species. This may have an adverse impact in the future, especially for the population of our native species since it can be difficult to create a young generation for the native species, which could mature so that they can compete in areas colonized by *Pholcus phalangioides*.

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MIGRATION FACTORS OF RURAL INHABITANTS LIVING IN THE INNER PERIPHERY OF THE CZECH REPUBLIC. A CASE STUDY OF THE MICRO-REGION BYSTŘICE NAD PERNŠTEJNEM

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Abstract: The paper deals with the migration factors (push and pull factors) defined and evaluated according to a questionnaire survey conducted among inhabitants (aged 18–30 years) of Bystřice nad Pernštejnem micro-region, situated in the inner periphery of the Czech Republic. Emigration leads to ageing and depopulation of rural areas. Leavers are mainly young and educated people representing the hope and prospect for the micro-region. Push factors are centrifugal factors, which make or motivate migrants to leave their native villages. Pull factors motivate migrants in their effort to move to certain target towns or regions. Young residents in the region miss more opportunities of entertainment, culture and sports, and subsequently also an offer of skilled jobs. They would also like to become independent, which is often easier in larger towns. Cities are attractive for young people by a wider range of entertainment, culture and sports (the strongest pull factor), by higher salaries and jobs that are more prestigious. Attractive for respondents is also the urban style of living, which provides greater anonymity.

Key Words: migration, push and pull factors, inner periphery, employment

INTRODUCTION

Rural areas currently feature a distinct phenomenon of young people leaving for larger towns both for education and for jobs. Willingness to change residence decreases with the increasing age and this is why the issue relates mostly to young persons. Departure of highly qualified young people has a negative impact on the development of rural areas. Living conditions in rural areas are considerably more challenging than in the cities, the more so in the peripheral regions. This has to do also with the problem of lower employment, particularly of females, school leavers and underprivileged population. Possibilities of using services are limited in dependence on the distance from towns. At the same time, young people are the only prospect for rural areas if we wish to stop their ageing and depopulation. The analysis of factors supporting/preventing the migration of young people to towns was conducted in the administrative district of Bystřice nad Pernštejnem municipality with extended powers (MEP) in the Vysočina administrative region.

MATERIAL AND METHODS

Bystřice nad Pernštejnem is a town located in the district of Žďár nad Sázavou, in the eastern part of the Vysočina Region. It represents a municipality with extended powers and its administrative district includes 39 communities, which corresponds to average in the Region. The administrative district of Bystřice nad Pernštejnem neighbours with the South Moravian (Jihomoravský) Region in the east, in the north-west it has a short border with the Pardubice (Pardubický) Region, in the west it neighbours with communities of the administrative district Nové Město na Moravě, and in the south with communities of the administrative district Velké Meziříčí. To the date of 1 January 2015, the official population of the town amounted to 8 343 persons, with the number of inhabitants slightly falling every year; for example in 2013, the population amounted to 8 822 (Czech Statistical Office 2016). The town Bystřice nad Pernštejnem has about 40% of the population of the administrative district. In 2015, the

age index (the number of people aged 65 and over per 100 youths under age 15) was 136.7 and unemployment in the same year reached 8.25%. Balance of migration and total population growth in individual administrative districts of municipalities with extended powers in the district of Žďár nad Sázavou in 2014 are shown in Figure 1. Balance of migration for 2015 was -43 and total population growth/loss was -41. The micro-region of Bystřice nad Pernštejnem can be characterized as an inner periphery in the Czech Republic. Emigrating from peripheral rural areas are mainly young and educated people who are lacking most good job opportunities as well as social and cultural contacts (Venhorst et al. 2010). With abundant nature and cultural monuments, the micro-region of Bystřice nad Perštejnem has a sound potential for the development of tourism.

Musil and Müller (2008) dealt with peripheries in connection with possible social exclusion. Most dictionaries explain the term periphery generally as "something on the edge". In the narrower sense, periphery means a marginal zone. In sociology, the meaning of periphery was until recently connected only with the outskirts of cities. In the contemporary sense, it has been considerably extended for use in sociology, political science and geography. The essence of the concept consists in discerning the economic and also the social space into the core and the periphery. Inner peripheries of the Czech Republic are usually continuous territories situated on the edges of metropolitan regions and on edges of the catchment areas of regional centres. They are characterized by high shares of residents working in agriculture and by low population density. These primary features bring about a relatively low education level of the local population, high proportion of commuters for work outside the community, low share of persons working in the tertiary sector, low percentage of foreigners in the population or low level of technical infrastructure.

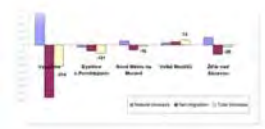
A great part of the economically active population in Bystřice nad Pernštejnem works in industries with problematic prospects for retraining. Compensation for jobs lost in connection with the closure of uranium mines in Rožná was sought firstly in the localization of new industries. However, the town could hardly compete with other regions featuring better transport infrastructure and traffic connections. In spite of this obvious disadvantage, it succeeded in having attracted several new enterprises (Vaishar et al. 2002). The location of plants of some other companies is currently negotiated. However, there are concerns that newly created jobs will require less skilled labour and will be remunerated by low wages.

Migration of young people is often explained in media as personal self-fulfilment or inevitable movement (Nugin 2014). Changes in the migration behaviour of population are affected by mutual preferences of the population and characteristics (conditions: pull and push factors) in the target and source areas of migration. Since these conditions are evaluated primarily in dependence on the life cycle of households and individuals, general changes in the society relating to population ageing, diversification of life styles and changes in the character of housing and work play a significant role, too (Ouředníček et al. 2013).

The research objective was to define so-called push and pull factors inciting young people to leave the countryside. Push factors are centrifugal and force or motivate the migrants to leave their native community (e.g. unemployment, poor quality of life etc.). Pull factors lead the migrants to the effort to move to certain target towns or regions (Dorigo and Tobler 1983). Nevertheless, there are also factors, which "hold" many residents in their original homes, e.g. family background, social ties with friends and acquaintances, asset situation, habit and adjustment to life in the countryside etc. If we could define such influences, we would be able to issue specific recommendations and measures leading to their enhancement and to subsequent maintaining the population in rural areas.

The research was conducted in the first half of the year 2016. We studied statistic databases of the Czech Statistical Office and based on the study, we established the migration growth and total population growth in the concerned area. In order to assess the push and pull factors, we conducted a questionnaire survey with young people up to 30 years. The most abundant group of respondents consisted of students from secondary schools in Bystřice nad Pernštejnem because this social group stands only before the decision-making as to rooting in the region or mobility. Other groups included university students and graduates, leavers from higher vocational schools, employees etc. The questionnaires were distributed both by the classic form and electronically via the Survio.com web interface.

Figure 1 Population increase in individual administrative districts and total in the Vysočina region, year 2014 (according to Czech Statistical Office 2016)



RESULTS AND DISCUSSION

The questionnaire was filled by 92 respondents of whom 65.2% were females and 34.8% males. More than a half of the respondents fell in the age category of 18–19 years, i.e. secondary school students on whom the research was primarily focused. Other respondents were aged 20–30 years.

The strongest push factor is according to the respondents the lack of prestigeous/well-paid jobs in rural areas (see Figure 2). The second strongest factor (44.4% of respondents) is the lack of cultural activities and entertainment. The option of moving away from the family, i.e. independence, gained nearly the same number of answers. We also recorded the option "other" represented by the factor of low number of friends in the village. Surprisingly, only a quarter of respondents mentioned the factor of "lower wage". In total, we evaluated six different factors.

The pull factors of migration were examined by asking the following question: "If you plan to live in a big city/medium-sized town, what reasons do you have for that?". Results showed that the strongest factor is "more entertainment, cultural activities and sports". This factor was followed by "better paid job" and "more prestigious job". A quarter of respondents find attractive the urban life style with greater anonymity. The answer "other" was not recorded; the respondents put up with the offered eight options. The results show that it is not only economic reasons that explain for migration. Many people would be able to find a corresponding job in the region but are lacking cultural and leisure time activities. This particularly applies to residents with higher education or people who are socially or culturally committed, artistic and creative people for whom rural regions do not offer a sufficiently diverse array of conditions for existence (Ouředníček et al. 2011).

The question "What reason you have to stay living in the rural area?" brought surprising results. The highest percentage of answers (nearly 75%) mentioned "nature and landscape", and this answer was not favoured in relation to the young generation of residents at creating the questionnaire. However, the "clean environment" related to this answer gained hardly 47%. The remaining respondents were either not convinced about the clean environment in the region or it was not greatly important for them. Other significant factors for the respondents were proximity to family, relatives, friends and acquaintances. The least frequented answers included satisfactory job and habit, which is not surprising because habit in general plays a greater role in older residents and the option of satisfactory job was less frequented since the majority of respondents were students.

Figure 2 Push factors of migration

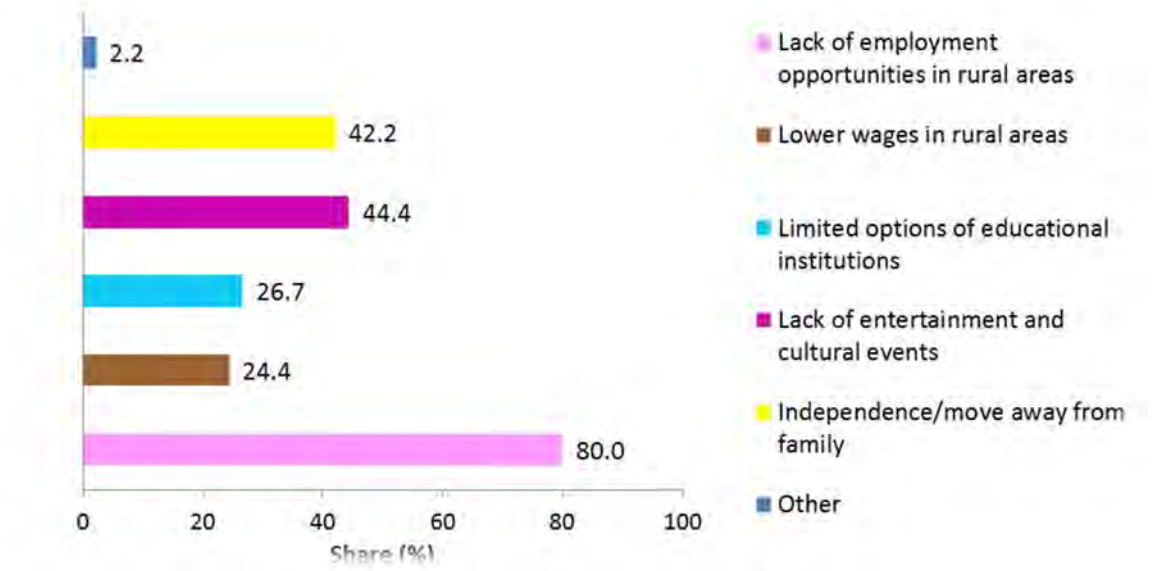
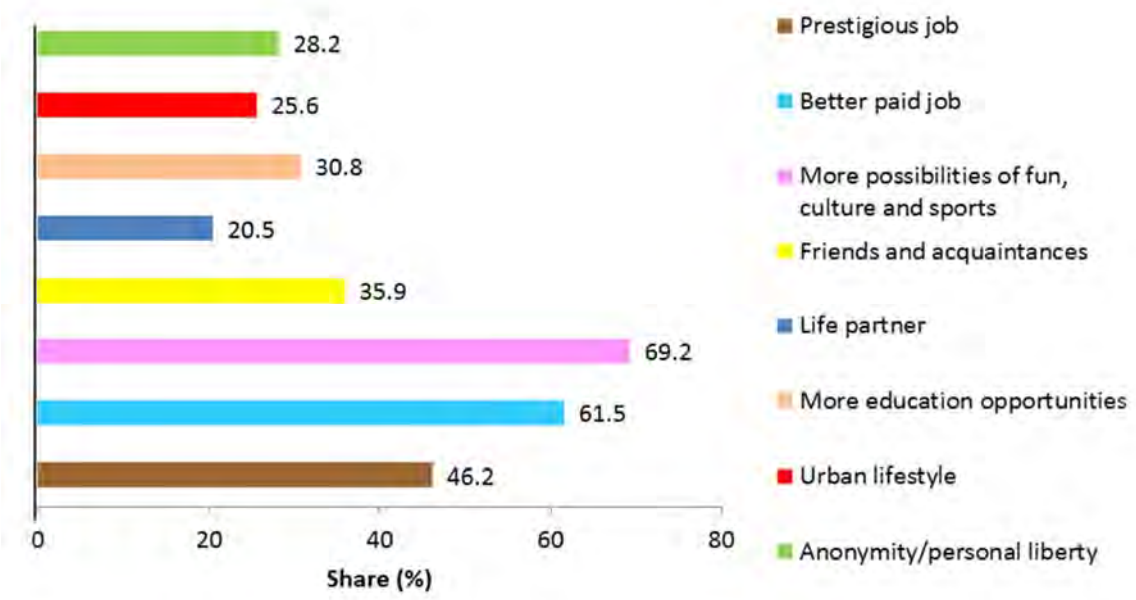


Figure 3 Pull factors of migration



Only 13.7% of respondents were decided to settle down in the town of their current/future university studies (question was targeted onto the secondary and university students). Nevertheless, the option of not settling down there was chosen by 42.5% of respondents, i.e. a considerably larger number. The general question "Would you like to move to a big town (over 100 thousand inhabitants) or a medium-sized town (20–100 thousand inhabitants)?" brought a similar result. A negative response was given by 40.2% of respondents; nearly 30% plan for such a departure mentioning however the option of "temporarily", which is likely relating to their study at university or secondary vocational school (Figure 4). These answers favour the interpretation that more young people have strong ties with their native region and do not long to move into the town, even at the cost of worse position on the labour market. This judgement cannot be considered unambiguous though since young people may frequently change their opinion, the conditions may change or the presented questionnaire cannot cover all eventualities.

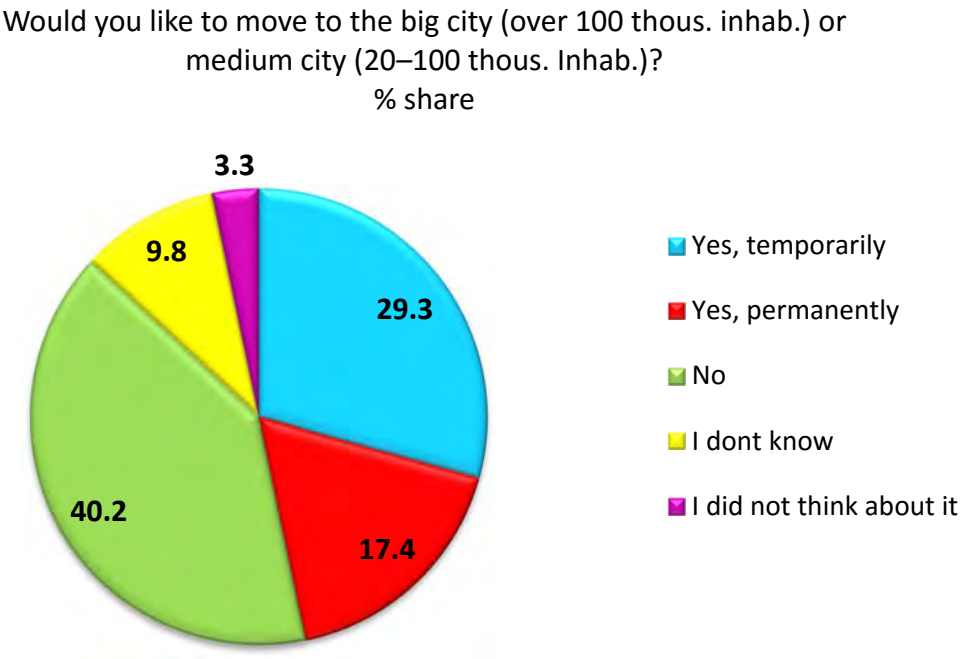
Migration has been and will be present because a part of young people will leave for the town either voluntarily or under the pressure of circumstances such as unemployment, offer of unskilled work etc.

A greater part of the inquired secondary school students prefers the city of Brno for their university studies, which is in the closest distance, readily accessible by traffic, and offers a wide range of schools and study lines. The Moravian metropolis is followed by Prague and popular Olomouc. Some answers mentioned study abroad (Bristol, Cardiff) and Opava, which is more distant in terms of traffic. Regions mentioned by the respondents as most attractive for living were Vysočina, South Moravian (Jihomoravský) Region and Prague, which was expected and corresponds with the above facts.

Willingness to move for work to an unknown town/community is relatively high in the respondents, which is apparently given by their age composition. "Yes" was declared by more than 60% of respondents. However, 33.7% of respondents would choose the option only in extreme cases. On the other hand, "no" was claimed only by 12% of respondents. In general, the willingness of the Czech population to move for work is relatively low and is often replaced by daily and non-daily commuting (Lux and Sunega 2007).

Migration may be caused by material poverty because of unemployment. Globally endangered are females more than males and their situation is worse than that of males also in terms of poverty level (Sundari 2005). The decision-making about moving is however affected not only by the economic factors. Many social, psychological and environmental reasons play a role, too. Important is a limit for acceptance of the load of commuting for work and services. If a majority of young people teeters on the edge of endurance, then the worsening of services and labour market in rural areas would further advance depopulation. However, Ouředníček et al. (2011) maintain that even a slight life quality improvement in rural areas may lead to the stabilization and activation of life even in small villages. As a great issue, they can see the poor traffic accessibility of villages, which did not show at all as a push factor in the results of our questionnaire and hence does not seem essential for young residents. The problem acquires importance rather with senior people. Other push factors mentioned by the authors include the already known "lack of jobs", namely skilled work, and the lack of cultural and leisure time activities and sports. Activities should be directed to the local and micro-regional level in order to activate the local human potential (Ouředníček et al. 2011).

Figure 4 Settlement preferences of respondents



CONCLUSION

The paper presents push and pull factors of migration according to the questionnaire survey conducted in 2016 in the micro-region of Bystřice nad Pernštejnem, which can be characterized as peripheral and depopulating. Emigration leads to the ageing and depopulation of rural areas, which often have to face cumulative circular causality (Myrdal 1957) when the worsening of one factor leads to the worsening of another factor, which again leads to the worsening of the first factor. Leaving are mainly young and educated people who are still not rooted in the region, have no commitments and often can compare life in other localities (towns) and in rural areas from where they come. These people lack more entertainment, cultural activities and sports as well as an offer of skilled work. The issue of emigration from rural regions should be dealt with by the society in order to prevent passivity and the gradual deterioration of living conditions in some regions.

ACKNOWLEDGEMENT

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ANALYSIS OF RURAL LANDSCAPE DEVELOPMENT IN THE SOUTH MORAVIAN BORDERLAND IN THE 20th CENTURY

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Abstract: Development of Moravian borderland landscape in the 20th century is a very moot point. Landscape changes caused by natural factors as well as by political and socio-demographic changes are apparent until today and the borderland landscape has often problems to cope with them. The paper presents detailed information and analysis of landscape in the western part of the Znojmo region, concentrating on the cadastral areas of Onšov and Lesná. The two neighbouring villages are situated north-west of the town of Znojmo. The study analyzes the historical development of the landscape and land use, focusing on the identification of problems in the landscape, which were caused by its inappropriate management. A source for the analysis was the method comparing maps and semi-standardized interviews with local old residents who were able to describe changes occurring in the landscape during the last fifty to sixty years. Results of the analyses show that significant changes in landscape use occurred particularly in critical periods due to the construction of dam, later after World War II and in the period of collectivization.

Key Words: landscape development, Lesná, Onšov, borderland, NP Podyjí

INTRODUCTION

The areas of interest in cadastres of villages Onšov and Lesná are situated near the Podyjí National Park. The buffer zone of the National Park Podyjí reaches into the southern part of both cadastres (Petrová et al., 2001, Kirchner et al. 2003) and neighbours with the NP Podyjí itself (Šťastná et al., 2015). Regarding higher altitudes, grape vine as a typical crop in the Znojmo region (Figure 1), surrounding a greater part of the south-eastern boundary of the National Park Podyjí, cannot be grown there. In 1938–1945, the Znojmo borderland was inhabited mostly by German population and belonged in the Reichsgau Niederdonau province with the capital Krems an der Donau. The subsequent evacuation of German inhabitants and their incomplete replacement with settlers of Slavonic origin with an entirely different relation to the landscape very significantly affected the further landscape development (Vaishar et al., 2000). Because of the above specific features and partial isolation, the villages rank with problematic recreational rural areas (Perlín and Kuldová 2008), which is why the local landscape is largely preserved.

The research aimed at finding main factors participating in land use changes happening in the concerned cadastres of NP Podyjí from the 20th century until the present.

MATERIAL AND METHODS

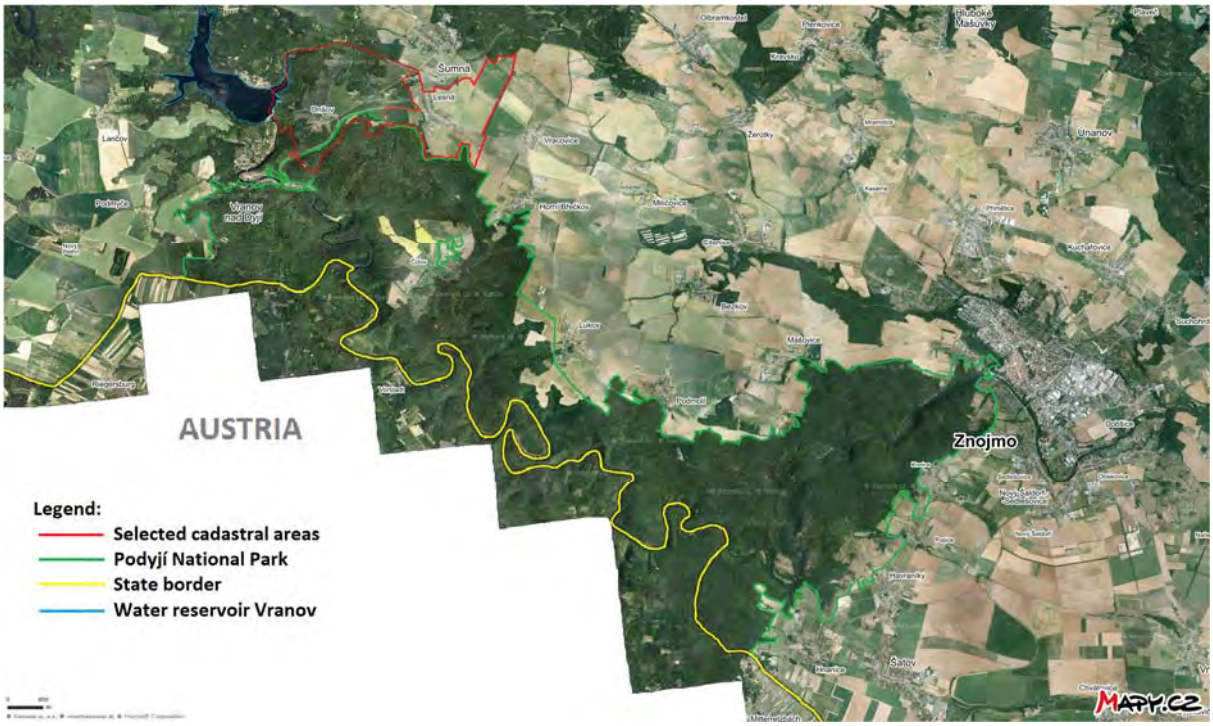
To obtain results, we used primarily the comparative method by which individual available maps were compared not only in the electronic form as orthophotomaps but mainly as prints from archives. Sources of information were national archives as well as municipal archives. Background materials included also reports on the construction of engineering works in the landscape. All this was supported by semi-standardized interviews with residents in the given locality, who were able to provide closer information about changes in the landscape happening in the last 50–60 years. Furthermore, we used available statistical data about the area size of individual lands and their use available mainly from the

Czech Statistical Office. All these information sources offer a comprehensive view of landscape development in the 20th century.

RESULTS AND DISCUSSION

The first and major change in the landscape was the construction of water works on the Dyje River. A small part of the Vranov water reservoir reaches into the Onšov cadastral area.

Figure 1 Overview map of the Znojmo region with the selected cadastres. Mapy.cz, 2016



The first plans for the water reservoir construction date back to 1908 when they were submitted as a student draft to prevent frequent inundations on the Dyje River. The draft was accepted in the 1920s. The construction was launched in 1930 and completed in 1933 (Vranovská přehrada 2016).

A part of the water reservoir is situated in the territory of Onšov. The construction affected the local landscape primarily by the need for flooding some forests and a farm building. Up the river, the future water reservoir took its toll in the form of flooding a whole village (Bítov) and a need for building a new one. Together with the necessary transportation of needed building material; this is why a narrow-gauge railway from Šumná was constructed. To build the railway, a part of the forest in the northern part of Onšov had to be felled on the site known today as Swiss Creek.

At that time, there used to be a small spa Quellenbad in Lesná-spring spa with a pool that was built in 1913. The spa was famous and well attended, which brought many advantages to the village. However, the favourable trend was brought to end by the construction of the water reservoir. The spa lost importance with the development of recreation at the dam lake and was closed in 1939. Contributing to the closure of the spa was also the population change after 1938 (Padalík 2009).

Since the two municipalities are of "borderland" character, their appearance was influenced also by both world wars. The villages used to be inhabited mainly by the German population and were both annexed to the Reich by Hitler in 1938. A greater part of the remaining population was German. After the war, the documents used in our research were settlement/allotment maps, the map of 1950, and the first aerial photographs made in 1953.

The detailed settlement map of Onšov provided a precise sum of the areas of lands and houses determined for new owners/settlers together with the list of their names. The map indicates that the confiscated property amounted to 219.70 ha while 385.07 ha remained to the original owners. Accurate data from Lesná are not known. The maps show however that the cadastre was considerably reduced to the benefit of Šumná. The maps from 1950 and the aerial photographs from 1953 clearly show that the

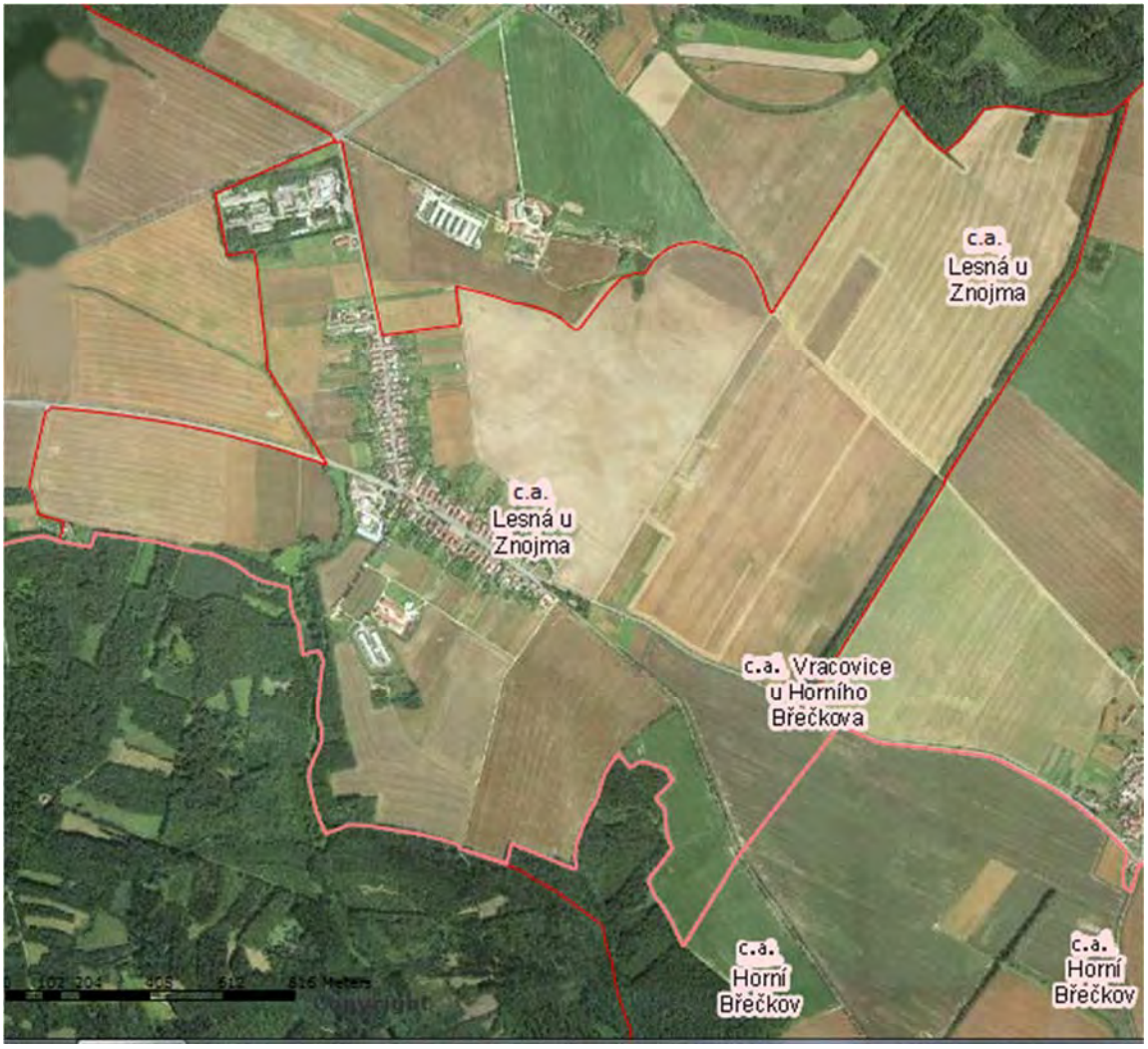
landscape was kempt and used for agriculture (Figure 2). The size of cultivated plots apparently corresponds to the size of land parcels of individual owners.

Information and maps on land use from the period 1951–1958 were preserved in both cadastres and clearly indicate the onset of collective farming and creation of extensive continuous fields for unified cultivation. In Onšov, 225.78 ha were used as arable land and 2.68 ha were occupied by orchards. Land determined for new construction occupied 5.70 ha, 13.52 ha were to be planted with forest and 5.62 ha were proposed to be used as meadows. The rest of the cadastre is maintained. In Lesná, 175.42 ha were used as arable land, 5.70 ha were orchards, 7.81 ha were meadows and 1.20 ha were meant to be planted with forest. There was also individual tenure in Lesná, which amounted to 34.70 ha. The maps show also a draft of "new" road from Lesná to Vranov nad Dyjí, which was built in the 1960s–1970s. Attempting at an extension of the existing communication, this road brought minimum changes into the landscape. Only a part of it is led newly. This part leads parallel to the original road serving today to hikers or only as a field road. A small part of the original road was completely cancelled and is used as arable land today.

Figure 2 Orthophoto Lesná year 1953. Orthophotomap of South Moravian Region, Ortofotomapa JMK, 2013

The onset of uniform agriculture decided about the use of hitherto unused land. Both in Onšov and in Lesná, drainage works were conducted in several phases until the 1980s and changed the landscape particularly around the Klaperův potok Brook on the borders of cadastres where the land started to be used as arable land for agriculture. At the same time, cowsheds, pigsties and centres of unified farmers' cooperatives are built on the outskirts of villages. Gradually improving mechanization led to the increasing size of lands with the same crop.

Figure 3 Orthophoto Lesná year 1998. Orthophotomap of South Moravian Region, Ortofotomapa JMK, 2013



Following the regime change, most lands are still under management of large agricultural operators and this is why major landscape changes or reduction of the size of cultivated lands have not occurred (Figure 3). On 1 July 1991, the National Park Podyjí was established by CR Government Regulation no. 164/1991 Sb. In the most valuable parts of the former Protected Landscape Area Podyjí whose area is 63 km^2 (PLA was decreed in 1978 on the area of 103 km²) (Lipský, 2006). On the Austrian side of the state border (Dyje River), it neighbours with the National Park Thayatal (Správa Národního Parku Podyjí, 2012).

Declaring the area a national park was possible thanks to the unspoiled landscape (Brzák and Kirchner, 2001), which was paradoxically preserved intact because its access was banned under the former regime. The whole area served as a border zone and was strictly guarded. The National Perk Podyjí reaches into both cadastres both by its core areas and by the buffer zone. The buffer zone coincides with the northern boundary of intravillans of both communities. By this way, the Administration of NP Podyjí attempts at maintaining the landscape and ensuring a continuous transition from agricultural arable land to forests in effort to maintain and form meadows, prevent soil erosion and preserve the typical landscape character of broad surroundings and villages.

CONCLUSION

One of important historical changes in the last 250 years of development of the studied territory can be considered foundation of Lesná community 220 years ago. The greatest observed change is probably the felling of the local forest and its conversion into building and agricultural land. In the course of the following more than a hundred years from the construction of the village to the beginning of the 20th

century, the landscape did not change much. Intravillans of communities are well preserved-village green in Onšov and modern development, more precisely parcelling street development in Lesná. In the community of Lesná, there is a double plot for the original yard in the street, which was built as the first one. If we consider the analyzed territory as a whole and not each cadastre separately, a matrix is arable land. Forest stands, solitary developments, the original quarry and water surfaces form enclaves. A natural corridor in the landscape is the spring area of Klaperův potok Brook and an artificial corridor is represented by road patterns. Transitions in the landscape are mostly sharp but even here the Podyjí NP endeavours after smoother transitions between arable land and forest.

At the beginning of the 20th century, the landscape was affected by the construction of water reservoir on the Dyje River, which resulted in a larger water surface area in the northwest of the Onšov cadastre. Considerable influence on the then landscape had also structures related to the dam construction, which were replaced by today's forest. The dam lake is clearly dominant in the contemporary landscape. Since it is very attractive for tourists, a great number of recreational and refreshment facilities emerged along the banks at the site of the original forest and changed the original character of the landscape.

In the second half of the last century, the population number decreased and new settlers replaced the original German population. The collectivization of agriculture featured the consolidation of land when individual small lots became large continuous fields under management of unified agricultural cooperatives. Since the road pattern was maintained, the landscape did not suffer major changes and remained relatively well preserved. At the present, the two villages are being slightly extended due to the construction of both new dwelling houses and farm buildings.

Lands are being drained, which brings changes in the use of hitherto unused plots. Unfortunately, the former drainage systems represent a big problem today because their service life is at the end. Thanks to political changes at the end of the 20th century, landowners often do not have a slightest idea about their existence because they were unkempt and subsequently lost their functionality. Today, wetlands emerge on the originally drained sites, which cannot be tilled. Such sites are situated mainly around the spring area of the Klaperův potok Brook. The historical maps suggest that the sites were unused before the drainage or used at minimum as meadows or pastures.

The development of the analyzed territory is characterized by areas of land types since 1845 listed in Table 1 Unfortunately, since the borders of the respective cadastres were changing during the history, some data are distorted and the table is therefore only of informative character.

Table 1 Land areas in 1845, 1948 and 2012. ČSÚ and ÚAZK, 2016

Land areas (ha)	**Village of Onšov**			**Village of Lesná**		
	1845	1948	31 Dec. 2012	1845	1948	31 Dec. 2012
Arable land	274.15	273.64	235	Data consolidated with c.a. Vracovice	118.90	257
Gardens	7.92	8.08	6		8.95	10
Meadows	*41.27*	*33.84*	-		*1.86*	-
Pastures	*53.67*	*23.51*	-		*2.39*	-
permanent grass stands	94.94	57.35	4		4.25	20
Agricultural land	**377**	**339**	**244**		**132.1**	**288**
Forest land	224.04	237.59	261		4.27	10
Water surface	0.06	12.14	13		0	1
Built-up area	3.84	2.54	4		4.94	9
Barren land	*0.33*	*0.05*	-		*0.05*	-
Road pattern	*11.18*	*13.39*	-		*4.90*	-
Other surfaces	11.51	14.44	36		4.95	35
Total area	**616.46**	**604.78**	**558**		**146.27**	**343**

Boundaries of cadastral areas were shifted towards the end of the last century. In the last twenty years, some areas of arable land along forests in the landscape changed into meadows and the percentage of forest stands exhibited a negligible increase. Maps of land use change indices in Czechia (Bičík 2008) corroborate the fact too. The maps indicate that the index of land use changes ranged from 10% to 20% in the period from 1845–2000. The studied period can include the construction of Lesná, construction of water reservoir and drainage of lands. In 1948–1990, the index ranged up to 10% in the respective municipalities, which apparently reflects a minimum change (drainage). The last map refers to the period from 1990–2000 where the index of land use changes was only up to 2%. Landscape development can be monitored also thanks to the Corine Land Cover, which shows land use and its changes from 1970 to 2006 (AOPK 2012). The maps indicate that the landscape did not experience too many changes. In the period from 1970–1990, we can see enlargement of the built-up area. In 1970, the forest cover was greater as compared with 1990, apparently due to forest harvesting. Before the year 2000, the situation returned to the same state as it was in 1970. The last change observed in the period 2000–2006 is a mildly increased forest area in the eastern part of the Onšov cadastre.

In the last about 5 years, new ornamental greenery was planted along field roads, those serving tourism in particular. Monuments (chapels) were repaired, especially along field roads. The activity was caused particularly by a higher number of hikers visiting the National Park Podyjí, the lake and the castle in Vranov nad Dyjí. Some beauty spots impressed into the landscape their specific genius loci and a factor of comfort (Sklenička 2003). In this respect, sites worth visiting include the Claryho kříž Cross, the Kumpova poklona Niche Chapel, the Lusthaus gazebo, windmill, church and the former spa site.

The development of the analyzed territory is characterized in Table 1 where areas of land types are listed since the year 1845. In spite of the fact that the Table of informative character only and one cannot take all data as 100% accurate, it points to trends, which represent an important indicator in this analysis. The results of our analyses show that significant changes in landscape use occurred particularly in critical periods due to the construction of water reservoir, later after World War II and in the period of collectivization.

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THE MOISTURE ENVIRONMENTAL CONDITIONS AND THEIR EFFECT ON THE YIELD OF WINTER WHEAT

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Abstract: Wheat grain yields and value of the effective drought index (EDI) in the critical period in terms of yield formation of winter wheat in the Czech Republic were evaluated for the period 1971–2015. The EDI was calculated for four different experimental stations. The EDI is based on the calculation of effective precipitation using only daily precipitation. At these stations were determined dry years: 1974, 1976, 1993, 2012, and humid years: 1987, 1996, 2006, 2010. The historical yield range from the experimental stations for the wheat was correlated with the values of the EDI across the stations for each decade (ten days). The statistically significant correlations ($\alpha = 0.01$, $\alpha = 0.05$) between the wheat yield and the value of the EDI were found in 1., 2., 3., 10. and 11. decade of vegetation.

Key Words: wheat, yield, drought, the effective drought index

INTRODUCTION

It is supposed that in consequence of the climate change there will be higher occurrence of floods as well as longer and more intensive drought periods (IPCC 2013). Drought, being one of the abiotic stress factors, can be considered a gradually growing natural threat in all the climatic zones.

In order to monitor drought and its effects, it is necessary to define the term of drought, which is, considering its nature, rather complicated. General concept of drought is fairly broad and each scientific and agricultural discipline has its own definition of this term.

According to Blinka (2004), drought is a phenomenon characterized by slow emergence which can take months, sometimes even the whole season, years and decades. Determination of the beginning and the end of the period of drought is a difficult process which requires application of a number of meteorological as well as hydrological variables. Drought effects have cumulative character: it intensifies every day. Drought effects can last up to several years after normal precipitation conditions were achieved.

Lack of water is one of the most significant stress factors in plant production worldwide and can affect both quantity and quality of the produce. The effect of each drought period is not only influenced by the length and intensity of the meteorological drought, but also by the time of its occurrence. Thus, the process as well as consequences of each drought period are unique (Brázdil and Kirchner 2007).

MATERIAL AND METHODS

The aim was to quantify the relationship between the EDI value and wheat grain yield. The relationship was demonstrated on common wheat (*Triticum aestivum* L.), the winter variety. Relation of EDI and yield was discovered at 4 experimental stations of Central Institute for Supervising and Testing in Agriculture (CISTA) during the period 1971–2015.

Experimental stations are located in different production and agroclimatological areas between 171 and 505 meters above sea level with the annual average temperature 7.4–9.6 °C and annual precipitation 461–616 mm (Table 1 and Table 2).

Table 1 Basic CISTA experimental stations characteristics

Station	Altitude (m)	Long-term average temperature t_{30} (°C)	Long-term average precipitation p_{30} (mm)	Soil type
Brno–Chrlice	190	9.0	451	FMm – h
Jaroměřice nad Rokyt.	425	8.0	481	HMm – jh
Lednice	171	9.6	461	CMm – h
Lípa	505	7.5	594	KMg – ph

Legend: ČMm – chernozem, FMm - fluvisol, HMm – brown earth, KMg – cambisol pseudo-gleyic, h – loam, jh – clay loam, ph – sandy loam, t_{30} and p_{30} – the long-term average temperature t_{30} and the long-term average precipitation p_{30} (1971-2000)

Table 2 Agroclimatic characteristics of CISTA experimental stations (Kurpelová et al. 1975)

Station	Agroclimatological macroarea	Agroclimatological area	Agroclimatological subarea
Brno–Chrlice	Warm	Warm enough	Mostly dry
Jaroměřice nad Rokyt.	Mildly warm	Slightly humid	Mostly dry
Lednice	Warm	Mostly warm	Mostly dry
Lípa	Mildly warm	Relatively mildly humid	Mildly humid

A unified kind of agricultural engineering was performed at the monitored stations (pre-crop, fertilization), the varieties used for the experiment were similar in the particular year. The yield was only evaluated if it followed a good pre-crop (mostly legume) from agronomic practice at optimal fertilization intensity and plant protection level according to the CISTA methods for experiment management.

Out of various characteristics, the EDI was selected for drought classification. Its result was a non-dimensional number which was found on the basis of effective precipitation method, which uses daily precipitation data of the particular experimental station for the last 365 days. The EDI was designed to detect the beginning and the end of periods of drought and also allows objective comparison of places regardless of their climatic conditions. One of the advantages of the effective precipitation method is the fact that it does not require accurate input data but is able to determine periods of drought precisely, which is possible due to the use of daily precipitation data of a particular locality (Byun and Wilhite 1999).

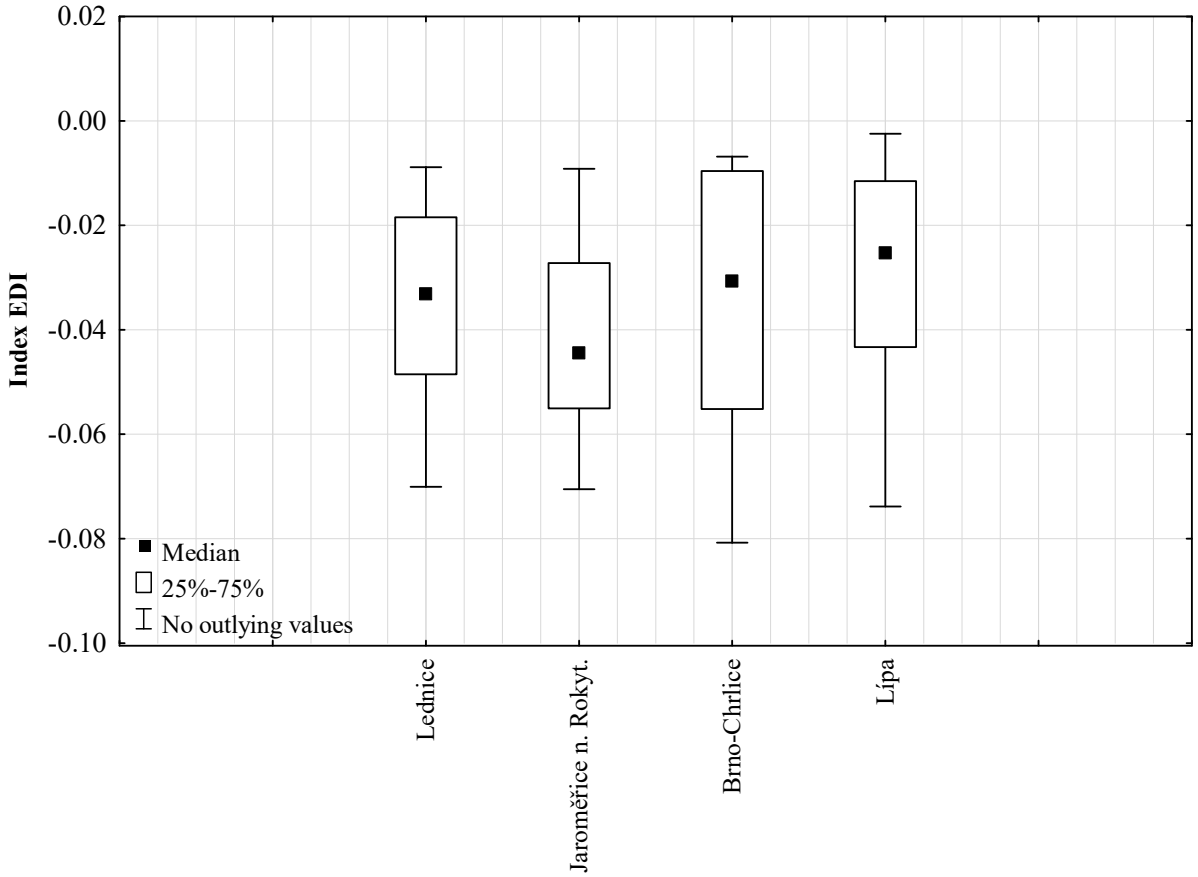
In the monitored period 1971–2015 annual yield values and ten-day EDI values between days 61 and 180 of each year were compared. Their relationship was described by the correlation coefficient.

RESULTS AND DISCUSSION

The EDI for every decade of the monitored years in every experimental station was calculated on the basis of the effective precipitation method. Average EDI seasonal value in the 4 selected stations in the years 1971–2015 was between -0.030 and -0.043. The highest average long-term EDI value of all decades was detected in the Lípa locality (-0.030) at higher altitude, while the lowest average value was detected in a lower altitude locality.

Maximum average EDI value of the monitored period 1971–2015 was reached between days 71 and 80 in Lípa (-0.002) and the lowest average value between days 121 and 130 in Lednice (Figure 1). The EDI was used to evaluate every year at every station. Rainfall conditions in years 1974, 1976, 1993, and 2012 were considered deficient, average EDI of all decades was lower than -0.2. In the years 1987, 1996, 2006, and 2010 the average EDI was the highest (above 1.0) and the rainfall conditions were the most favourable.

Figure 1 Average values of EDI at CISTA stations in 1971–2015 per decade



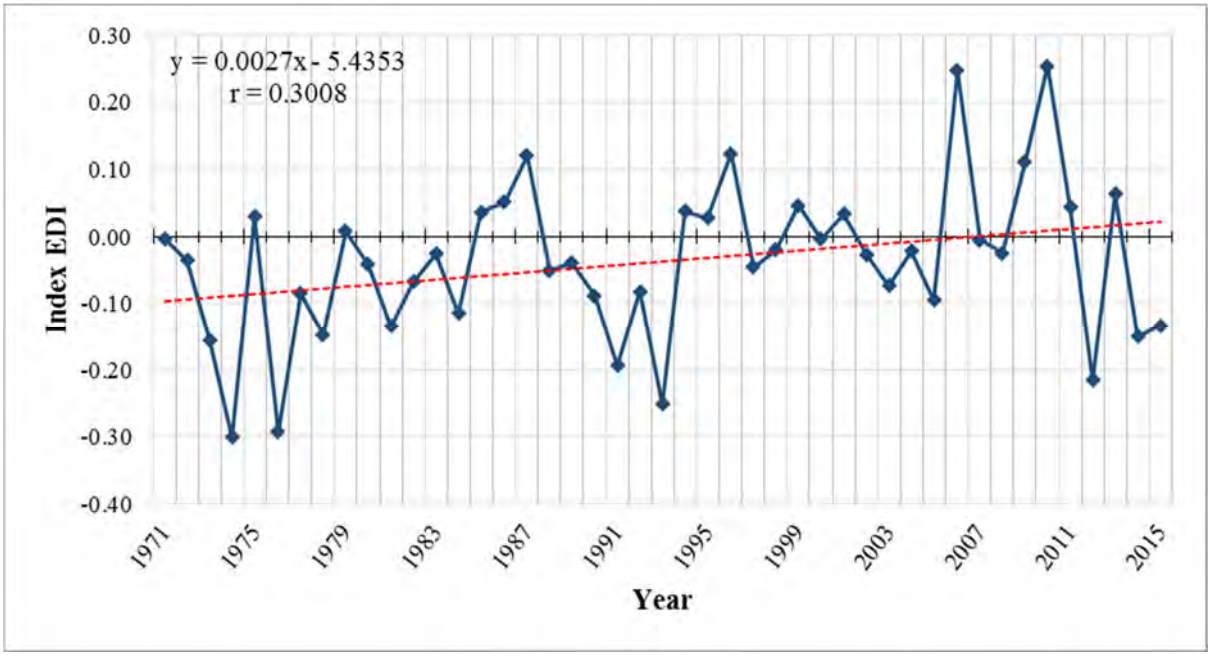
Considering the grain yield (Table 3), the year 2014 seems to be the best, since the highest yield was recorded at 2 localities, despite the average annual EDI value being lower (-0.151). It is difficult to pick the worst year, because it was different for every station. The lowest yield was recorded it the years 1974, 1976, and 1993, when also the average annual EDI value was low.

Table 3 Average, highest and lowest yields of winter wheat at selected stations in 1971–2015

Stations	Altitude (m)	MAX	Year	MIN	Year	Average
Lednice	190	11.19	2004	2.74	1993	7.609
Jaroměřice	425	11.07	2014	3.08	1976	7.386
Brno–Chrlice	171	11.23	2014	3.90	1974	7.691
Lípa	505	10.11	2011	3.51	1972	6.628

Figure 2 shows the progress of average annual EDI values throughout all the monitored CISTA stations. With correlation coefficient $r = 0.3008$ it shows statistical significance ($n = 45$) at the significance level $\alpha = 0.05$. Based on the low average EDI values per decade (ten days) at all experimental stations, rainfall in year 2012 was deep below average and the lowest value -0.538 was recorded between days 71 and 80. In this year, the soil moisture supplies in South Moravia as well as in Central Bohemia were very low. The average value of available water holding capacity (VVK) did not exceed 50% (Mužíková et al. 2013). According to Doorenbos a Pruitt (1984), the value without negative effect on yield and its quality is 55% VVK during all the growth stages except for flowering (45%) and ripening. The results of Středová and Středa (2015) and Středová et al. (2011) suggest an increase of potential evapotranspiration and thus higher susceptibility of agricultural intense areas of southern and central Moravia and Central Bohemia to dryness in 1961–2010 compared to the mean of 1901–1950.

Figure 2 Progress of average annual EDI values throughout all stations during 1971–2015



On the basis of average annual EDI calculated for all decades (days 61 to 180) throughout CISTA stations, years were divided into 2 categories: dry (EDI < 0) and humid (EDI > 0). EDI values per decade were correlated with annual grain yields of each experimental station (Table 4). Their relationship was defined by the correlation coefficient. Statistically significant (α = 0.05) or highly significant (α = 0.01) relationship was recorded in 1., 2., 3., 10., and 11. decade of the vegetation period. Highly significant relationship (α = 0.01, n = 27) during dry years was recorded at Lednice and Jaroměřice nad Rokytnou stations in 2., and 3. decade of vegetation period (between days 71–90), and in humid years (α = 0.01, n = 18) at Lípa station in 3. decade of vegetation period. In some vegetation periods (1., 2., 3., 10., and 11. decade), statistically significant relationship was recorded at each station.

Table 4 Relationship between EDI and wheat yield expressed by the correlation coefficient at selected stations during 1971–2015

Decade (ten days)	LED		JAR		CHR		LIP	
	Dry	Humid	Dry	Humid	Dry	Humid	Dry	Humid
61–70	0.254	0.063	0.260	-0.005	* 0.436	0.053	* 0.399	-0.282
71–80	0.189	-0.269	** 0.487	0.357	0.284	0.025	0.370	0.037
81–90	** 0.586	0.105	* 0.389	-0.298	* 0.393	-0.024	0.239	** -0.654
91–100	0.147	-0.148	-0.012	-0.112	-0.168	-0.413	0.047	-0.217
101–110	0.080	-0.239	0.099	-0.014	0.060	0.068	-0.054	0.012
111–120	0.057	0.001	-0.098	-0.038	-0.020	-0.254	-0.253	-0.109
121–130	0.272	-0.221	0.101	-0.111	-0.027	0.177	-0.016	-0.021
131–140	0.250	0.048	-0.074	0.300	-0.096	0.291	-0.209	-0.338
141–150	0.100	0.134	0.021	0.056	0.022	-0.089	-0.010	-0.116
151–160	0.122	0.037	* 0.423	-0.455	0.365	-0.184	0.244	-0.114
161–170	0.019	* -0.560	0.069	-0.200	0.046	* -0.492	0.001	-0.369
171–180	0.181	0.450	0.081	0.175	-0.034	0.042	0.103	-0.411

*Legend: ** statistically highly significant relationship, * statistically significant relationship, LED – Lednice, JAR – Jaroměřice nad Rokytnou, CHR – Brno–Chrlice, LIP – Lípa*

Statistically significant relationship was mainly recorded in March (decades 1–3) in the tillering stage, when number of spikes is based along with the foundation of secondary roots (Haberle et al. 2008), and in June (decades 10 and 11), when delicate flowering stage is in progress. According to Hlavinka et al. (2009) during the April–June period plant sensitivity to drought is the highest and can have a negative effect on the wheat yield.

CONCLUSION

Average, minimum and maximum yields of winter wheat were calculated for 4 selected CISTA experimental stations during 1971–2015. Oscillation of the EDI in the period critical for development and yield of winter wheat in the Czech Republic (days 61–180) was also calculated. Significant variability of moisture conditions at experimental stations was recorded during the vegetation periods of monitored years. Dry years with EDI < -0.2, were 1974, 1976, 1993, and 2012 (sorted by drought intensity, 1974 being the driest). Years with the best moisture conditions (EDI > 0.1) were 2010, 2006, 1996 a 1987 (sorted by moisture level, 2010 being the wettest).

Relationship between effective precipitation total and grain yield was demonstrated on the basis of correlation of the EDI values with winter wheat grain yields per hectare. For the dry and humid categories correlation coefficients were calculated at all experimental stations for each decade (days 61–180). Statistical significance was found at all experimental stations with 99% or 95% probability in particular stages of the vegetation period (1., 2., 3., 10. and 11. vegetation decade). This suggests use of the EDI for simulation of weather influence on yield formation in significant area of the Czech Republic and possible use for yield simulation on the basis of scenario data.

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MONITORING OF WATER QUALITY IN THE UPPER BASIN OF LITAVA RIVER

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Abstract: Morava River Board, s. e. as the governor of Litava basin is monitoring deteriorated physical and chemical indicators of main river in long term period, however exact source of pollution whether point or nonpoint is not known. Annually observed increased concentrations of phosphorus rate the Litava according to ČSN 75 7221 in V. class which means very heavily polluted water. The aim of this research is monitoring and chemical analysis of surface water quality in the upper part of Litava river basin in order to identify potential sources of pollution, which should be examined in detail within the scope of further investigation. Twelve sampling profiles were selected in the treatment area – eight profiles are directly on the stream of Litava river, four are on main tributaries. Monitoring program is performed by above-and-below approach, where sampling profiles are placed upstream and downstream of treatment area section. During three-month period of monitoring four water quality indicators were being measured in the field monthly: pH, temperature, dissolved oxygen and electrical conductivity. Also the samples were being taken and further analysed in the laboratory of water management for determination of total phosphorus, total nitrogen, nitrate nitrogen, ammonium nitrogen and chemical oxygen demand (COD). Results has been graphically demonstrated and evaluated according to Government order No. 401/2015 Coll., as amended and ČSN 75 7221. Chemical analysis confirmed that high concentration of total phosphorus is the major issue in upper basin of the Litava river, except spring areas.

Key Words: Litava river, water quality, water pollution, laboratory analysis, phosphorus

INTRODUCTION

Securing clear water will always be a primary goal of watershed management in order to keep aquatic ecosystem and ecological balance in landscape, ensure water supplies for drinking and service water, etc. River monitoring stays at the beginning of complex repetitive process of water quality maintenance or improvement which should be in human best concern as the anthropogenic activity is generally main cause of pollution.

Upper part of Litava basin which ends in front of Bučovice town is placed east of Brno on the border between South Moravian Region and Zlín Region. Litava, formerly known as Cézava, flows into the Svratka left tributary near Židlochovice. Basin of Litava river belongs to the Black Sea drainage. The catchment area has 136.83 km^2 and consists of seventeen subbasins. By reason of lower altitude, warm climate and fertile soils, the original riparian forest in the surrounding floodplain was cut down and all the floodplain now serves for agricultural production. Arable land is formed by large blocks of estates, it covers 59% of basin and forests only 23%. The basin is overall densely populated. Length of main channel which was in the past inappropriately narrowed and entrenched is 23.64 km. Average annual flow in no. 381 in Brankovice is 0.22 m^3/s.

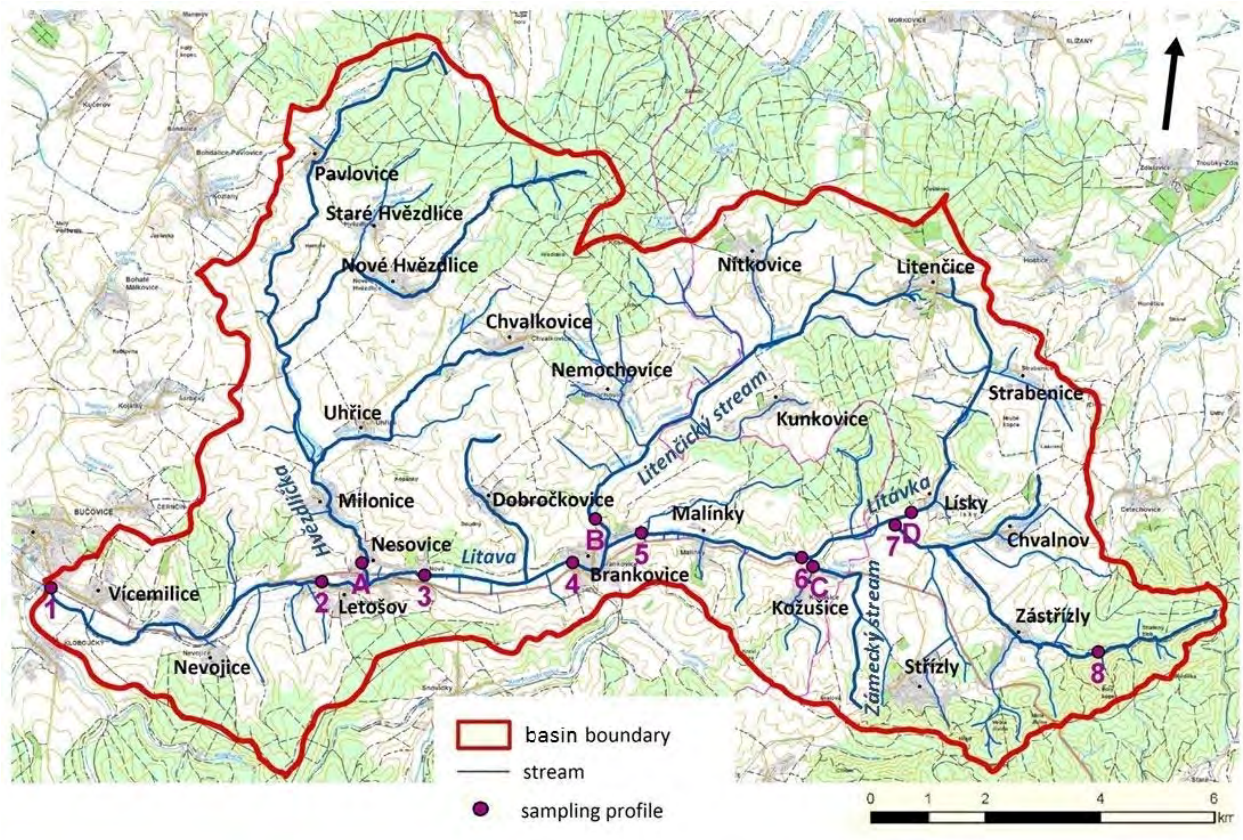
MATERIAL AND METHODS

Monitoring program

The design of the monitoring program must locate appropriate sampling profiles taking to account objectives and the type of monitoring planned (Kunkle et al. 1987). Monitoring program is performed by above-and-below approach, where sampling profiles are placed upstream and downstream of

treatment area section. Sampling profiles are located to determine if particular activity is causing the water quality standards to be exceeded. (Brooks et al. 2003). In the treatment area has been determined twelve sampling profiles (see Figure 1), from which eight are directly on the river Litava stream (station no. 1–8) and four are on main tributaries in front of mouth into the Litava stream – Hvězdlička (A), Litenčický stream (B), Zámecký stream (C), Litávka (D).

Figure 1 Location of the twelve sampling profiles in upper basin of Litava river (source: author, basemap: GeoPortal INSPIRE – cenia_t_podklad)



Measuring process

Monitoring of the Litava water quality was being performed monthly since June to August 2016 and it will continue until May 2017. Four water quality indicators are measured on each sampling profile directly in the field using portable multimeter Sension 156 (HACH LANGE Company): pH, temperature, dissolved oxygen and electrical conductivity. In addition water sample is collected from each profile in form of single „grab" sample by hand in to plastic bottle.

However, a single sample is representative of the stream discharge only at the time of sampling (Brooks et al. 2003). Samples are analyzed within 24 hours in the laboratory of water management of the Departures of Applied and Landscape Ecology at the Mendel University in Brno. Determination of total phosphorus, total nitrogen, nitrate nitrogen, ammonium nitrogen and chemical oxygen demand (COD) is performed according to HACH LANGE Company standardized methods using spectrophotometer HACH DR 4000 a mineralization thermostat HACH DRB 200.

Evaluation of indicators

Results collected for last three months were graphically demonstrated and evaluated according to ČSN 75 7221 and Government order No. 401/2015 Coll., on the indicators and values of permissible pollution of surface water and wastewater, mandatory elements of the permits for discharge of wastewater into surface water and into sewerage system, and on sensitive areas, as amended (further in text „GO No. 401/2015 Coll.") – Annex 3A. The attachment of government order contains 125 indicators divided into 6 groups with permissible surface water pollution requirement specified for each one of them.

RESULTS AND DISCUSSION

PH, dissolved oxygen

Surface waters in upper part of Litava basin are mildly alkaline with pH in range 7.32–8.39 meeting average value between 6 and 9 specified by GO No. 401/2015 Coll. Highest values were ordinarily measured in profiles no. 2, 5, 6 and A. Values of pH exceeding 8 are probably caused by higher photosynthetic activity of green organisms which occurrence is also accompanied by higher concentration of dissolved oxygen (11–14 mg/l). Limit for dissolved oxygen (over 9mg/l) was fulfilled just on mentioned profiles with higher pH. The lowest concentration of dissolved oxygen (4–5mg/l) was usually measured in profile B – Litenčický stream, which is placed under water reservoir Hlavatka. Dissolved oxygen concentration drop could be caused by fish farming focused on leisure time and sporting activities.

Temperature, electrical conductivity

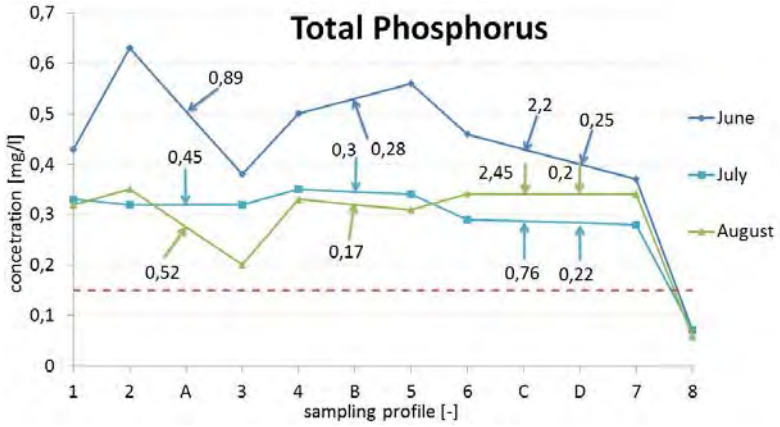
Water temperature during the flow through was mildly increasing every month. Highest temperature was recorded in the profile 1 in June (26.8 °C), thus the maximum limit specified by GO No. 401/2015 Coll. (29 °C) was not exceeded. Electrical conductivity results provide quick information about content of anions and cations dissolved in the water and the resulting concentration of dissolved minerals (salts), ergo pollution. Highest values of conductivity was recorded in A and C profiles. Kopp (2015) states, that might be caused by both lower flows in summer season or runoff from agricultural land or anthropogenic activity.

Total phosphorus

Phosphorus is an essential element for plant life and its compounds play key role in cycles of matter. This indicator is most importantly monitored because of eutrophication as the most common cause of unsatisfactory water quality. Natural origins of phosphorus are certain soils, weathered rocks and minerals. Among anthropogenic origins belong washing, cleaning and degreasing lotions, phosphate fertilizers utilized in agricultural production, intensive livestock and sewage. (Pitter 2009)

Only the profile no. 8 fulfills the limit allowed by GO No. 401/2015 Coll. – 0.15 mg/l, see Figure 2. The highest concentration of phosphorus in Litava river was recorded in June in profile no. 2. This concentration increase was probably caused by discharging of domestic sewage waters from Milonice village located on the tributary A. Nesovice town lying between profiles no. 2 and 3 has wastewater treatment plant since 2013 including additional phosphorus withdrawing and sewerage. Potential source of pollution appears Milonice village itself which is indicated by one of highest values of phosphorus concentration in the tributary A (Hvězdlička stream). Also Milonice is not equipped with wastewater treatment plant neither sewerage. (DPWSS 2016)

Figure 2 Total phosphorus values in profiles (1–8) on the Litava river and its tributaries (A–D is graphically demonstrated by arrows with concentration values (source: author)



On the contrary the lowest concentration of phosphorus is recorded in the profile no. 3 upstream of Nesovice town. River is passing agricultural area between profiles no. 3 and 4 however it is covered in riparian vegetation all along this section which probably positively influences the water quality by self-cleaning ability i. e. natural removal of pollutant. The highest value was permanently measured

in profile C (Zámecký stream) downstream Kožušice village. The village is equipped with combined sewerage directing domestic sewege directly into watercourse (DPWSS 2016). In this case the origin of phosphorus is again in waste water; however the impact on the profile no. 6 lying downstream is minimal considering low flow ratio between Litava river and Zámecký stream.

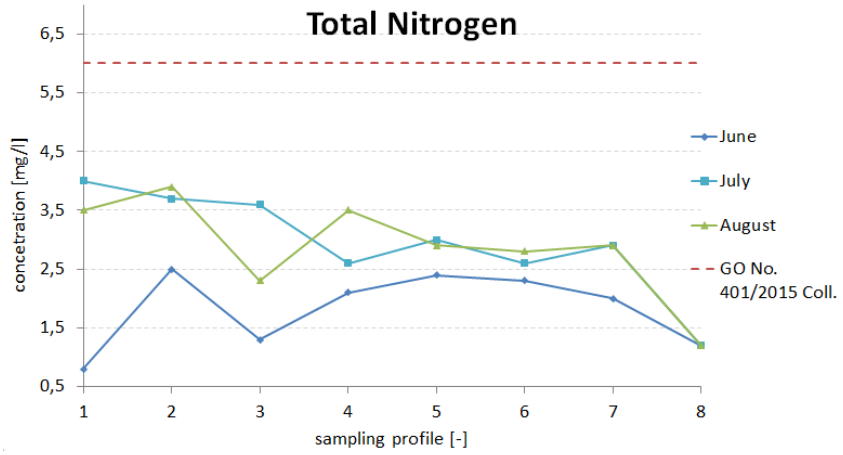
Nitrogen compounds

Nitrogen, similarly to phosphorus, belongs among essential elements and occurs in several forms. Organic nitrogen breaks down into ammonia nitrogen, which is furthermore oxidized to nitrite nitrogen. Nitrite nitrogen is however unstable therefore the process of oxidization continues into stable nitrate nitrogen as the final product of the decomposition of the nitrogenous organic matter (Brooks et al. 2003).

Natural sources of nitrogen are decaying of vegetable and animal matter, fixation of aerial nitrogen, and precipitation. Anthropogenic sources are then sewage, outwash of nitrogenous fertilizers from agricultural soils, agricultural wastes and particular industrial wastewater (Pitter 2009).

Concentration of total nitrogen in Litava river has never exceeded limit specified in GO No. 401/2015 Coll. – 6 mg/l, see Figure 3. Trend of nitrogen nitrate values matches with the total nitrogen. None of the values have exceeded limit specified in GO No. 401/2015 Coll. – 5.4 mg/l. Highest values were measured in profiles A and C with maximum of 2.8 mg/l. These low concentrations indicate that the influence of nitrogenous fertilizers from agricultural land on surface water quality can be excluded.

Figure 3 Total nitrogen values in profile on Litava river (source: author)

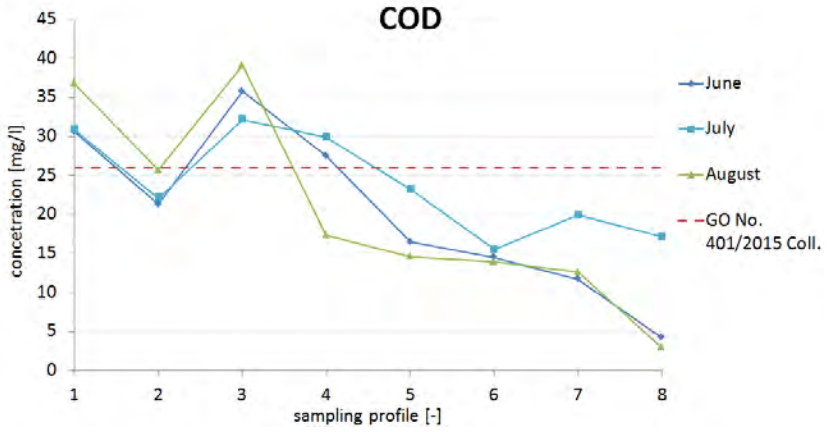


Higher concentrations of ammonia nitrogen have been observed in lower profiles of the main stream. Only profiles no. 6, 7, 8 and profiles on tributaries B and D have met tight limit GO No. 401/2015 Coll. – 0.23 mg/l. According to expectations the highest concentrations has been recorded in profile C, placed 50 m downstream Kožušice village, where the average value of all 3 records is 18.2 mg/l. Because the ammonia nitrogen is generally considered as "indicator of fecal contamination", it is obvious that pollution has anthropogenic origin particularly domestic sewage. It is paradoxical, that high concentrations of ammonia nitrogen from profile C do not influence downstream profile no. 6 which is attributed to surface waters dilution. Highest increase of concentrations is usually observed between profiles no. 5 and 6, however still within the required boundaries. Between these profiles is located Malínky village without sewerage and wastewater treatment plant and with livestock *(*DPWSS *2016).*

COD

Chemical oxygen demand can be defined as „amount of oxygen expended under given conditions into chemical oxidation, using strong oxidizing agents" (Heteša and Kočková 1997). COD is very important indicator for surface waters by which is possible to determine organic pollution caused by wastewater, livestock waste or other decomposing organic matter in the water. Highest COD values exceeding limit GO No. 401/2015 Coll. – 26 mg/l, have been measured in profiles no. 1, 3 and 4, see Figure 4. Even though both villages lying between monitored profiles are equipped with sewerages and wastewater treatment plants, growth of COD concentrations can be still assigned to anthropogenic pollution caused by large areas with livestock.

Figure 4 COD values in profile on Litava river (source:author)



Czech national standard 75 7221

Surface waters quality is for basic information defined by quality classes listed in ČSN 75 7221 Classification of Surface Water Quality. Reference to this standard is only orientation as the required compared value is average of the 3 worst records for whole monitoring period. Indicators are classified into 5 groups. COD, ammonia nitrogen ($N\text{-}NH_4^+$), nitrate nitrogen ($N\text{-}NO_3$), total phosphorus (TP) and dissolved oxygen (DO) belong into group „General, physical and chemical indicators“. Table 1 contains classification of monitored profiles within the scope of physical and chemical indicators.

Table 1 Classification of monitored profiles according to ČSN 75 7221(units: mg/l)

Classification	Profile	COD	$N\text{-}NH_4^+$	$N\text{-}NO_3$	TP	DO
I.	–	< 15	< 0.3	< 3	< 0.05	> 7.5
II.	8	< 25	< 0.7	< 6	< 0.15	> 6.5
III.	1, 3, 4, 6, 7, B, D	< 45	< 2	< 10	< 0.4	> 5
IV.	2, 5, A	< 60	< 4	< 13	< 1	> 3
V.	C	≥ 60	≥ 4	≥ 13	≥ 1	≤ 3

Results plainly show significant influence of water quality in Litava river basin by settlement. Especially high concentrations of total phosphorus and ammonium nitrogen point to missing system of sewerages and related wastewater treatment plants. Only 34% from total of 8125 inhabitants living in monitored basin are connected to wastewater treatment plant of who are most large town citizens of Brankovice, Nesovice and Vícemilice (part of Bučovice). According to Development Plan of Water Supply and Sewerage for period after 2015 should be each municipality equipped with sewerage draining wastewater into downstream local or neighboring wastewater treatment plant (DPWSS 2016). Smaller villages under 500 inhabitants will probably have difficulties to fulfill the plan considering large economic burden connected with such ecological investments. At present, the most of the residents are using cesspits, septic tanks or they are discharging wastewater into municipal sewerage leading directly into watercourse. Another potential source of surface water pollution is large areal with livestock, place almost all around Litava's alluvial plain.

However, wastewater from municipalities and livestock are not the only originators of deteriorated water surface water quality in Litava basin, other contributors might be cumulative effects caused by anthropogenic and natural impact. Litava basin has the long term lowered flow caused by lesser precipitation of 500–600 mm/year leading to minor dilution of point sources of pollution. Higher concentration causes poor rating of most indicators (e.g. total phosphorus and ammonia nitrogen), which in connection with reduced self-cleaning ability by reason of previously improperly technically treated river channel significantly worsen surface water quality. Last but not least negative factor is higher water erosion of agricultural land in the basin bringing sediments (undissolved solids) into the water course.

CONCLUSION

Presented results show, that the biggest impact on worsen water quality in upper part of Litava basin have point sources of pollution, especially human residences. Only about 34% of basin inhabitants are connected to wastewater treatment plant while the rest is discharging pre-treatment or non-treatment wastewater directly into the surface water or subsurface water. This is the reason why high concentrations of total phosphorus overcoming limits defined by GO No. 401/2015 Coll. have been recorded in the main stream. However critical values (up to sextuple of limit) of total phosphorus have been measured in tributaries Hvězdlička (A) and Zámecký stream (B). Zámecký stream also contains extremely high concentrations of ammonium nitrogen which indicates presence of fecal contamination. Water quality monitoring of Litava basin will continue in contemporary month period and addition of several profiles is presumed for more detail identification of sources of pollution, especially on tributaries Hvězdlička and Zámecký stream.

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CHANGES IN CONTENT OF SOIL MINERAL NITROGEN AND UTILIZATION OF MINERAL NITROGEN BY SOIL MICROORGANISMS DUE TO APPLICATION OF DIFFERENT FERTILIZERS

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Abstract: Today in many parts of Europe including Czech Republic, the soil cover is damaged by intensive application of mineral fertilizers containing mineral nitrogen (N_{min}). On the other hand, the increasing number of biogas plant stations and composting plants has been put into operation in CZE since 2002. Therefore, the present work deals with application of digestate and compost as potential alternative organic sources of N which can be used in Czech agriculture without negative effects on soil properties. The potential effect of these organic fertilizers and one mineral fertilizer was studied by pot experiment. The highest level of N utilization was found in variants where digestate (150 kg/ha N) and compost (150 kg/ha N) were applied, about 20% higher in comparison with control variant (without fertilizers) and 35% with variant fertilized by mineral N. Above all, application of digestate had positive effect on plant biomass production and compost application on development of potential soil fertility.

Key Words: digestate, mineral fertilizer, compost, mineral nitrogen, soil fertility

INTRODUCTION

The agriculture is currently dependent on external fertilizer inputs. The input could be organic, e.g. manure, digestate, compost or inorganic, e.g. mineral fertilizer.

Digestate (DG), the semi-solid residue obtained after biomass extraction in anaerobic digestation, is considered as a vital source of organic matter and nutrients, nitrogen in particular (Tiwary et al. 2015). Dealing with digestate is currently gaining great importance as it has certain amount of plant nutrients and organic matter, and can be used as organic fertilizer or soil conditioner (Massaccesi et al. 2013). Compost (CP), the main product of composting, can by defined as the stabilized and sanitized product of decomposition of organic matter (Diaz et al. 2007).

Nitrogen (N) is a very important nutrient for growth and development of plants. Excessive N application accelerates its loss through ammonia volatilization, denitrification, surface runoff and leaching (Wang et al. 2014). These processes have a result in significant source for water contamination (Zhu et al. 2005). Nitrate leaching is influenced by environmental and management factors, such as climate (Smith et al. 2013), soil properties (Qiu et al. 2009) and management practices (Li et al. 2006), for example kind of fertilizers. The presented study evaluates the potential use of digestate and compost as alternative source of N_{min} for soil microorganisms and increasing the N_{min} content in the soil without the above negative effects.

MATERIAL AND METHODS

Design of experiment

To study and demonstrate effect of DG and CP application on content of mineral nitrogen (N_{min}) in soil and microbial biomass, the pot experiment was established according to Elbl et al. (2014a): eighteen plastic experimental containers were filled with 800 g of soil with or without addition of CP, DG and mineral fertilizer (NPK). Soil sampling was done on the 10th of November 2014 by ČSN ISO

10 381-6 in the field experimental station "Vatín" (49° 31´N, 15° 58´E). CP samples came from company "CKB a.s." and were collected on the 30th of November in accordance with ČSN EN 46 5735. Experiment was conducted from the 1st of March 2015 to the 3rd of May 2015. *Lactuca sativa* L. was selected as indicator plant. During whole experiment, plants were grown in the determined conditions at 22 °C with a day length of 12 h and light intensity of 300 μmol/m^2/s; for 63 days. After this period, containers were dismantled.

Fertilizers

For the laboratory experiment, these fertilizers have been used: DG, CP and mineral fertilizer GSH. Applied DG came from biogas plant Nové Město na Moravě. Input material in this biogas plant is bovine manure, farmyard manure, grass silage, and maize silage. The properties of applied digestate and compost are summarized in Table 1.

Table 1 The properties of used soil and applied organic fertilizers

Properties	Soil	Properties (wt%)	Digestate	Compost
		Dry matter	9.20	55
C_{org} (wt%)	1.25	C_{org}	0.31	16.8
N total (wt%)	0.14	N total	0.31	1.31
C_{tot}/N_{tot}	11.9	C_{tot}/N_{tot}	2.15	13.9
Ca (mg/kg)	735	Ca	0.19	1.12
K (mg/kg)	278	K	0.49	0.64
Mg (mg/kg)	176	Mg	0.08	0.12
P (mg/kg)	39	P	0.08	0.05
pH ($CaCl_2$)	4.7	pH	8.17	8.31

Special type of CP (organic waste compost) "Black Dragon" was used as a second organic fertilizer. Black Dragon meets all the parameters (humidity, phytotoxicity and nutrient contents; see Table 1) stated in ČSN EN 46 5735. Moreover, mineral fertilizer GSH-NPK containing N, P, K and S in the ratio 10:10:10:13 was used as soil amendment. All used fertilizers are registered (the Fertilizers Act no.: 156/1998) for agriculture use in the Czech Republic.

Six variants of experiment with or without addition of fertilizers were prepared, each one with three repetitions. Complete overview is presented in Table 2. The dose of individual fertilizers was calculated according their N content.

Table 2 Overview of laboratory experiment

Variants	Designation	Dose of nitrogen (kg/ha)
Control	C0	0
Digestate	DG220	220
Digestate	DG150	150
Digestate	DG80	80
Mineral fertilizer	NPK150	150
Compost	CP150	150

Determination of N_{min} content in soil and availability of N for soil microorganisms

N_{min} (consisting of ammonium and nitrate N) was extracted from soil samples (after termination of experiment) by 2M KCl according to Bundy and Meisinger (1994) and concentration of N_{min} in extract was determined using distillation-titration method by Peoples et al. (1989).

Method for determination of N availability was characterized and described by Bundy and Meisinger (1994) and used for example in laboratory experiment Elbl et al. (2014b): soil N availability for microbes is estimated from ammonium produced during 7 day waterlogged incubation. The method is divided into two parts. (I) determination of the content of ammonium and nitrate N (N_{min}; described

in the previous paragraph) before incubation and (II) determination of ammonium N which is floated out of the microbial cell after incubation. Soil samples were placed into incubation bottle with distilled water and located in thermostat. After incubation at 40 °C, only the content of ammonium N was determined (because the content of nitrate N is unchangeable) by the same procedure as the determination of the N_{min}. The principle of this method is described in detail by Bundy and Meisinger (1994) and application in Elbl et al. (2014b).

Statistical analysis

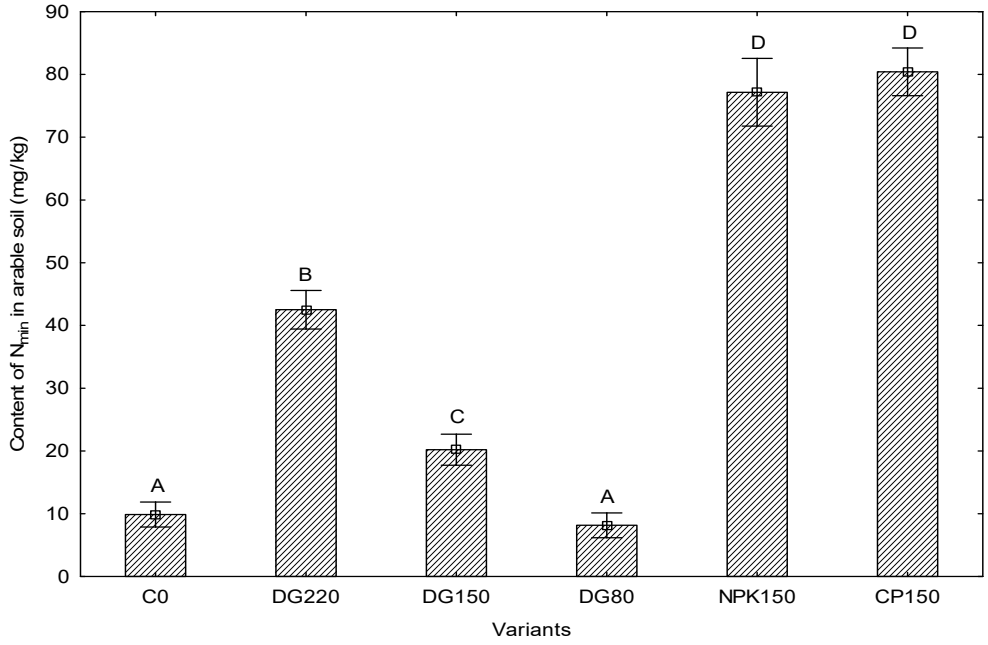
The potential differences in content of N_{min} in soil, availability of N_{min} for soil microorganisms and plant biomass production between individual variants were analysed by ANOVA ($P < 0.05$) with post – hoc LSD Fisher test.

RESULTS AND DISCUSSION

Amount of N_{min} in soil

Sutton (2011) states the content of N_{min} in soil represents an important indicator of the agriculture (arable) soil which directly indicates level of soil fertility. The Figure 1 shows the effect of individual fertilizers composition on N_{min} content in soil. The statistical analysis demonstrated that there were significant differences ($P < 0.05$) between individual variants.

Figure 1 Amount of N_{min} using individual treatments (mean ± SD; n = 3)



Legend: Different uppercase letter indicates significant differences

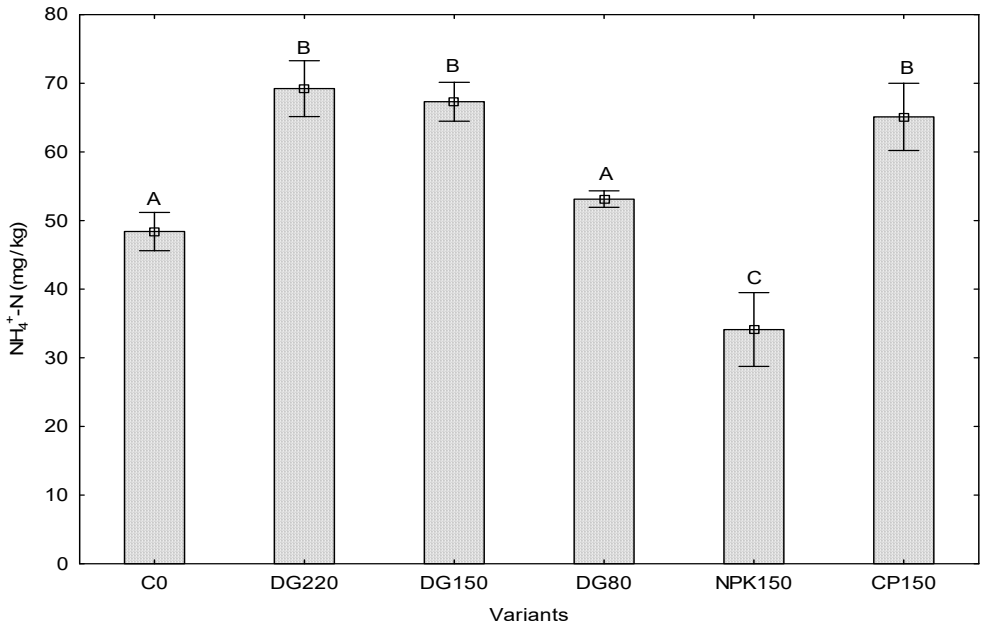
The application of DG (DG220 and DG150) to arable soil brought an improvement in concentration of N_{min} in arable soil, in comparison with control variant (except DG80). On the other hand, application of NPK (NPK150) and CP (CP150) has better effect on N_{min} content in soil – the significantly highest amount was found there, but in the case of NPK (NPK150) its N was not utilized by soil microorganisms (consider Figure 2). The application of DG (García-Sánchez et al. 2015) and CP (Diaz et al. 2007) affects an increase in N_{min} concertation in soil and its utilization by plants and soil microbes due to their composition (Renneberg et al. 2009, Makádi et al. 2012).

Index of nitrogen availability: Ammonium production during waterlogged incubation

Index of N availability represents a specific indicator, which shows the quantity of N in microbial biomass (Elbl et al. 2014b). Consequently, this method indicates the availability of N for soil microbes (Bundy and Meisinger, 1994) because concentration of N is measured in soil extract before/after 7 day incubation at 40 °C and only anaerobic thermopiles (minority group of soil microorganisms) can survive these temperatures. There is a presumption that index of N availability correlates with the ability of soil

microbes utilizing N_{min} (Goloran et al. 2013). In general, the measured results of N availability show large differences between individual variants of experiment which indicates the different effect of organic and inorganic fertilizers on availability of N for soil microorganisms.

Figure 2 Availability of N_{min} for soil microorganisms (mean ± SD; n = 3)



Legend: Different uppercase letter indicates significant differences

The availability of N in variant with DG and CP was higher than in the control (C0) and variant with NPK application. Consider Figure 1 and 2, the significantly highest nitrogen availability was found in variant where DG or CP was applied (DG220 = 69.2 mg/kg; CP150 = 65.1 mg/kg), in comparison with control variant and variant with NPK. Scientific works of Nevens and Reheul (2003) and Weber et al. (2007) confirm that CP addition has a positive influence on the availability of N in the soil (for plant and microbes) due to its composition. Moreover according García-Sánchez et al. (2015) and Alburquerque et al. (2012), application of DG contributes to increase in soil physical and biochemical properties and subsequently in availability of N in soil.

Plant biomass production

Lactuca sativa L. (salad) was chosen as the indicator for the evaluation of DG, CP and NPK addition effect on plant biomass production and it was grown for 63 days. The significantly highest plant biomass production was found in variant where 150 kg/ha of N by digestate was applied (DG150). Conversely, the significantly lowest values were detected in control (C0) variant and variant with CP addition (CP150). Consider, the dose of N, which was applied in variants DG150, NPK150 and CP150, was 150 kg/ha but in different forms. These results indicate the direct effect of fertilizers composition on plant production (Table 3) and utilization of N in soil by soil microorganisms (Figure 2).

Table 3 Plant biomass production

Treatment	Mean of biomass production (g)	Standard Deviation	Mean Differences
C0	1.89	0.19	A
DG220	2.90	0.47	B
DG150	3.57	0.23	C
DG80	3.28	0.39	B,C
NPK150	3.04	0.43	B
CP150	1.79	0.18	A

Legend: Different uppercase letter indicates significant differences

The salad was chosen as indicator plant on the basis of D´antuona and Neri (2001) study, where authors confirmed the positive correlation between production of salad biomass and availability of N in soil due to different kind of fertilizer application. According Makádi et al. (2012), DG has specific composition – lower ratio of C and N. Therefore, N is easily accessible to plant (supporting the production of plant biomass) in comparison to CP (see Table 1). On the other hand, CP application affects soil properties in long term (Diaz et al. 2007) and higher ratio C/N results in improving the content of soil organic matter (Nevens and Reheul 2003) which is necessary for development of long-term soil fertility (Sutton 2011).

CONCLUSION

The main problem lies in the fact that the inorganic nitrogen from mineral fertilizers is hardly sustainable in the soil environment. Therefore, there is a gap for using organic fertilizers. The presented results indicate that the application of DG and CP may represent opportunity to mitigate the negative influences of extensive agriculture – depletion in soil fertility, but only if the dose of DG and CP is proportionate to the state of arable soil because inappropriate DG/CP leads to leaching of nutrients from soil (nutrients – especially N cannot be used by organisms and plants), disruption of microbial activity in soil, and subsequently to soil degradation.

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DYNAMIC OF SOIL TEMPERATURE WITH DIFFERENT FERTILIZERS MANAGEMENT

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Abstract: Soil temperature is very important, it affects not only the soil environment but also the plants. But, scientists have not devoted big attention yet. Long-term field experiments started in 2014 on the experimental plots of Mendel University (Vatin, the Czech Republic). The calcium ammonium nitrate and digestate were applied into the soil with aim to study their effect on soil temperature dynamics and to compare differences in the effect of industrial and organic fertilizer on the course of soil temperature. We evaluated changes in different depth (0.05, 0.10 and 0.15 m) by vegetation cover corn. Measurements were carried by temperature sensors. Better (for limits extreme temperature) was digestate with lower (almost 4 °C) variation range in 0.05 m. Results indicated different variation ranges and different delays mainly in surface layer. The main difference was in the depth of 0.05 m. The soil temperature amplitudes with mineral fertilizer in the depth of 0.05 m were slightly bigger than within the digestated plots, and they came about one hour earlier.

Key Words: field trial, digestate, mineral fertilizer, cambisol, vegetative season

INTRODUCTION

Soil temperature is an important agro-climatic indicator. It influences plant growth, development of all kinds of soil organisms and, indirectly, the soil fertility (Petr 1987, Probert 2000, Steiner et al. 2009), it controls the decomposition of organic compounds (Davidson and Janssens 2006), mineralisation of nitrogen (Gonçalves and Carlyle 1994). On the other hand, the temperature is depended on different facts, for example topography, soil texture or soil water content (Zheng 1993), vegetation cover or tillage method (Drury et al. 1998).

Temperature changes are caused by changes of soil thermal balance, and by heating and cooling of the soil body, which comes always from the surface. The daily amplitude of soil surface temperature is significantly reduced by clouds, density of vegetation, or by snow (Havlíček et al. 1986). Heating of the soil surface is also significantly affected by the heat capacity: higher values of soil specific heat capacity means the slower heating up of the soil. It is supposed that dry soils have 3–5 times lower values of specific heat capacity than water. It means that the thermal soil capacity depends on water content. Wet soils, which are in the same conditions, have lower temperature than dry soils and their daily temperature amplitude is lower. Dry soils are heated more quickly and have higher daily temperature amplitude. Usually having higher specific heat conductivity, heat is more easily dissipated deeper into the soil and therefore the surface area is heated up slowly. Thermal conductivity is directly dependent on porosity and soil moisture. High porosity caused the conductivity values, because of air, which is a poor heat conductor. Time shift of temperature maximum and minimum also increases with a soil depth. Delay of the day cycle is approximately 2.5 to 3.5 hours at every 0.10 m of depth, delay of the annual periods of temperature is from 20 to 30 days for the depth of one meter (Králová and Zvěřina 2002).

Digestate the semi-solid residue obtained after conversion of biomass by anaerobic–digestion is considered as a vital source of organic matter and nutrients, especially nitrogen in particular (Tiwary et al. 2015). Dealing with digestate is currently gaining great importance as it has certain amount of plant nutrients and organic matter and can be used as organic fertilizer or soil conditioner (Massaccesi et al. 2013).

Many scientific papers point to the long-term lack of interest in the study of soil temperatures by scientific community (Buchan 2001, Možný 1991, Nosek 1972, Lehnert 2014).

In our investigation the field trial was focused on recording of temperature changes by 2 fertilizer management during vegetation season 2015. The fertilizer managements were digestate (D) and mineral fertilizer (CAN). The vegetation cover was corn.

MATERIAL AND METHODS

Characterization of growing locality

Long-term field experiment was located at Vatin (Zdar nad Sazavou county, the Czech Republic) on the Mendel University´s experimental plots. Each experimental plot had 10 m^2. Soil was classified according to Němeček et al. (2011) as haplic Cambisol, eutric variety (KAme'), sandy-loam textured, on weathered gneiss. Average annual rainfall is 594 mm; average annual temperature is about 6.1 °C. The experiment was established in 2014 and we evaluated period in 2015 (from 30th April to 20th October 2015). Soil properties of the field trial are presented in Table 1. We used for measure of the soil temperature datalogger with 6 chanels (AMET association Litschmann and Suchy) in 10min. steps.

For measure of the soil moisture we used VIRRIB probe (AMET association Litschmann and Suchy). The depth imposition of sensors was 0.15 m. All values of soil moisture (in variant with digestate) were lower than values in variant with mineral fertilizer.

Table 1 Selected soil prosperities of the field trial in Vatin before the start, the depth scale from 0 to 0.20 m

Soil properties	Value	Soil properties	Value
Particle size		Porosity (% vol.)	54.4
Clay (<2 μm, wt%)	11.7	Field capacity (% vol.)	29.3
Silt (50–2 μm, wt%)	34.9	Max. capillary water cap. (% vol.)	39.9
Sand (2 000–50μm, wt%)	53.4	Minimum air capacity (% vol.)	14.5
		Oxidizable carbon (wt%)	1.8
Bulk density (kg/m^3)	1197.1	pH/H_2O	5.8

Legend: Values shown for individual properties are averaged from all of the samplings taken from the research plot.

Experimental design

Two different fertilizers on monoculture corn (*Zea mays L.)* were applied - calcium ammonium nitrate (CAN) and digestate (D). The amount of used fertilizer was calculated in accordance with nitrogen content. Applied value of nitrogen was 150 kg/ha. All fertilizers were applied in two doses during the vegetative season (spring – 60% of total delivered N and June – 40% of total delivered N). The mineral fertilizer was applied as calcium ammonium nitrate with single superphosphate and muriate of potash. Digestate was made of farmyard manure, cereals´ silage, corn silage, green grass, grass silage and was reached from biogas plant station in Pikárec (the Czech Republic). The harvest residues were left on the soil surface and were incorporated into the soil during the cultivation by disking to the depth 0.16 m. First dose of digestate and mineral fertilizer was applied during the spring on cultivated parcel before the maize sowing. Digestate was applied into the furrow and mineral fertilizer on the parcel surface with subsequent covering by soil. Second dose was applied between the row spacing on the surface.

The soil temperature was carried by datalogger device. The sensors were in 3 different depths: 0.05, 0.10 and 0.15 m. The temperature was recorded in the period 30th of April to 20th October 2015. It was after sowing and harvest of maize. The air temperature was carried by local weather station in Vatin.

RESULTS AND DISCUSSION

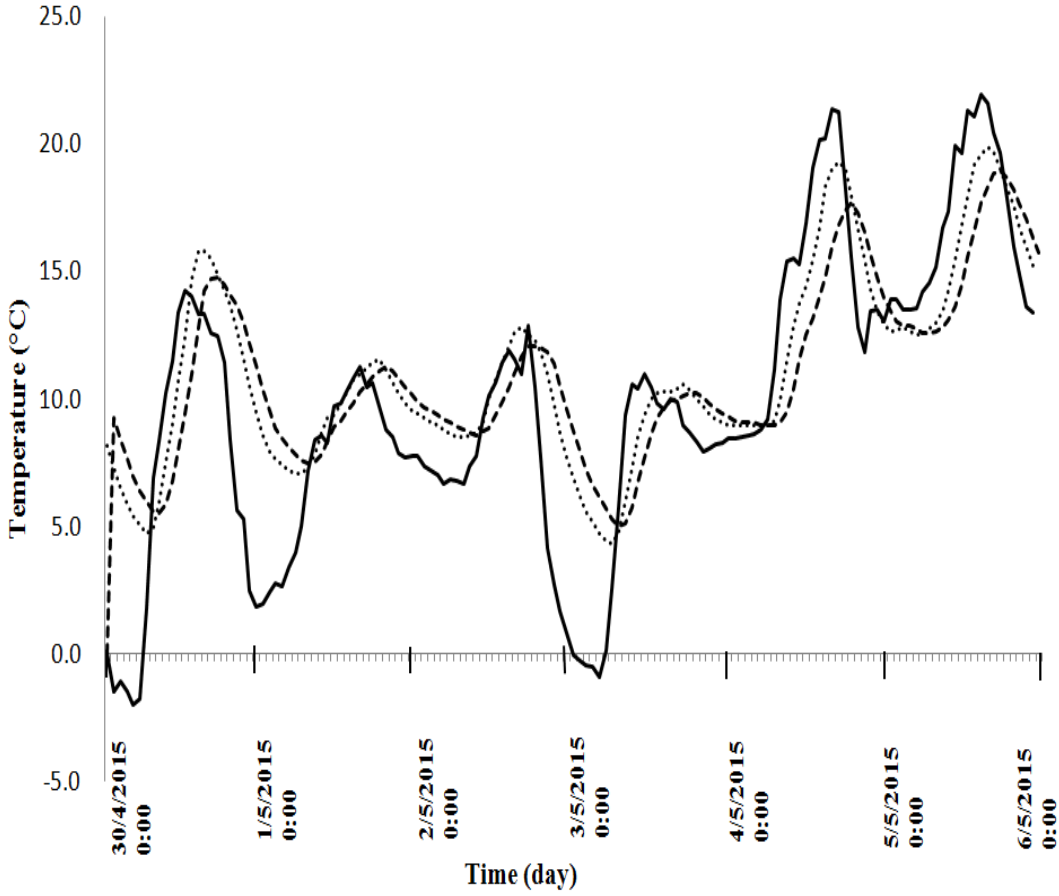
The temperature changes by the depth 0.05 m

The average air temperature in 2 meters above the ground was 14.7 ± 0.1 °C during the observed period (30th of April 2015 to 20th October 2015). The variation range was from -3.4 to 34.6 °C.

Ground average air temperature was in the same period 14.9 ± 0.1 °C. The variation range was from -4.2 to 35.8 °C. Variation range was approximately 2 °C greater than the air temperature in 2 m. But, it is very important to become aware that this temperature is without vegetation. The temperature from field trial is after corn vegetation. So, the differences between weather station and fertilizer management had to be not from different fertilizer (Zheng 1993).

The average soil temperature by D management was 16.1 ± 0.1 °C; variation range was from 3.4 to 31.3 °C. After mineral fertilizer application (CAN variant) the average temperature was equally 16.1 ± 0.1 °C, but the variation range was different: from 2.0 to 33.8 °C. Among the variances, there was a statistically significant difference at a significance level of 0.05. Maximum and minimum temperatures were lower after digestate application, and were approx. 1 hour delayed. The detail period of 30th to 6th June 2015 you can see at Figure 1.

Figure 1 Detail of the fluctuations of hourly temperature for the period of 30th April to 6th June 2015 at the depth 0.05 m

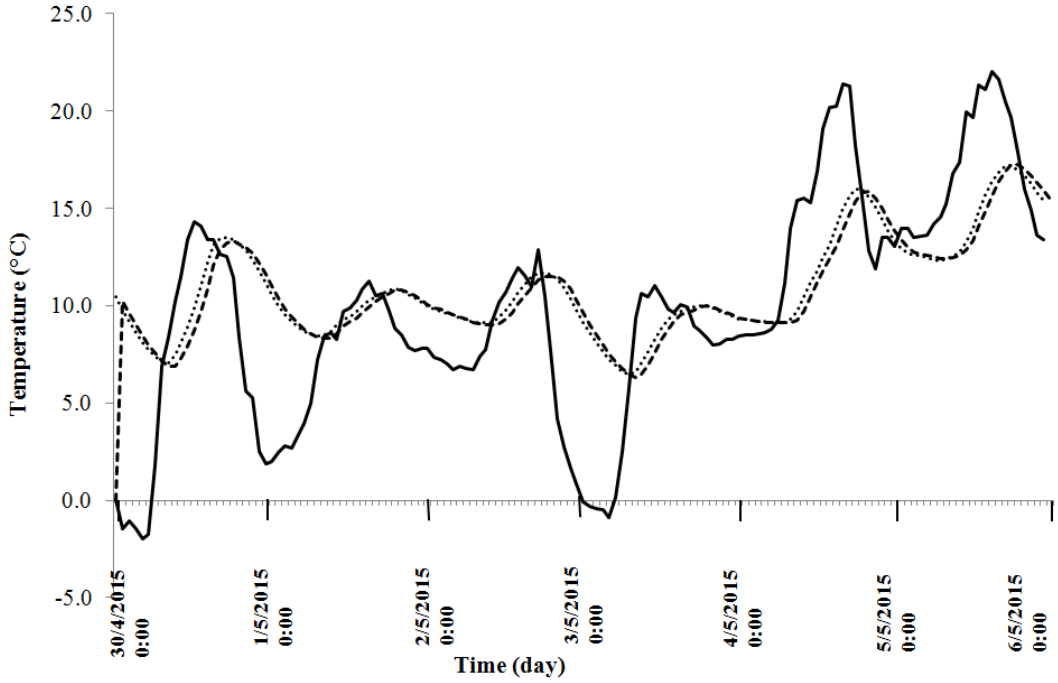


Legend: ····· CAN, - - - D, —— air temperature

The temperature changes by the depth 0.10 m

The average soil temperature by digestate application was 15.9 ± 0.1 °C with the variation range from 4.8 to 27.9 °C. For plot with applied mineral fertilizer the average temperature was equally 15.9 ± 0.1 °C, but variance range was wider again, from 4.3 to 28.5 °C. Among the variances, there was not the statistically significant difference at the significance level of 0.05 and even at the significance level of 0.01. Maximum and minimum temperatures were after digestate application lower and delayed too, but not so significant. The detail period of 30th to 6th June 2015 you can see at Figure 2.

Figure 2 Detail of the fluctuations of hourly temperature for the period of 30th April to 6th June 2015 at the depths 0.10 m

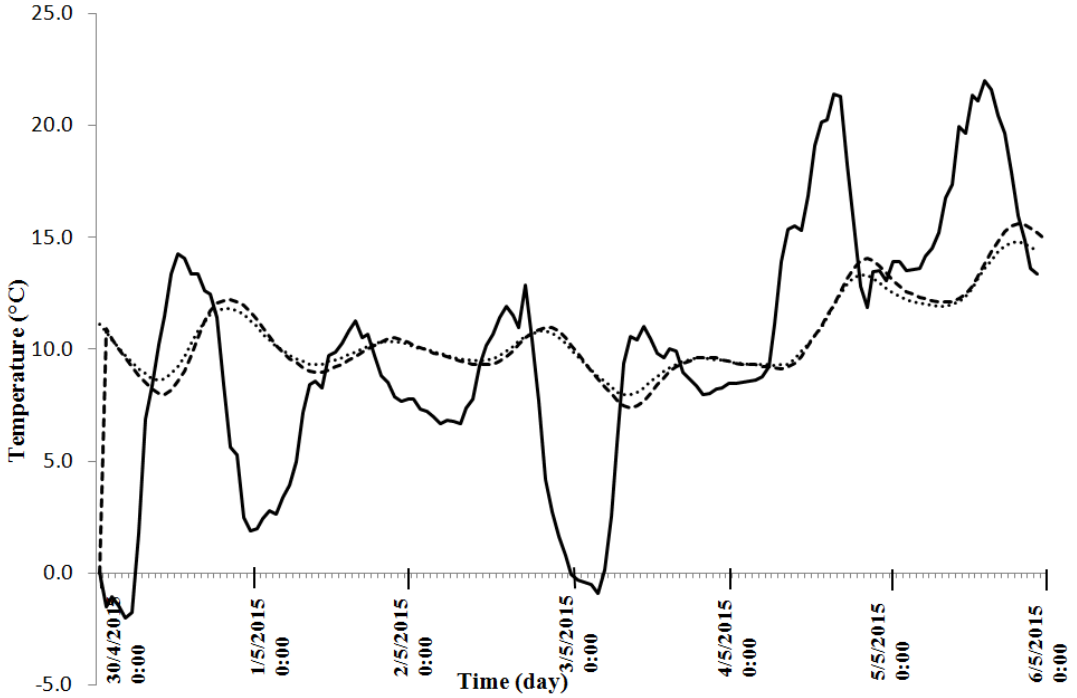


Legend: ····· CAN, - - - D, —— air temperature

The temperature changes by the depth 0.15 m

The average soil temperature by digestate application was 15.8 ± 0.1 °C with the variation range from 6.3 to 25.5 °C. The plot with mineral management was the average temperature same 15.8 ± 0.1 °C but with wider variation range: from 6.0 to 25.5 °C. Among the variances, there was not the statistically significant difference at the significance level of 0.05 but there existed at the significance level of 0.01. The detail period of 30th to 6th June 2015 you can see at Figure 3.

Figure 3 Detail of the fluctuations of hourly temperature for the period of 30th April to 6th June 2015 at the depths 0.15 m



Legend: x – time, axis y – temperature (°C) , ····· CAN, - - - D, —— air temperature

The temperature and fertilizer management

By mineral fertilizer with depth (0.05, 0.10 and 0.15 m) the kurtosis grew (-0.18; -0.54; -0.78). Normal distribution had (generally) the zero kurtosis. Negative kurtosis was indicated that the distribution was uniform, and the density curve was flatter than the normal distribution. The variance was not affected by improbable outlaying values. With the depth the smoothing of temperature extremes occurred. On the contrary, in the system the skewness decreased (0.31; 0.13; 0.01) for the same depth. The skewness of zero or approaching to zero indicated that the values of the random variable were uniformly distributed to the left and to the right from the mean value. The positive skewness signified that on the right side from the average appeared more remote values than on the left, and most values were close to the left from the average.

By digestate with increasing depth (0.05, 0.10 and 0.15 m) the kurtosis was growing again (-0.42; -0.61; -0.74). The situation was very similar to the applied mineral fertilizer, but the values of kurtosis were more significantly in the first two depths. This signified that at the depth of 0.10 m and 0.05 m the frequency distribution of the set of measured temperatures was more uniform and the density curve was more flat than within the normal distribution. In the system, again, the skewness decreased with increasing depth (0.21; 0.08; 0.01). Around 0.15 m depth it showed that random variable values were uniformly distributed to the left and right from the mean value. For comparison, we state the values of the skewness and the kurtosis for the air temperature at 2 m above the ground (kurtosis 0.01 and skewness 0.43) and surface soil temperatures (kurtosis 0.01 and skewness 0.45).

The problem is that there are not enough scientific papers with temperature problematic. In case those scientists engaged soil temperature, then mainly they generate models mainly (Lehnert 2014, Zheng et al. 1993). But, the influences of different properties, for example type of fertilizer applied, are not shadow.

In the average temperature was recorded the same trend as by Hora (2011). The average soil temperature by both fertilizer managements was higher than the average air temperature.

CONCLUSION

Not only the air temperature but also the soil temperature is very important for good plant growth. Both fertilizer variant was implying restrictions of the extreme temperatures (low, and also high). However, the digestate variant was slightly favourable. The transitions between the extremes of highs and lows were lower dissipation than with mineral application. Obtained results also indicated that soil temperature amplitudes in the depth of 0.05 m for the plots with applied mineral fertilizer were slightly bigger than within the digestated plots, and they came about one hour earlier. It could be due to different humidity between variants digestate and mineral ferlizer, which are in the extremes ranged in the order of a few percent (but soil moisture was not aim of this study).

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THE CHANGES OF PHYSICAL SOIL PROPERTIES DEPENDING ON APPLIED FERTILISER

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Abstract: Agricultural production is very demanding in nutrients. Fertilisers are applied to aid this compensation. The paper introduces the development of basic physical soil properties (bulk density, total porosity, field capacity and minimum air capacity), depending on applied fertilizers – digestate and mineral fertilizer - during the growing corn crop season. The soil properties were monitored from the beginning of the growing period in the year 2014 up to 2016, in two depths. The changes in physical soil properties were not the same during the years and the soil properties difference was found through depths. Significant change of physical properties was observed in the case of mineral fertilizer application.

Key Words: mineral fertilizer, digestate, bulk density, total porosity, field capacity, minimum air capacity

INTRODUCTION

The renewable energy resources are supported by the current world energy policy, especially European countries. Biogas plants are one of these. The by-product – digestate – is used as an agricultural fertilizer after the input material transformation by fermentation. The Czech legislation classifies digestate as an organic fertilizer (low no. 156/1998 collection).

Some scientific papers highlight the positive impact of organic fertilizer on different soil properties (Celik et al. 2004, Leroy et al. 2008). It is primarily the soil organic matter content and physical soil properties (aggregation, field capacity, hydraulic conductivity or bulk density) which have such a positive effect (Franzluebbers 2002, Quin et al. 2014, Zebarth et al. 1999).

Digestate, the semi-solid residue obtained after conversion of biomass by anaerobic–digestion, is considered as a vital source of organic matter and nutrients, especially because of nitrogen (Tiwary et al. 2015). Dealing with digestate is currently gaining great importance as it has certain amount of plant nutrients and organic matter. Also it can be used as organic fertilizer or soil conditioner (Massaccesi et al. 2013).

Despite these advantages, one major problem arises. Fertilizer with unknown potential negative consequences is applied to the soil. Long-term monitoring and in-depth analysis of soil fertility which is amended with digestates is required (Bachmann et al. 2014). The literature dealing with digestate is focused nutrients impact, fate of N and also soil organic matter (Bachmann 2014, Galvez et al. 2012, Grigatti et al. 2015, Walsh et al. 2012). Unfortunately there is lack of literature focused on the soil physical properties changes caused by digestate application (Beni et al. 2012).

In our investigation the field trial was aimed on observing of physical soil properties changes (bulk density, total porosity, field capacity and minimum air capacity) caused by two fertilizers (digestate and mineral fertilizer).

MATERIAL AND METHODS

Experimental area description

The field trial was established on the area of Research grassland station Vatín – Faculty of Agronomy, Mendel University in Brno, the Czech Republic in the spring of 2014. Vatín is located

around 60 km NW of Brno (exactly 49° 31' 01.5" N and 15° 58' 22.2" E). The elevation of the research station is 540 m above the sea level. Soil properties of the field trial are presented in Table 1.

Table 1 Selected soil properties on locality Vatín before establishment of field trial (0–0.20 m)

Soil properties	Value	Soil properties	Value
Particle size		Porosity (% vol.)	54.36
Clay (<2 μm, wt%)	11.69	Field capacity (% vol.)	29.34
Silt (50-2 μm, wt%)	34.87	Field capacity (% vol.)	39.87
Sand (2 000-50μm, wt%)	53.44	Minimum air capacity (% vol.)	14.49
		Oxidizable carbon (wt%)	1.8
Bulk density (kg/cm^3)	1197.12	pH/H_2O	5.79

Legend: Values shown for individual properties are averaged from all of the samplings taken from the research plot.

Experimental design

Two fertilizers were applied on the vegetation corn cover (*Zea mays*). One plot had 10 m^2. The fertilizer managements were: calcium ammonium nitrate (CAN) and digestate (D).

Amount of fertilizer which was applied was derived from the N content. Each fertilizer supplied 150 kg /ha of N. The CAN and D were applied in two dates during year 2014 and 2015 (60% of N dosed by spring and 40% of N was dosed during June). The digestate was from biogas plant Pikárec.

The crop residues were left on the soil surface and they were buried into the soil during cultivation by disking into the depth of 0.16 m. First dose of D and CAN was applied during the spring on cultivated parcel before the corn sowing. D was applied into the furrow and CAN on the parcel with subsequent covering by soil. Second dose was applied between the row spacing in surface.

The soil samples were taken in the spring of years 2014, 2015, 2016. The year 2014 represents zero or control variant (the samples were taken from the entire field trial from 2 depths – 0.03–0.07 m and 0.13–0.17 m. These samples described the root zone. Core soil samples were taken before application of fertilizers each year.

Laboratory

The core samples were sampled by Kopecky rollers (volume of 100 cm^3). The depths of samplings were 0.05 m and 0.15 m (the middle of rollers). Soil samples were taken in row spacing within 5 repetitions for each fertilizer management. Samples were processed according to the methodology of the Central Institute for Supervising and Testing in Agriculture (Zbíral 2002).

The laboratory results identified physical properties of soil like bulk density, total porosity, field capacity and minimum air capacity. These physical soil properties are used for evaluation of the soil physical condition in the Czech Republic.

Statistical analysis

Obtained data were processed by Shapiro-Wilk W test for the identification of data normal distribution. Results of each physical soil properties obtained from years of 2014–2016 were statistically compared. The depths were compared separately. The different values of physical soil properties were analyzed via ANOVA with interactions. Post-hoc tests were carried out on all ANOVAs using Tukey HSD test at the level $p < 0.05$ using the Statistica 12 software (StatSoft, USA).

RESULTS AND DISCUSSION

According to bulk density and total porosity changes, the changing effect during the years was possible to deduce (see Figure 1 and Figure 2). The development of reserved soil properties at the depth 0.05 and 0.15 m between seasons was observed by comparing the fertilizers management. Between 2014 and 2015 (the first year of the experiment), the decrease of bulk density and porosity increase (D variant application) was detected. The values of both described physical properties have changed during the second year of the experiment (i.e. 2015 and 2016). The development of CAN variant was exactly the opposite.

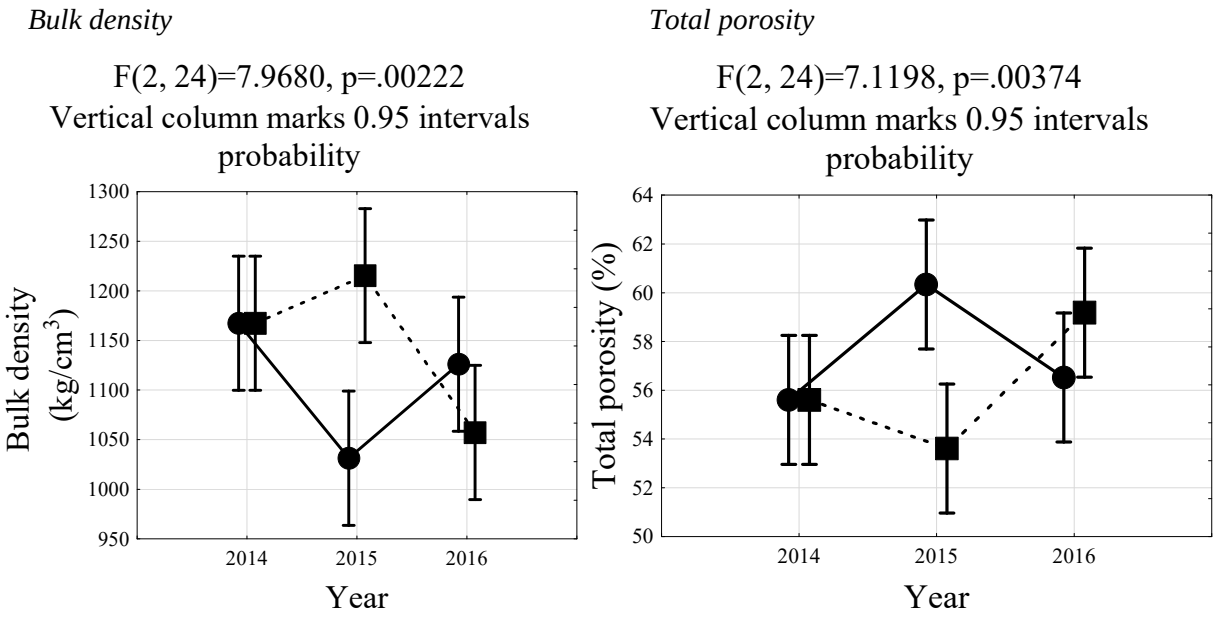
These changes could be attributed to adverse weather conditions. Among the sampling carried out in 2014 and 2015 rainfall of 928.2 mm was measured. Between sampling years 2015 and 2016, rainfall was only 541.2 mm. The data were obtained from meteorological station on Research grassland station Vatín. Bodner et al. (2013), Pires et al. (2008) and Silva et al. (2010) concluded that the cycle of drying and humidification of the soil environment may affect the seasonal changes in the soil physical properties. Schiettecatte (2005) mentions the influence of rainfall on the bulk density too.

The field capacity course was practically identical during the years. The lines at the first depth (see Figure 1) were almost superimposed. The difference was already between the fertilizers at the depth of 0.15 m (see Figure 2). All values were falling, but D application showed slower changing trend. The statistically significant differences between the variants in individual years were not found. However, the statistically significant difference was found between years 2014 and 2016 – at the depth of 0.05 m in both variants of fertilization and at the depth of 0.15 m in CAN variant. From the perspective of assessing the physical state was the application of mineral fertilizer considered as better one. The value found below the reference was in year 2016 and reached the level of 36%.

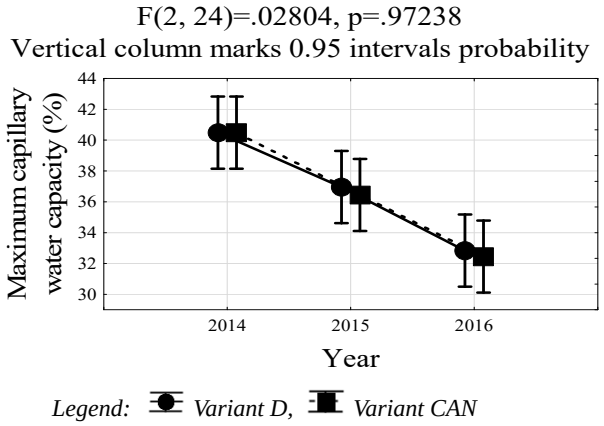
The situation with minimum air capacity was different. It was possible to monitor the increase in value over the years for both fertilizers at the depth 0.05 m (see Figure 1). But, the statistically significant difference between them was not found. However, the noticeable increase was observed only in the CAN variant at the depth of 0.15 m (see Figure 2). This property stagnated among the years 2014–2015. But values increased sharply between the years 2015–2016. However, the slight decline occurred for D in the last year. In year 2016, the statistically significant difference was found between the variants values.

The slower decline in maximum capillary field capacity and minimum air capacity at the depth 0.15 m the variant D could be caused to the depth of the digestate application. It was applied to approximate the depth of 0.15 m. Therefore, the material was accumulated there, if not decomposed by microorganisms.

Figure 1 The changes of soil physical properties during the years 2014–2016, depth 0.05 m



Field capacity



Minimum air capacity

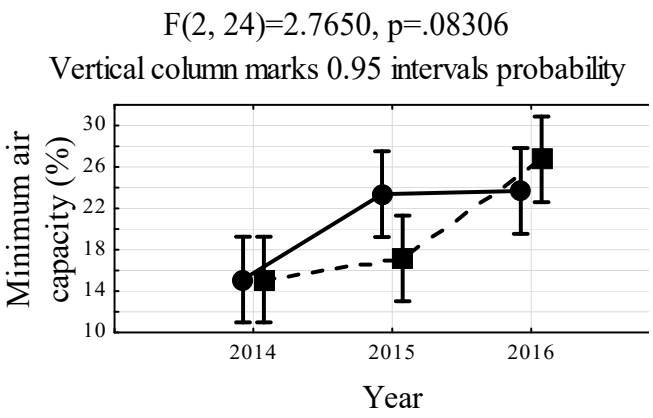
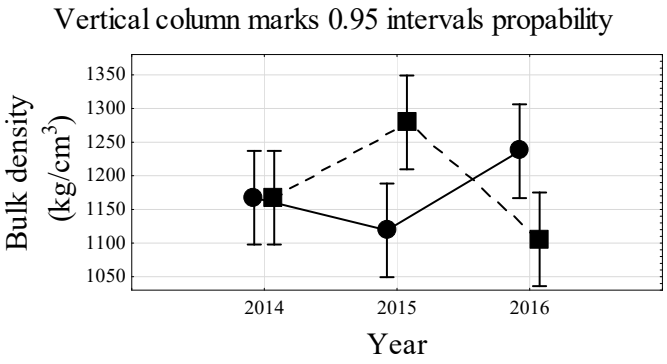
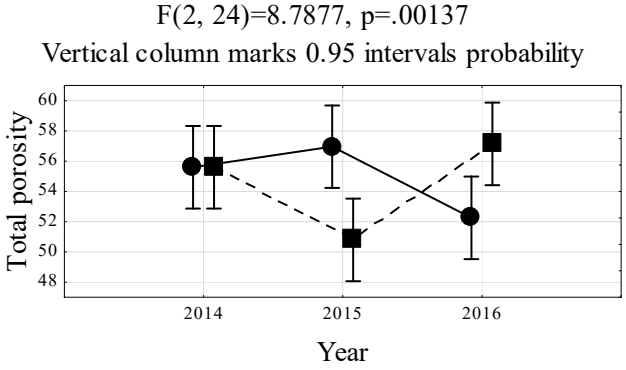


Figure 2 The changes of soil physical properties during years 2014–2016, the depth 0.15 m

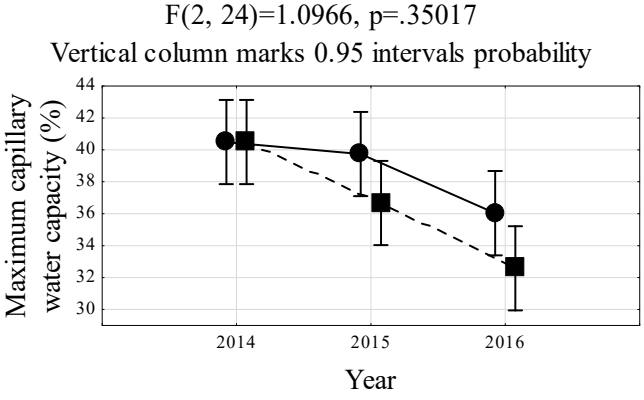
Bulk density



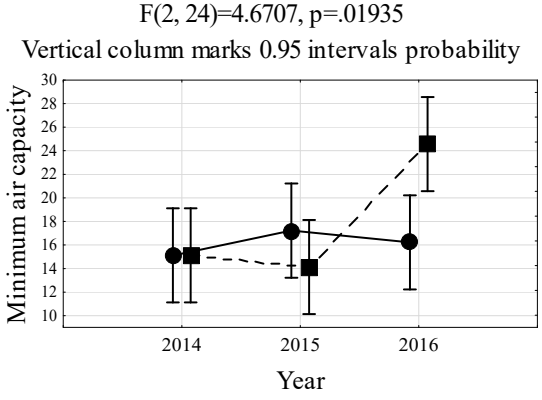
Total porosity



Field capacity



Minimum air capacity



Legend: Variant D, Variant CAN

The scientific articles agree that organic fertilizers should generally contribute to the good condition of the soil (Celik et al. 2004, Leroy et al. 2008, Xin et al. 2016), especially if it is compared with the effects of organic fertilizer and mineral fertilizer. However, the general opinion does not follow from our results. The Czech legislation based on the contents degradable substances and the amounts of nitrogen ranked the digestate as organic fertilizer (low no. 156/1998 collection). Some soil physical properties in comparison with mineral fertilizer were deteriorated by our field trial.

CONCLUSION

We cannot derive binding conclusion from the results for the time being. However, based on the results obtained until now, we will need to monitor the influence of digestate during adverse weather conditions, especially in the absence of sufficiently amount rainfall which is in our climatic conditions more and more often. Two years digestate application led to the deterioration of the monitored soil physical properties, compared to application of mineral fertilizer.

ACKNOWLEDGEMENTS

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PHENOLOGICAL PHASES AND METEOROLOGICAL ELEMENTS INTERACTION

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Abstract: The paper deals with evaluation of phenological data series of sugar beet, sum of daily average active air temperature (5, 10 and 15 °C) and length of the vegetation season. The average value of phenological phases onset and the beginning of agronomic practices of two long-term periods (1931–1960, 1960–1990) were compared. A prolongation of the interval between sowing and emergence was proved. The length of growing season was different at analyzed stations. Sowing of sugar beet is on average performed in earlier time. We are not able to determine significant trend. This may be due to high-performance agronomy machines that are used in agriculture. A thirty-year trend analysis (1961–1990) of sugar beet phenological phases shows significant changes in phenological phases sowing (20 days) and emergence (14 days). Significant trend was found in case of phenological phases sowing ($r = 0.510$) and emergence ($r = 0.445$). Active sum of daily average temperature above 5, 10 and 15 °C and length of the vegetation season which is given by values of the daily average values of air temperature exceeding given threshold (5, 10, 15 °C) are addressed in this work. The average values of four long-term periods (1931–1960, 1961–1990, 2021–2050, and 2071–2100) were compared to one another. The comparison of sum of active air temperature and vegetation season length proved their gradual increase and prolongation from 1931–1961 to 2071–2100.

Key Words: phenology, vegetation season, sugar beet, growing season, Czech Republic

INTRODUCTION

The biological temperature minimum, that is the value indicating the beginning or the ending of growth and limiting of metabolism, is important in the life of plants. Vegetation of moderate climate is generally affected by the temperature of 5 °C. Temperature suitable for the period from sowing to heading varies from 9 to 16 °C. In case that temperature exceeds 20–26 °C, the growth is being retarded (Petr 1987).

The onset of individual developmental phases is driven by temperature expressed as the sum of active or effective temperature. The active temperature is defined as the temperature exceeding the biological minimum which is the value of the decline of the ability to vegetate. The sum of all daily average temperature values exceeding the biological minimum (or the threshold temperature) is called the sum of active temperature (SAT). Daily average values of 0, 5, 10, and 15 °C are considered as the temperature thresholds. The effective temperature then means also the sum of the daily/hourly average temperature exceeding the threshold when the value of the threshold is subtracted.

The on-going increase in temperature which is, according to Lobell and Field (2007), put down to the climate change, will also cause the increase in the sum of the effective temperature that would induce shortening of phenological phases, which has unfavourable impact on the yield. Research confirms that global warming has an impact on the timing of phenological phases (Hudson 2010).

An assessment of phenological data from the period 1940–2008 detected an earlier onset of the phenophase beginning of flowering in case of apricot by about 13 days, i.e. 2 days per decade under the climatic conditions of the Czech Republic (Středa et al. 2009).

Sobíšek (1993) states, that we can distinguish the great vegetation season, the main vegetation season and the vegetation summer. The great vegetation season determined by the beginning and the

end of the season with the daily average temperature above 5 °C (VO 5). The main vegetation season is defined temperature of 10 °C (VO 10), and the vegetation summer with temperature of 15 °C (VO 15).

The vegetation season could be prolonged to up to 21 days by the year 2020 and up to one month by the year 2050 because of the temperature increase. The analysis of the data from International Phenological Gardens (IPG) from 1969–1998 proved 8 days acceleration of the beginning of the vegetation season in Europe (Šiška and Takáč 2008). The observed trend corresponds with the changes of the air temperature and is considered to be a consequence of the global warming. According to Chmielewski and Rötzer (Chmielewski and Rötzer 2001), the increase in the average January and February temperatures (by 1 °C) causes a 7 days earlier beginning of the vegetation season.

MATERIAL AND METHODS

Analysis of selected phenological phases of the sugar beet

The average value of phenological phases onset and the beginning of agronomic practices of two long-term periods (1931–1960, 1960–1990) have been compared. Data from the publication Kurpelová et al. (1975) were used for a long-term retrospective comparison (1931–1960). These phenological stations were analyzed: Tvrdonice (162 MASL), Branišovice (170 MASL), Hodonín (190 MASL) and Napajedla (240 MASL). These stations are situated in South Moravia, Czech Republice, Central Europe. Apart from that, the trend analysis of individual phenological phases and practices onset during 1961–1990 was carried out. Data for the period 1961–1990 were obtained from direct observations of Czech Hydrometeorological Institute (CHMI). Observed phenological phases and their description are given in Table 1.

Table 1 Description of the phenological phases (Pifflová 1956)

Phenological phases	Abbreviation	Description	Phenological phases (PP)/Agronomic practices (AP)
Sowing	SD	The seed are worked into the field.	AP
Emergency	EM	Hypocotyl is emerge above soil surface. The crop beginning to create visible rows.	PP
First pair of true leaves	FPTL	Plant has the first pair of true leaves.	PP
Harvest	HD	The day when the harvest begins.	AP

Vegetation season (VS)

Characteristics of VS:

- Sum of active temperature exceeding 5, 10, 15 °C (SAT 5, SAT 10 a SAT 15).
- Length of the great and the main vegetation season and the length of the vegetation summer (VS 5, VS 10 a VS 15).

SATs and VSs were evaluated during four long-term periods (1931–1960, 1961–1990, 2021–2050, and 2071–2100). The evaluation is based on „technical data series“ (TDS) that are created in the CHMI. TDS represents a fully homogenized database of daily values of climatic elements (such as mean, minimum and maximum air temperature, precipitation total, mean air humidity etc.) in a 10 km grid (i.e. 787 points) for the entire area of the Czech Republic from 1961 up to the present. TDS is based on the data from standard measurement on the climatologic station network and deals with the regional climatic model (RCM) ALADIN – Climate/CZ which is driven by the global climatic model ARPEGE – Climate (Skalák et al. 2008).

Moreover, future climate conditions by up to the year 2100 can be predicted by means of the “scenario data series” (SDS) created in CHMI. SDS deals also with RCM ALADIN – Climate/CZ, and besides that, with the emissions scenario A1B (according to IPPC). The SDS network corresponds with the TDS network (i.e. 787 points in a 10 km grid).

The grid creation and all data processing including TDR and SDS have been implemented by the Pro ClimDB software (Štěpánek 2007).

The reference point for the SAT and VS evaluation is the grid point situated in the town of Břeclav at the altitude of 197 MASL. For a longer retrospective comparison (1931–1960), data from the publication „Agroclimatic conditions“ (Kurpelová et al. 1975) for the Židlochovice station in 185 MASL were employed. The distance between the Židlochovice station and the town of Břeclav is about 30 km.

RESULTS AND DISCUSSION

Analysis of the onset of phenological phases

Growing season (GS) is interval between SD and HD. This interval is the same at Tvrdonice in period of 1931–1960 and 1961–1990 (GS is 183 days–Figure 1). This trend is the same at station Napajedla, where GS is 177 days in both periods. On the one hand, prolongation of GS was found out at the station Branišovice, on the other hand shortening of GS was found out at station Hodonín. Prolongation of interval SD–EM was determined for all analyzed stations (Table 2). Interval between EM and HD is getting shorter at station Napajedla, Tvrdonice and Hodonín. At all stations occurs earlier sowing of sugar beet in the period 1961–1990 (Table 2). Harvesting was carried out earlier than in the period 1931–1960.

Figure 1 The onset of phenological phases of sugar beet, Tvrdonice locality

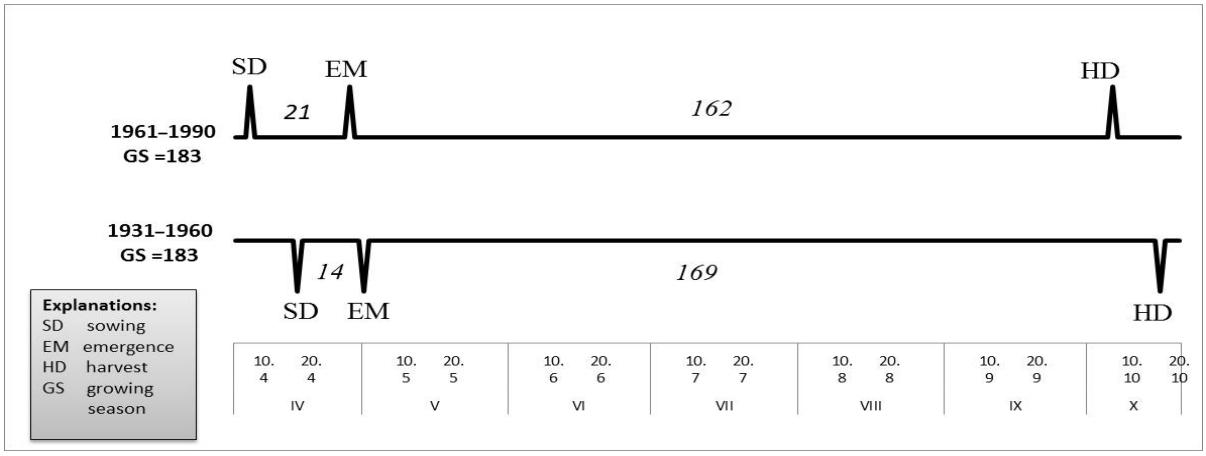
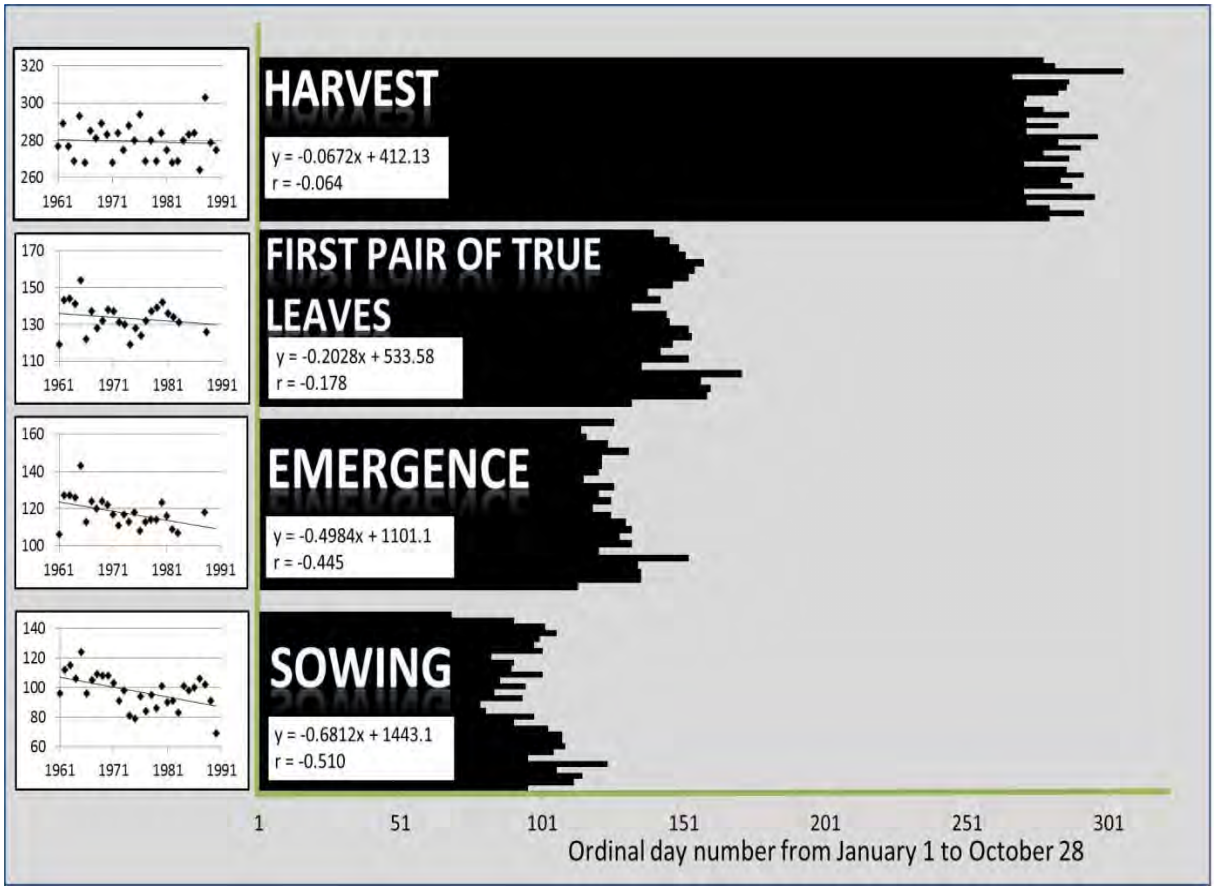


Table 2 The onset of phenological phases of sugar beet for station Branišovice, Hodonín, Napajedla and Tvrdonice

Locality	Period	SD	EM	HD	SD–EM	EM–HD	GS
Branišovice	1931–1960	104	121	283	17	163	180
	Date	14. IV	1.V	10.X			
	1961–1990	97	115	280	18	166	183
	Date	7. IV	25. IV	7.X			
Hodonín	1931–1960	107	121	289	14	169	183
	Date	17.IV	1.V	16.X			
	1961–1990	104	124	275	20	152	172
	Date	14.IV	4.V	2.X			
Napajedla	1931–1960	105	120	281	15	165	177
	Date	15.IV	30.IV	8.X			
	1961–1990	101	119	277	18	159	177
	Date	11.V	29.IV	4.X			
Tvrdonice	1931–1960	107	121	289	14	169	183
	Date	17.IV	1.V	16.X			
	1961–1990	97	118	279	21	162	183
	Date	7.IV	28.IV	6.X			

The trend analysis of phenological phases and practices onset proved their significant earlier shift (20 days earlier of SD, 14 days earlier of EM, 6 days of FPTL and 2 days of HD) in 1961–1990 (Figure 2). It might be explained by the climate change but rather due to planting of new sugar beet varieties. These new varieties are more resistant to weather factors, diseases etc. Significant trend in onset of agronomic practices of SD (r = 0.510) and phenological phases of EM (r = 0.445) was found out (Figure 2).

Figure 2 The onset of phenological phases of sugar beet, Tvrdonice locality



Vegetation season

The comparison of three long-term periods proved gradual increase of SAT 5, 10, 15 in all months (January to December), in all seasons (DJF: December, January, February, MAM: March, April, May, JJA: June, July, August, SON: September, October, November) and also in the whole year (YEAR) see Figure 3. The SAT 5 of 1961–1990 was by about 204 °C higher in comparison with 1931–1961. Its further 644 °C or 1250 °C increase has been predicted for 2021–2050 or 2070–2100 respectively (again, compared with the period of 1931–1961). Středová and Středa (2015) determinate increase of temperature sum above 10 degrees C and annual air temperature in 1961–2010 compared to the mean of 1901–1950 probably due to climate the mean of 1901–1950 probably due to climate change in the Central Europe.

An analogous rise was also proven in case of SAT 10 (by 193 °C or 597 °C or 1210 °C higher values in 1961–1990, 2021–2050 and 2070–2100 respectively) and SAT 15 (75 °C or 416 °C or 1053 °C higher values in 1961–1990 or 2021–2050 or 2070–2100 respectively).

Similarly, Figure 3 presents the prolongation of all VSs in comparison with 1931–1961. VS 5 was longer by three days, VS 10 by nine days and VS 15 by twelve days. The change will probably be confirmed in the future. According to the predictions, VS 5 will be prolonged by about 14 days, VS 10 by about 27 days and VS 15 by about 15 days in 2021–2050. The values for 2071–2100 are as follows: VS 5 46, VS 10 56, and VS 15 44 days.

Figure 3 Comparison of SAT 5, 10, 15 for three or four long–term periods, Břeclav locality

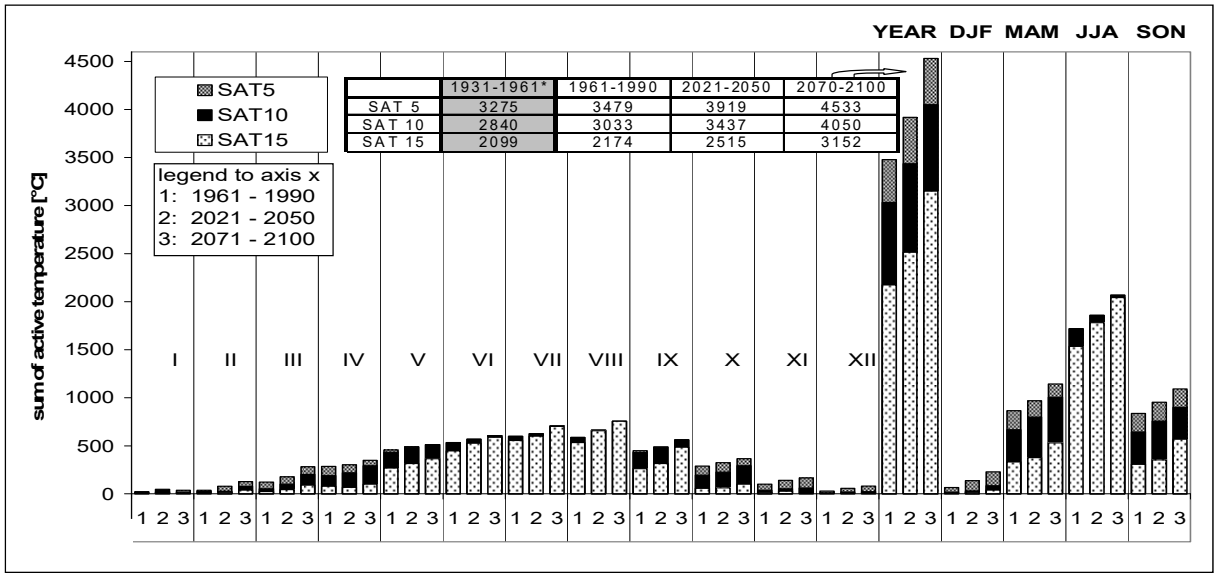
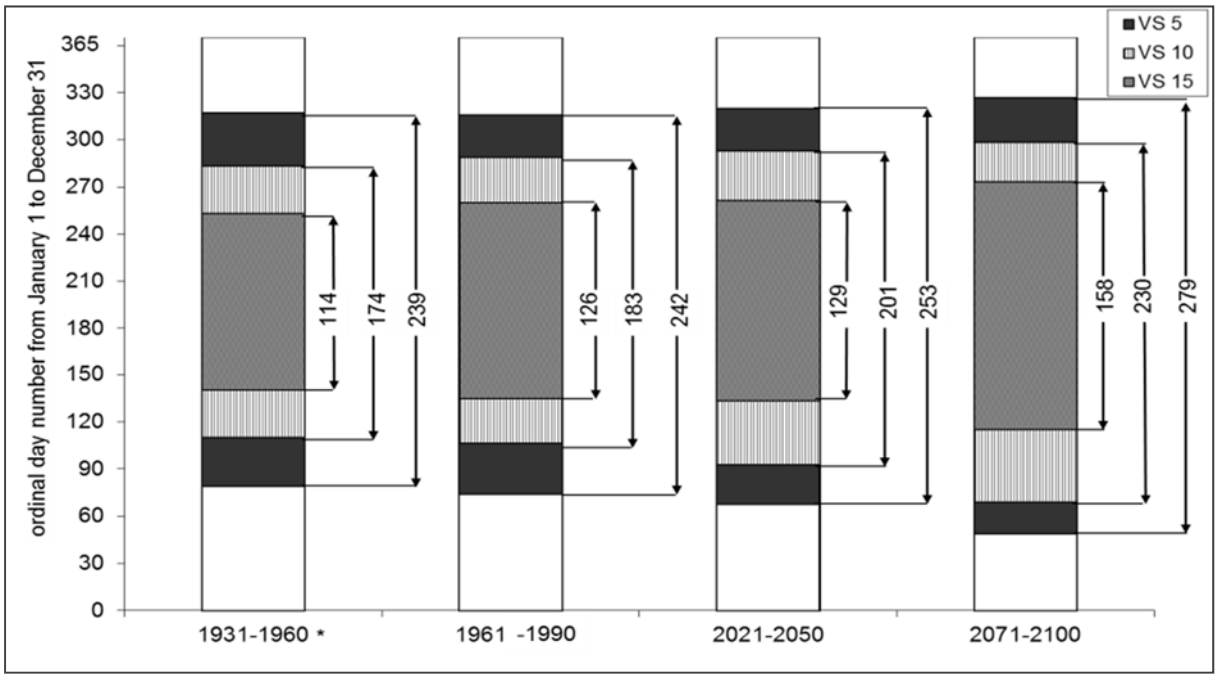


Figure 4 Comparison of the length, the beginning and the ending of VS 5, 10, 15 for four long-term periods, Břeclav locality



CONCLUSION

The comparison of SAT and VS length proved their gradual increase and prolongation from 1931–1961 to 2071–2100.

The findings were subsequently confronted with the results of the phenological phases onset of sugar beet based on the phenological data from CHMI. There wasn't found out evident trend of GS. In the period 1961–1990 sowing was carried out earlier at all stations. The analyses of phenological phases onset confirmed the prolongation of the interval between SD and EM. Interval between EM and HD is shorter at station Napajedla, Tvrdonice and Hodonín. A thirty-year trend analysis (1961–1990) of sugar beet phenological phases shows significant changes in phenological phases SD (20 days earlier) and EM (14 days earlier). Significant trend was found out in term of agronomic practices of SD ($r = 0.510$) and phenological phases of EM ($r = 0.445$).

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RETROSPECTIVE ANALYSIS OF THE PHENOLOGICAL PHASES OF SPRING BARLEY AND ITS IMPACT ON SOIL EROSION

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Abstract: The paper deals with analysis of phenological data of spring barley for the Tečovice station and Luhačovice station. This analysis is carried out for the period 1931–1960 and 1961–1990. When comparing these two periods growing season getting longer about 3 days in 1961–1990 in station of Tečovice. In this period a prolongation of the sowing term was found out (4 days). A shortening of the interval between emergency and heading (9 days), and a prolongation of the interval between heading (9 days) and harvest was also proved. Statistically significant linear trend was found out only for agronomic practice of harvest (r = 0.503) in station of Tečovice. The trend analysis of phenophases and practices onset proved a significant 14 days delay of the harvest beginning in the frame of a 30 years period 1961–1990. The period 1961–1990 was divided into decades in the paper. Subsequently the trend analysis was perfomed. Statistically significant linear trend was found out for phenophases heading in the third decade (r = 0.754) and for agronomic practice in the second decade (r = 0.783) in the Tečovice station. Significant linear trend was discovered in the second decade for all monitored phenophases in Luhačovice station. After calculation the cover and management factor on the base of Universal Soil Loss Equation (USLE) was performed for extreme years. Value of the cover and management factor in Tečovice station was 0.1474 in 1974 and 0.1886 in 1964. The protective effect of vegetation in Luhačovice station was 0.1327 in 1974 and 0.2411 in 1983.

Key Words: phenophases, C factor, spring barley, Czech Republic

INTRODUCTION

Phenological database is very valuable source of data. This data is used for prediction of pathogens (Středa et al. 2013), for application of preparations to plants protection (Krédl et al. 2012), in breeding practices (Heřmanská et al. 2015), for optimization of irrigations during critical phases of crops growth and monitoring of occurrence of dry seasons (Kohut et al. 2014), for medical purposes (determining of pollen allergens) and last but not least for bioindication and classification of climate change (Škvarenina et al. 2009).

The paper deals with relationship between phenological phases of spring barley and their effect on soil erosion. Spring barley is narrow-row crop. It isn't erosion risk plant, though erosion is found out for particular cereal. Soil erosion threatens productive and nonproductive function of soil (Janeček et al. 2012). Erosion is a significant global problem. The annual loss of agricultural land is three million hectares per year (Janeček 2002). The Czech Republic is threatened by water erosion over 50 percent of arable lands of areas (Novotný et al. 2014). Erosion processes include soil disturbance, carrying the soil particles and store them. This is caused by natural processes for example rain, wind, gravity, etc. or anthropogenic activities for example tillage, harvesting etc. (Boardman and Poesen 2006).

The calculation of soil loss is carried out based on formula of long-term soil loss according to Wischmeier and Smith (Wischmeier and Smith 1978). The value of the cover and management factor (C factor) enters into this formula. The C factor dependents on vegetation cover and agronomic management. Soil cover plays an important role for soil protection from the erosion. The cover of grass has a significant hydrological function. This vegetation cover has a high rate of infiltration of precipitation (> 50 mm per hour) and also is very resistant to soil erosion (Boardman and Poesen 2006).

The C factor can be calculated as the ratio of soil loss from estate with individual crop to soil loss from aerated black waste land. The value this ratio is higher, the erosion effect is lesser. The C factor for cereals ranges from 0.04 to 0.75 (Witschmaier and Smith 1978, Janeček et al. 2012). The erosive efficiency rain is taken into account for calculation of the protective effect of vegetation. The most erosion dangerous rains occur in the summer months (about 90 percent). If 60 percent of land become covered with spring barley, it's necessary to protect land from erosion (Klima et al. 2016).

MATERIAL AND METHODS

Analysis of the spring barley phenophases

The paper deal with phenological data of 1931–1960 and 1961–1990. Data for the period 1961–1990 were obtained from direct observations of Czech Hydrometeorological Institute (CHMI). Data for the period 1931–1960 were taken from the publication „Agroclimatic conditions of ČSSR" by Kurpelová et al. (1975). Detailed data analysis was carried out for the station Tečovice (260 MASL). This station was compared with the station Luhačovice (285 MASL). These stations were the nearest according to the phenological net of stations. Distance between these stations was 20 km. Tečovice and Luhačovice are situated in Zlín Region, Czech Republic, Central Europe.

In this paper detailed analysis of the onset of selected phenophases/agronomic operations (sowing SD, emergency EM, heading HE and harvest HD) was performed for the periods 1931–1960 and 1961–1990. And then the two periods and length GS were also compared.

Subsequent trends analysis if phenophases SD, EM, TI, HE, FR and HD was carried out for period 1961–1990. Descriptions of these phenological phases (PP) and agronomic practices (AP) are stated below (Table 1). The trend analysis was performed for the decades (1961–1970, 1971–1980 a 1981–1990) and for 30-year period (1961–1990) for the Tečovice station and Luhačovice station (Table 3). The paper deals with relationships between SD–EM, SD–TI, SD–HE, SD–FR a SD–HD under the terms of trends analysis. The results of these two stations were also compared.

Table 1 Monitored phenophases and agronomic practices and their description (Pifflová et al. 1956)

Phenological phases (PP)	Abbreviation	Description	PP/AP
Sowing	SD	The seeds are worked into the field.	AP
Emergency	EM	The first aboveground parts of plants could be observed in the field. The crop is beginning to create visible rows. Young plants of barley are yellow-green.	PP
Tillering	TI	More than a half of all plants have been tillering already. In the axil of the lower leaf, an approximately 1 cm long peak of the coiled leaf has developed. The tillering begins several days after the third leaf is uncoiled.	PP
Heading	HE	A half of an ear is taken out from the sheath of the highest leaf.	PP
Fully ripening	FR	More than a half of all plants are in the following state: The grains are dry, heavy and hard to break. The grain matter is rough, mealy or glassy.	PP
Harvest	HD	The day when the harvest begins.	AP

Soil erosion – C factor calculation

C factor was calculated for spring barley for two extreme years from the viewpoint of GS (with the longest and the shortest GS) for Tečovice and Luhačovice stations (%R × C, and this value divided by 100). The year 1974 was a year with the longest GS in both stations. And 1964 was a year with the shortest GS in Tečovice station and 1983 was a year with the shortest GS in Luhačovice station. The calculation of C factor was performed according to Janeček (Janeček et al. 2012). Example of calculation is stated in Table 6. Values of C factor is presented in Table 7. C factor values are stated below for individual periods (Table 2). Percentage distribution of erosion dangerous rain in the Czech Republic is used for calculation (Table 3). The dates of agronomic practices (SD and HD) are necessary for C factor

calculation. This data is possible to adopt from phenological observation. Table 2 Value C factor (Janeček et al. 2012).

Table 2 Value C factor (Janeček et al. 2012)

Period	Soil conservation effect	C factor
1	Stubble and rough furrow.	0.65
2	From preparing the land for sowing within one month after sowing.	0.80
3	During the second month after sowing.	0.65
4	To harvest	0.30
5	From harvest to seedbed preparation subsequent crops	0.70

Table 3 The percentage distribution of erosion dangerous rains (Janeček et al. 2012)

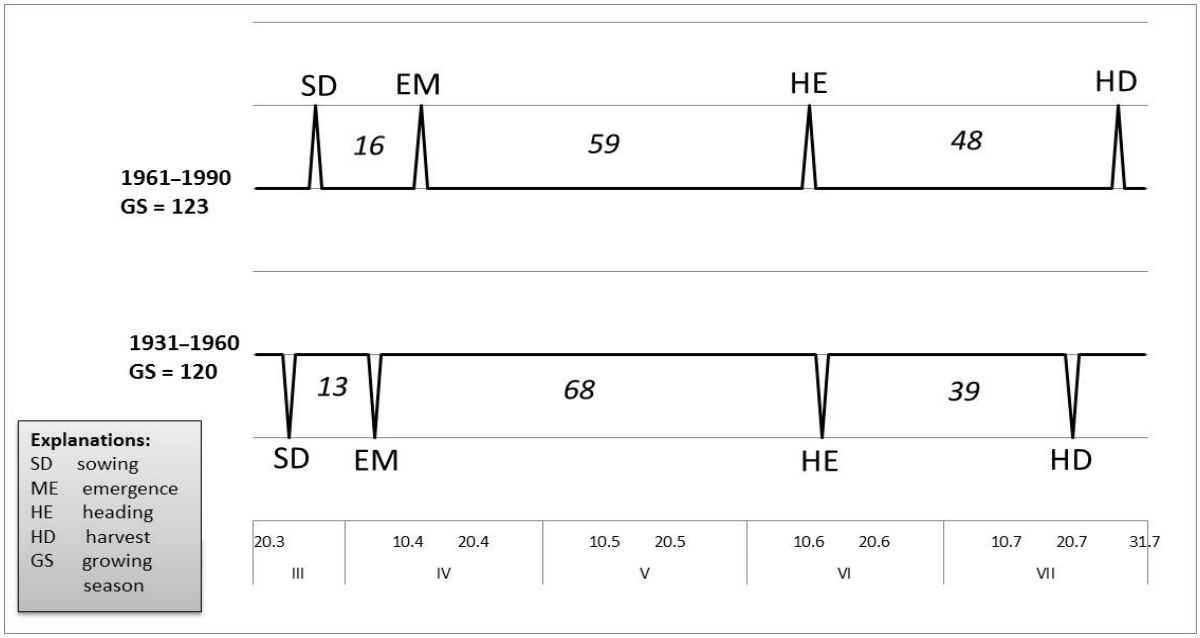
Month	IV	V	VI	VII	VIII	IX	X
% R factor	0.5	7	26.8	32.2	31.1	2	0.4

RESULTS AND DISCUSSION

Phenological analysis

The evaluation of the average onset of phenophases (EM, HE) and the beginning of agronomic practices (SD, HD) proved prolongation of the interval between SD and HD (GS) from 1931–1960 to 1961–1990.When comparing these two periods, GS was lengthened (3 days) in 1961–1990. In this period sowing of spring barley is prolongation about 4 days. A shortening of the interval between ME and HE (9 days) was proved and a prolongation of the interval between ME and HD (9 days) was also proved (Figure 1).

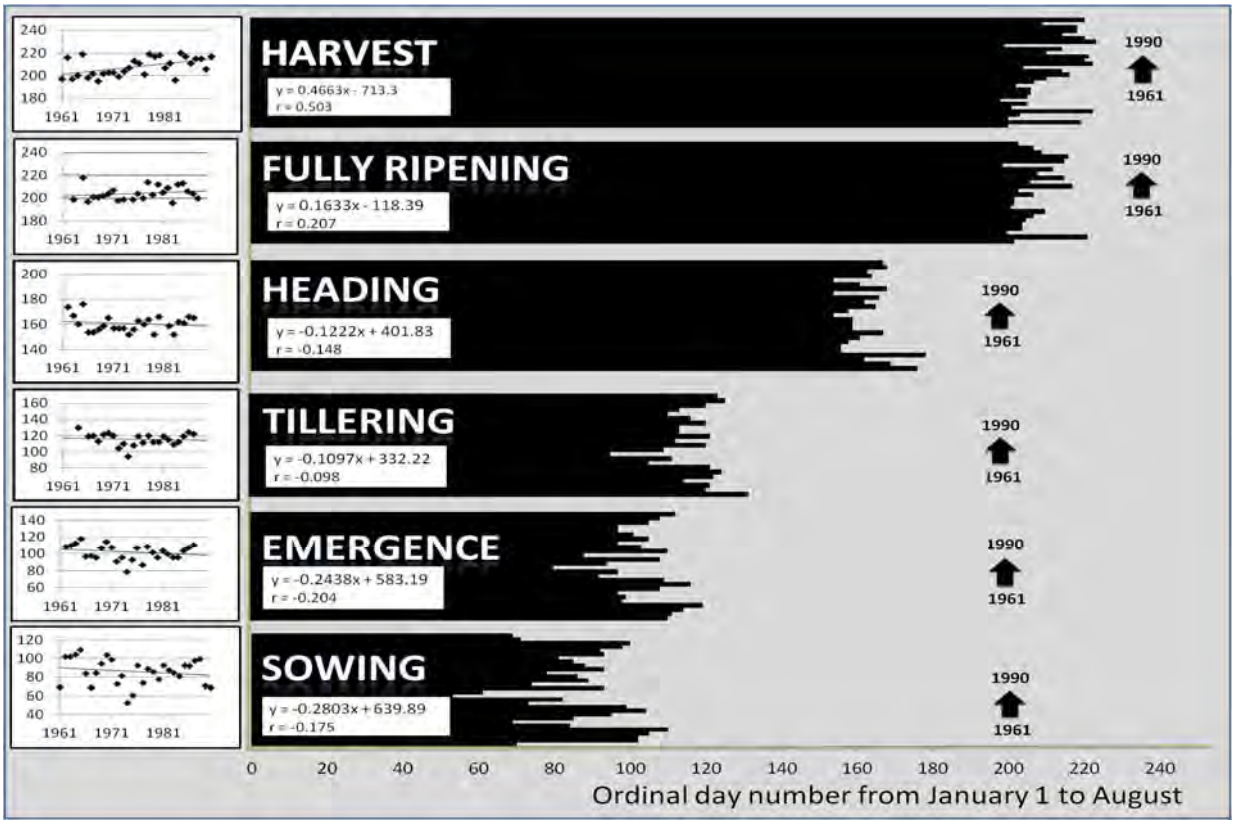
Figure 1 Analysis of the onset of phenophases for the periods 1931–1960 and 1961–1990, Tečovice locality



In trend analysis statistically significant linear trend was found out only for agronomic practice HD (r = 0.503) for period 1961–1990. The statistically significant linear trend was not proved for another agronomic practice and phenophases (Figure 2): SD (r = -0.175), EM (r = -0.204), TI (r = -0.098), HE (r = -0.148) and FR (r = 0.207).

The trend analysis of phenophases and practices onset proved a significant 14 days delay of the HD beginning in the frame of a 30 years period 1961–1990 (Figure 2).

Figure 2 The trend analysis (1961–1990) of phenophases and practices onset, Tečovice locality



Statistically significant linear trend was proved for phenophase HE in the third decade (r = 0.754) and for agronomic practice HD in the second decade (r = 0.783) in Tečovice station. Significant linear trend was also proved for all monitored phenophases in the second period in Luhačovice station (Table 3). The biggest movement of phenophases and agronomic practices was detected in the third decade in Tečovice station (Table 5 and 3). The sowing was carried out about 11 days earlier, but date of emergency was lengthened about 14 days in this period (1961–1990). Tillering of spring barley was delayed about 12 days to later date. A prolongation of phenophases HD (18 days) was also proved. The significant period was proved in the second decade (1961–1990) in Luhačovice station (Table 3). Important changes in movement of individual phenophases were identified in this period (1961–1990). The later onset of these phenophases: SD (34 days), EM (31 days), TI (28 days), FR (18 days) and HD (20–day) was proved. The earlier onset of phenophases HE (18 days) was also discovered.

Table 3 Analysis of the phenophases of the station Tečovice and Luhačovice

Station	PP	SD		EM		TI		HE		FR		HD	
	Period	r	Days	r	Days	r	Days	r	Days	r	Days	r	Days
Tečovice	1	0.047	2	0.217	-6	0.450	-10	0.497	-14	0.088	-2	0.156	-4
	2	0.041	2	0.135	4	0.220	5	0.429	6	0.498	8	**0.783**	17
	3	0.364	-11	0.593	14	0.529	12	**0.754**	18	0.146	-3	0.335	7
	61-90	0.175	-8	0.204	-7	0.097	-3	0.148	-4	0.207	5	**0.502**	5
Luhačovice	1	0.117	5	0.057	-9	0.033	1	0.302	5	0.323	-7	0.119	-2
	2	**0.644**	34	**0.683**	31	**0.651**	28	**0.788**	-18	**0.707**	18	**0.677**	20
	3	0.257	-10	0.317	-10	0.485	18	0.346	5	0.207	-4	0.040	1
	61-90	0.103	5	0.074	-8	0.219	10	0.199	4	0.310	7	0.357	12

Statistically significant linear trend was found out for relationships between SD–HD (r = 0.424) in Tečovice station. A prolongation of the interval between SD and HD (about 22 days) was found out (Table 4).

Table 4 Analysis of the relationshpis between selected phenophases

Station	PP	SD-EM		SD-TI		SD-HE		SD-FR		SD-HD	
	Period	r	Days	r	Days	r	Days	r	Days	r	Days
Tečovice	1	0.372	8	0.283	13	0.022	1	0.139	6	0.156	-6
	2	0.109	2	0.151	3	0.113	4	0.399	11	0.344	15
	3	0.511	4	0.145	2	0.117	-3	0.489	-17	0.509	18
	61-90	0.160	4	0.171	-6	0.202	8	0.140	6	**0.424**	22
Luhačovice	1	0.030	-4	0.136	-4	0.272	-10	0.042	-2	0.185	-7
	2	0.239	-3	0.361	-6	0.435	-16	0.123	-5	0.352	14
	3	0.165	-2	0.251	-7	0.304	-9	0.206	9	0.283	11
	61-90	0.212	-17	0.210	-6	0.017	1	0.067	-3	0.146	7

Table 5 Legend of phenological data for Table 3, 4

1	1961–1970	2	1971–1980	3	1981–1990	-	Shortening the interval
r	Indicates statistical significance linear trend (statistically significant trends in bold)						

Soil erosion in crop of spring barley

Table 6 Tečovice 1974 C factor

Tečovice (1974)	Sowing	22.2.	Harvest	25.7.
Month	% R	Period	C	%R.C
IV.	0.37	3	0.450	0.1665
	0.13	4	0.080	0.0104
V.	7.00	4	0.080	0.5600
VI.	26.80	4	0.080	2.1440
VII.	26.83	4	0.080	2.1464
	5.36	5	0.250	1.3400
VIII.	31.10	5	0.250	7.7750
IX.	2.00	5	0.250	0.5000
X.	0.40	5	0.250	0.1000
C factor			**0.1474**	

Table 7 Values C factor (Tečovice, Luhačovice)

Tečovice	1974	1964		Luhačovice	1974	1983
	0.1474	**0.1886**			**0.1327**	**0.2411**

GS and date of sowing have the essential influence for value of C factor. The sooner the spring barley is sowed, the value of C factor is lower. The C factor is influenced date of harvest. The sowing and emergency of plants of spring barley are characterized as a crisis period due to soil erosion. In this phenophases the soil is insufficiently protected by vegetation and could happen the big loss of soil. Erosion dangerous rains are characterized by big soil loss. Erosion dangerous phases of plants (emergency) had to occur in period when erosion dangerous rains do not occur. Sowing catch crops after harvest of spring barley is proper due to erosion dangerous rains. Catch crops protect soil from erosion dangerous rains and also enrich with organic material.

CONCLUSION

The paper deals with analysis of phenological data and calculation of value of C factor for spring barley in extreme years. Phenological analysis was carried out for stations of Tečovice station (MASL 260) and Luhačovice station (MASL 285). Analysis was made for period 1931–1960 and 1961–1990. A 3 days prolongation of GS was found out in period 1961–1990.

On average later sowing of spring barley was identified also in this period (4 days). A shortening of the interval between EM and HE (9 days), and a prolongation of the interval between EM and HD (9 days) was proved. The detailed trend analysis of phenophases and agronomic practices was performed

for Tečovice station. In this station the statistically significant linear trend was identified only for agronomic practice SD (r = 0.503). The period 1961–1990 was divided into decades and subsequently trend analysis was performed for individual decades.

Value of C factor in Tečovice station was 0.1474 in 1974 and 0.1886 in 1964. Difference between values of C factor is 22 percent in 1974 and 1964. The length of GS was 154 days in 1974 and 95 days in 1964. The protective effect of vegetation in Luhačovice station was 0.1327 in 1974 and 0.2411 in 1983. Difference between these years is 45 percent. The length of growing season and date of sowing affect a value of C factor. The sooner the spring barley is sowed, the value of C factor is lower. The sowing and emergency of plants of spring barley are characterized as a crisis period due to soil erosion. In this phenophases the soil is insufficiently protected by vegetation and could happen the big loss of soil.

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DEVELOPMENT OF HUMIDITY CONDITIONS OF NATURAL LANDSCAPE IN THE CZECH REPUBLIC

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Abstract: The main aim of this work is demarcation of protected localities in the Czech Republic, which may be threatened by drought, including the prospect until the year 2100. ArcGIS program was used to calculate the results, thus the results are presented as maps provided with a commentary. The drought threat is viewed from the aspect of climatic drought, which is defined by the basic water balance of grass cover. As protected localities the protected areas and Natura 2000 areas are considered.

Key Words: drought, ecosystem, protected areas, Natura 2000

INTRODUCTION

Drought is one of the extreme weather conditions which contribute to the environmental degrading and cause problems in all agricultural sectors. One of the approaches to this phenomenon is its evaluation and monitoring, considering the threat to valuable ecosystems.

Model projections of possible future climate development predict a temperature increase by 3.2–3.3 °C in the area of the Czech Republic by the end of the 21st century, while model ALADIN – 10 shows a slight decrease in precipitation (Brázdil et al. 2015). Moisture certainty analyses in the Czech Republic proved an increase of the driest areas and drought event probability increased in the 1961–2010 period (Středová et al. 2011). The results of Středová and Středa (2015) suggest an increase of potential evapotranspiration and thus higher susceptibility of agricultural intense areas of southern and central Moravia and Central Bohemia to dryness in 1961–2010 compared to the mean of 1901–1950.

Overall, drought in the Czech Republic may be considered a presumable demonstration of a climatic change. Regardless of the kind of drought, it will affect biodiversity, ecosystem resilience, and ecosystem services (supporting, provisioning, regulating and cultural). One of the presumable effects of climatic change is production ecosystem service influence followed by major socio-economic problems.

In the area of the Czech Republic there are a lot of valuable localities with specific protection levels. They are especially valued for their scientific and aesthetic uniqueness and in many there are ecosystems dependent on the presence of water.

Negative effects of drought imply an importance of its further study. Understanding this phenomenon will then help its evaluation and increase the environmental and overall safety.

MATERIAL AND METHODS

As a method for demarcation of protected areas potentially threatened by drought with the prospect until the year 2100 work in geographical information Arc GIS system was selected. First it was necessary to obtain the map layers, which were then processed in ArcGIS and interpreted.

Resources for map layers of valuable ecosystems

As valuable ecosystems, localities protected by The Act of Protection of Nature and Landscape are considered in this work. These resources for the work were provided by Nature Conservation Agency of the Czech Republic. Specifically, in this work they were obtained and used the following map layers:

- Small protected areas with protection zones (last update 29. 1. 2016).
- Large protected areas with protection zones (last update 26. 1. 2016).
- Sites of Community importance – Natura 2000 (last update 11. 12. 2015).
- Special protection area – Natura 2000 (last update 11. 12. 2015).

Drought map layer resources

The data for this work were provided by Czech Hydrometeorological Institute. As the drought layer, the basic water balance layer was selected, where eventual climatic drought is suitably described. In the final maps, the threat map layers are elaborated for two time periods:

- Basic water balance map layer for present (entry data from years 1961–2010).
- Basic water balance map layer for prospect until the year 2100 (entry data from years 2072–2100).

Both map layers show climatic drought in the Czech Republic described by basic water balance of grass cover in the vegetation period. It is a mutual difference of precipitation and potential evapotranspiration from the referential surface, in this case grass cover. Potential evapotranspiration represents the total amount of water (in mm), which can evaporate from subsurface, while still being saturated with water, under particular climatic conditions (Kohut 2007).

Modified Penman-Monteith method, which is worldwide acknowledged and recommended by FAO, was used for calculating potential evapotranspiration from grass cover for present (1961–2010), which allows calculation of water vaporization from different surfaces. Modified algorithm based on the Penman-Monteith method serves as the basis of AVISO model, which is being used at CHMI branch since 1992. It was modified and adjusted to the conditions in the Czech Republic and is regularly updated and optimized.

Map layer of basic water balance for prospect until 2100 is projected on the basis of A1B emissions scenario. For future climatic trend simulation, scenario data provided by CHMI were evaluated by regional climatic model ALADIN – Climate/CZ, which was controlled by global climatic model ARPÉGE. Final map layer was simulated on the basis of A1B emissions scenario for years 2071–2100 (Štěpánek 2007).

RESULTS AND DISCUSSION

Evaluating of the resulting maps was made on the basis of their visual evaluation. Behind the ear endangered sites are considered those in which moisture balance reaches values of -50 mm and below. First stage (0 mm to -49.9 mm) is considered like borderline. For sites that belong to this stage, it may approach the moisture balance equal to 0 mm, which is not considered as degradation factor.

Due to the large number of small protected areas and sites of community importance, area of these categories was not possible to specify. At this point it was described orientation in the context of threats to individual regions and their geographical affiliation to any large protected areas. On the following page are the final maps and next their description.

Figure 1 for present and Figure 2 for prospect until the year 2100 show that the largest area with the most negative humidity balance (below - 250 mm) is in present located in the South Moravian region. Significant is also the effect of drought in CentralBohemian and Ústecký region. In these cases the humidity balance only decreases below - 250 mm in very small areas, thus the most drought threatened areas are in South Moravian region.

Figure 1 Protected areas in the Czech Republic potentially threatened by drought – present

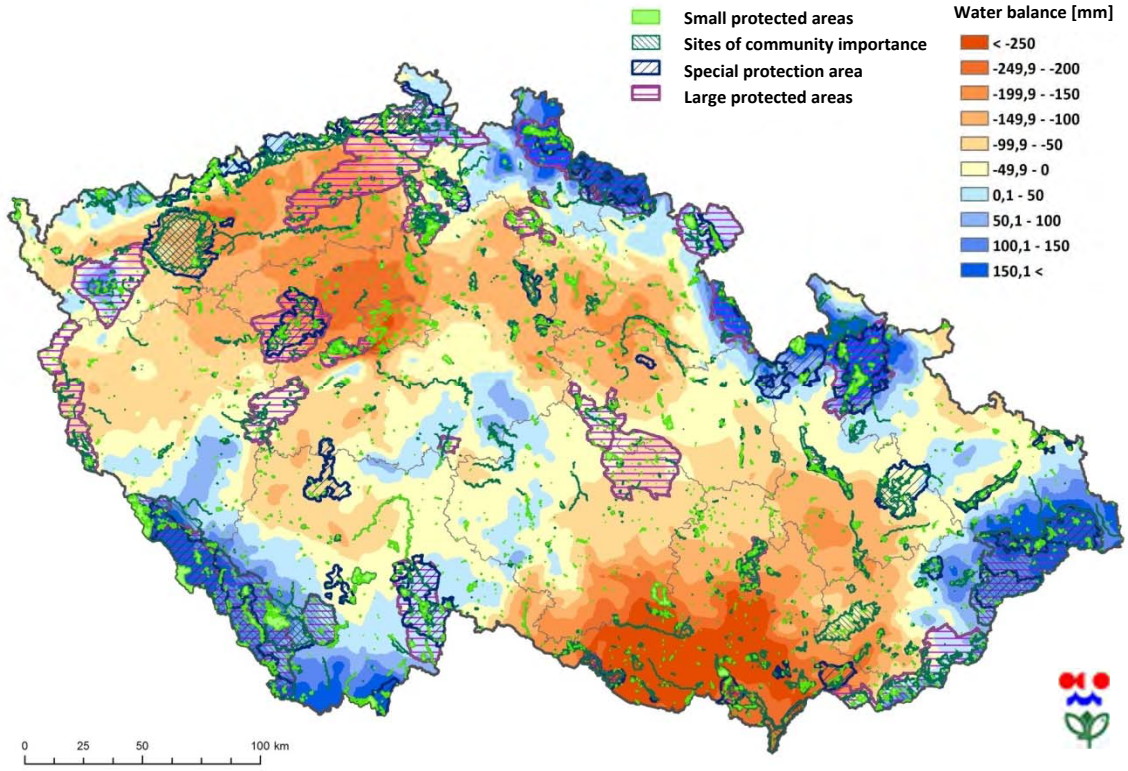
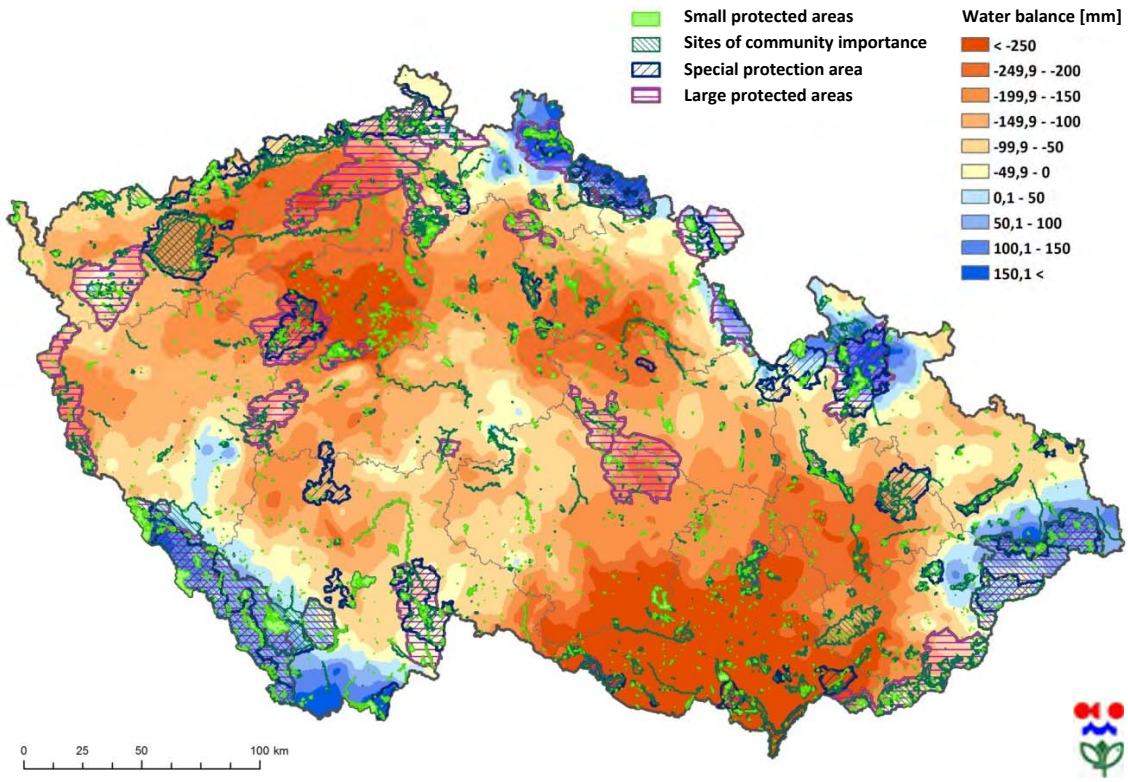


Figure 2 Protected areas in the Czech Republic potentially threatened by drought – the prospect until the year 2100



From large highly protected areas the most threatened is the Podyjí NP, the Pálava PLA, and the Moravian Karst PLA. The highest protection level is applied in the first zones of these categories.

In protected areas localities were demarcated, where occurrence of rare bird species is dependent on the presence of water habitats. Localities in the South Moravian region are thus the most threatened. There are 6 localities concerned: Pálava, Jaroslavické rybníky, Střední nádrž vodního díla Nové Mlýny, Lednické rybníky, Soutok – Tvrdonicko and Bzenecká Doubrava – Strážnické Pomoraví.

Because of a number of small protected areas and localities of European significance, it was impossible to provide full list of potentially threatened areas in his category. To sum it up, the protected localities, which are the most affected by drought, are in southern part of South Moravian region, southern part of Central Bohemian region, Ústecký, Královéhradecký and Olomoucký region.

Prospect until the year 2100 shows expansion of the drought as well as increase in its intensity, which will cause the raise in number of protected areas threatened by this factor. These localities along with the contemporary ones can be found in the final maps (small protected areas and localities of European significance can also be found on the attached CD). According to the prospect, positive humidity balance will only be preserved in a few borderline localities. From small protected areas and Natura 2000 areas, positive humidity balance will only be preserved in the ones located directly in or very near these large protected areas: Beskydy PLA (Moravian-Silesian and Zlínský region), Jeseníky PLA (Moravian-Silesian and Olomoucký region), Orlické Mountains PLA (Královehradecký region), Broumovsko PLA (Královehradecký region), Krkonošský NP (Liberecký and Královehradecký region), Jizerské Mountains PLA (Liberecký region), NP a PLA Šumava (Plzeňský and South Bohemian region) and Blanský les PLA (South Bohemian region).

Final maps show the protected areas threatened by drought. It is however necessary to differentiate areas for which drought does represents a real threat and those, for which it does not. We are thus able to compare such localities, as Moravian Karst PLA and national nature preserve Mohelenská hadcová step, where the effect of drought is quite different.

CONCLUSION

In this work, drought was evaluated from the aspect of threat to valuable ecosystems. A significant amount of protected localities are threatened by drought nowadays and the prospect until the year 2100 shows that major part of the Czech Republic will be affected by it, while positive humidity balance will be preserved at only a few localities. The results should not, however, be only perceived in a negative way. There is a number of different ecosystems with different humidity demand. That's why it is important to view each protected area individually and adjust the care to their specific needs.

Considering the threat to valuable ecosystems, there should be further study of the drought problem and this work should be seen as a base for following scientific works.

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EFFECTS OF APPLICATION OF BIOCHAR TO WINTER WHEAT (*TRITICUM AESTIVUM* L.) IN LONG-TERM DROUGHT CONDITIONS

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Abstract: The main aim of this work was to evaluate the effects of the application of biochar and activated carbon to winter wheat (*Triticum aestivum* L.) in conditions of simulated drought. The experimental pots were built so that the root system of one and the same pre-grown plant was divided by plastic foil into two compartments, one with and one without the addition of biochar (active carbon) in order to differentiate the reactions of the root system to stress created by drought. The cultivation tests of the soil-microorganism-plant (winter wheat) system were focused on understanding the reactions of soil microbial communities and experimental plants on the plant growing measures in combination with model effects of drought. Activated carbon had no demonstrable effect on the production of biomass, but the application of biochar aided significantly in the production of aboveground biomass in winter wheat (*Triticum aestivum* L.) in the stressful conditions of simulated drought. The plants preferred to grow their root system in the half with added biochar and activated carbon.

Key Words: rhizotron, mineral nitrogen, ammonium, nitrate, soil

INTRODUCTION

In recent years, there was an increase in the frequency of extreme climatic conditions occurring, especially in the form of long droughts or high temperatures (Liu and Allan 2013, Rahmstorf and Coumou 2011). The occurrence of these situations can negatively affect yields of agricultural crops, including winter wheat (Kolář et al. 2013, Hlavinka et al. 2009), which is a cereal vitally important in the field of human nutrition. It is likely that due to the growing population, it will be necessary to increase the production of winter wheat while maintaining its qualitative parameters. Many studies also report a likely increase in extreme climatic conditions in the future, which will also affect cultivation of winter wheat (Gourdji et al. 2013, Trnka et al. 2014). A significant opportunity to reduce the extent of negative climatic conditions can be found in maintaining or even improving soil fertility due to the improvement of biological and physico-chemical properties of the soil. In this regard, the application of biochar to the soil is an interesting option.

Biochar is a fine-grained material similar to wood coal which is produced out of plant biomass through pyrolysis. Pyrolysis is the thermal decomposition of organic materials without the presence of agents containing oxygen. Pyrolysis is based on the heating of materials above the limit of thermal stability of the organic compounds present, which leads to their cleavage all the way to stable low-molecular products resistant to microbial decomposition. The advantage of biochar lies in the wide range of materials which can be used to produce it (residues from forestry, agriculture, animal husbandry, municipal biological waste). Biochar is up to two orders of magnitude more stable than other forms of carbon in the soil derived from biomass. Adding biochar to soil can also be used as a method of Carbon Capture and Storage (Amonette et al. 2007); in some cases, carbon can be stored in the soil for many centuries (Kuzyakov et al. 2009, Downie et al. 2009, Lehmann et al. 2009). In terms of soil properties, biochar improves soil fertility (Liang et al. 2006), increases the cumulative area for the interaction of all living components of the soil, increases soil pH and increases cation exchange capacity (Lehman et al. 2011, Elad et al. 2011). The addition of biochar also significantly increases the content of available

water in the soil by increasing the amount of water retained in the soil (field water capacity) and allowing plants to draw the soil water content and lower it before wilting (Koide et al. 2015). The result is an increased yield from plants grown, increased accessibility of nutrients as well as an increase in microbial activity in the soil (Biedermann and Harpole 2013). After the application of biochar, mineralisation of nitrogenous matter in the soil is also reduced (Prayogo et al. 2014) and the speed of nitrification and denitrification processes is increased (Xu et al. 2014).

To assess the effects of the addition of biochar to improve the retention and accumulation capabilities of the soil and thus increase the resistance of plants to drought, we initiated a pot experiment. The cultivation tests of the soil-microorganism-plant (winter wheat) system were focused on understanding the reactions of soil microbial communities and experimental plants on the plant growing measures in combination with model effects of drought.

Material and Methods

Definition of the Sampling Site

Soil sampling was performed on the cadastral area of village Banín, where the soil has suffered long-term degradation and lack of organic substance input into the soil. At the sampling site, the main soil unit (MSU) is 12, i.e. medium-heavy brown earth with a soil substrate of mixed diluvium. The sampling site is located in climatic region MT2 (Vesecký 1961), with average annual temperature of 7–8 °C, and total rainfall of 550–700 mm/year.

Design of Experiment

The experimental pots were built so that the root system of one and the same pre-grown plant was divided by plastic foil into two compartments, one with and one without the addition of biochar in order to differentiate the reactions of the root system to stress created by drought. The root system of both portions was separated from the experimental soil mixture by polyamide netting which does not allow the roots to grow into the soil (mesh size of 34 µm). Each experimental pot (rhizotron) was planted with two plants of winter wheat. The experimental plants developed two different root systems based on their own preferences in the vertical slits between the soil compartment and the impermeable plastic foil. Contact with the particular variation of experimental soil was mediated primarily by the microbial activity through the polyamide netting. Retaining chambers (volume ca 4 cm^3) were placed into the rhizotron at a depth of 15 cm to measure the emission of gaseous microbial metabolites. The experimental pots were built based on the (Table 1) in four repetitions.

Table 1 Overview of experimental variants

Variant	Variant description
Ks	Control in drought mode
Km	Control in irrigation mode
As	Variant with the application of activated carbon in an amount of 50 t/ha in drought mode
Am	Variant with the application of activated carbon in an amount of 50 t/ha in irrigation mode
Bs	Variant with the application of biochar in an amount of 50 t/ha in drought mode
Bm	Variant with the application of biochar in an amount of 50 t/ha in irrigation mode

Both halves of the experimental pots of variants Ks and Km were only filled with experimental soil. In the other variants, one half was always filled with experimental soil (control) and the other with soil with admixtures based on the particular variant. The activated carbon used had particle size of 2.36–4.75 mm and was made of coconut shells, water content max. 5%, ash content max. 5%, bulk density 500 ± 50 g/l, pH 8–10. The application of activated carbon was chosen to simulate biochar which has been in the soil for a longer period of time. The biochar used was produced from waste biomass. Each half of the experimental pot carried mixed ion exchange polymers for capturing mineral nitrogen leaking from the system. The discs were filled with a mixed ion exchange polymer with cation : anion ratio of 1 : 1. We used AER grains type A520E (anion exchange resin) and CER grains type C100E (ion exchange resin) made by Purolite. The ion exchange discs were covered with a layer of sand, which was then covered by homogenised soil with additions based on the variant. Variants in drought mode and in

irrigation mode were regularly irrigated so that the dry variant was kept at 30% of available water capacity (AWC). AWC is the maximum amount of water which a plant is able to utilise in the given soil profile; mathematically, it is a difference between the field water capacity and wilting point. Variants in irrigation mode were kept at 70% AWC. Emissions of gaseous microbial metabolites were measured over the course of the experiment. The experiment was concluded upon the experimental plants reaching maturity.

Plant Biomass Production

After the conclusion of the experiment and the performance of the above analysis, both the aboveground and underground portion of the plants was dried at 105 °C to a constant weight.

Determination of Mineral Nitrogen

Discs containing the mixed ion exchange resin (IER) were dried at laboratory temperature. The dried ion exchange resin (IER) was transferred into plastic containers. Subsequently, ammonium (NH_4^+-N) and nitrate (NO_3^--N) nitrogen were extracted from the IER structure with NaCl at exact substance concentration by shaking in a laboratory shaker for one hour. The amount of ammonium (NH_4^+-N) and nitrate (NO_3^--N) nitrogen in the infusions was determined via a distillation-titration method in accordance with (Peoples et al. 1989). Distillation was performed on a Behr S3 device and titration on automatic burette Titronic 96.

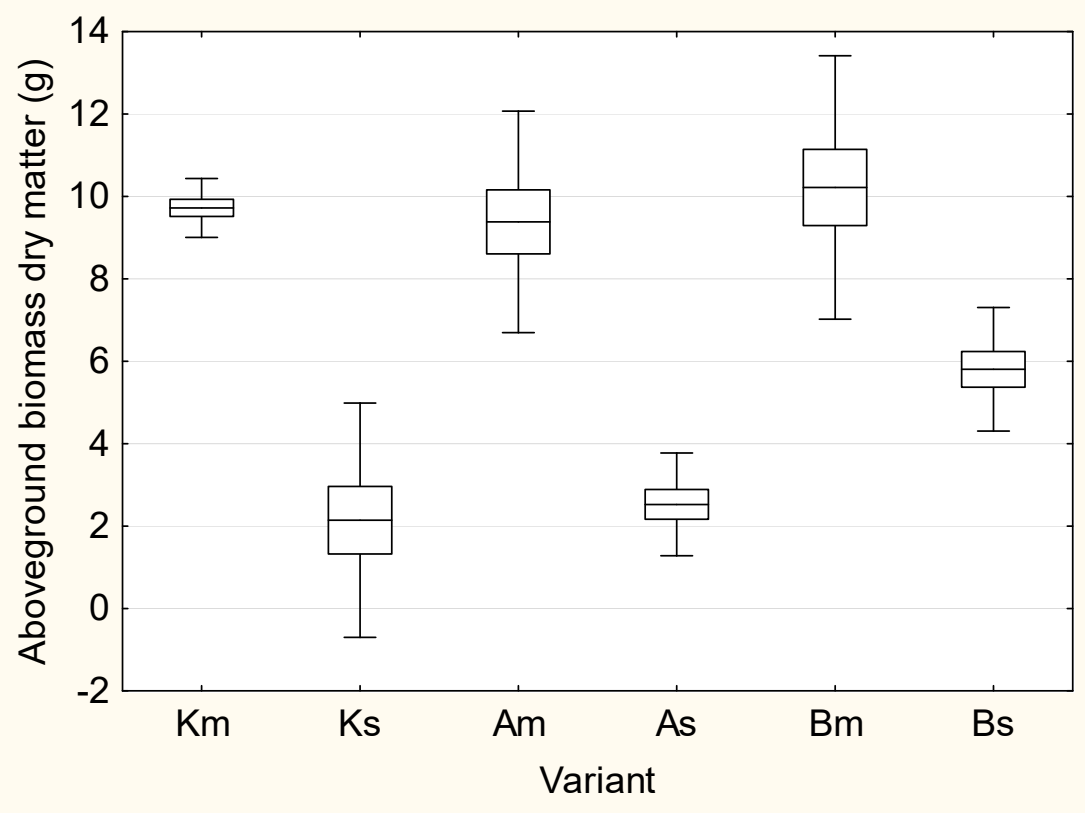
Statistical Analysis

All results were subjected to one-way analysis of variance (ANOVA) in combination with post-hoc Tukey's test ($P < 0.05$). All analyses were performed with the use of Statistica 12 software.

RESULTS AND DISCUSSION

The main objective of the experiment was to evaluate the effects of the application of biochar and activated carbon to winter wheat in conditions of simulated drought.

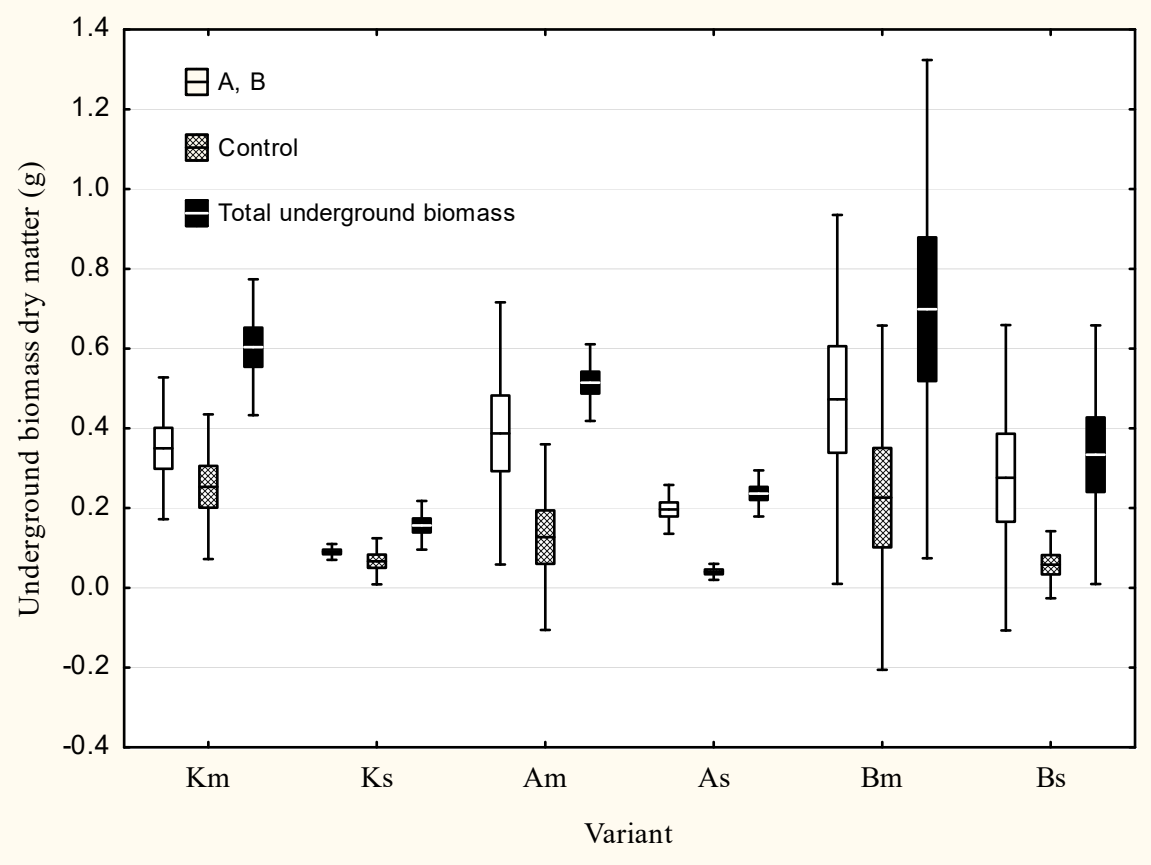
Figure 1 Graph of aboveground biomass



Legend: Ks – Control in drought mode, Km – Control in irrigation mode, As – Activated carbon 50 t/ha in drought mode, Am – 50 t/ha in irrigation mode, Bs – application of biochar in an amount of 50 t/ha in drought mode, Bm – of 50 t/ha application of biochar in irrigation mode.

Figure 1 shows the graph of total dried aboveground biomass. A statistically significant difference was found between variants in drought mode and variants in irrigation mode. No demonstrable statistical difference was found between the variants in irrigation mode (Km, Am, Bm).

Figure 2 Graph of underground biomass



Legend: Ks – Control in drought mode, Km – Control in irrigation mode, As – Activated carbon 50 t/ha in drought mode, Am – 50 t/ha in irrigation mode, Bs – application of biochar in an amount of 50 t/ha in drought mode, Bm – of 50 t/ha application of biochar in irrigation mode. A, B represent those halves of the experimental pots where activated carbon or biochar was applied; in variants Km and Ks, these only indicate the second half of the control variant

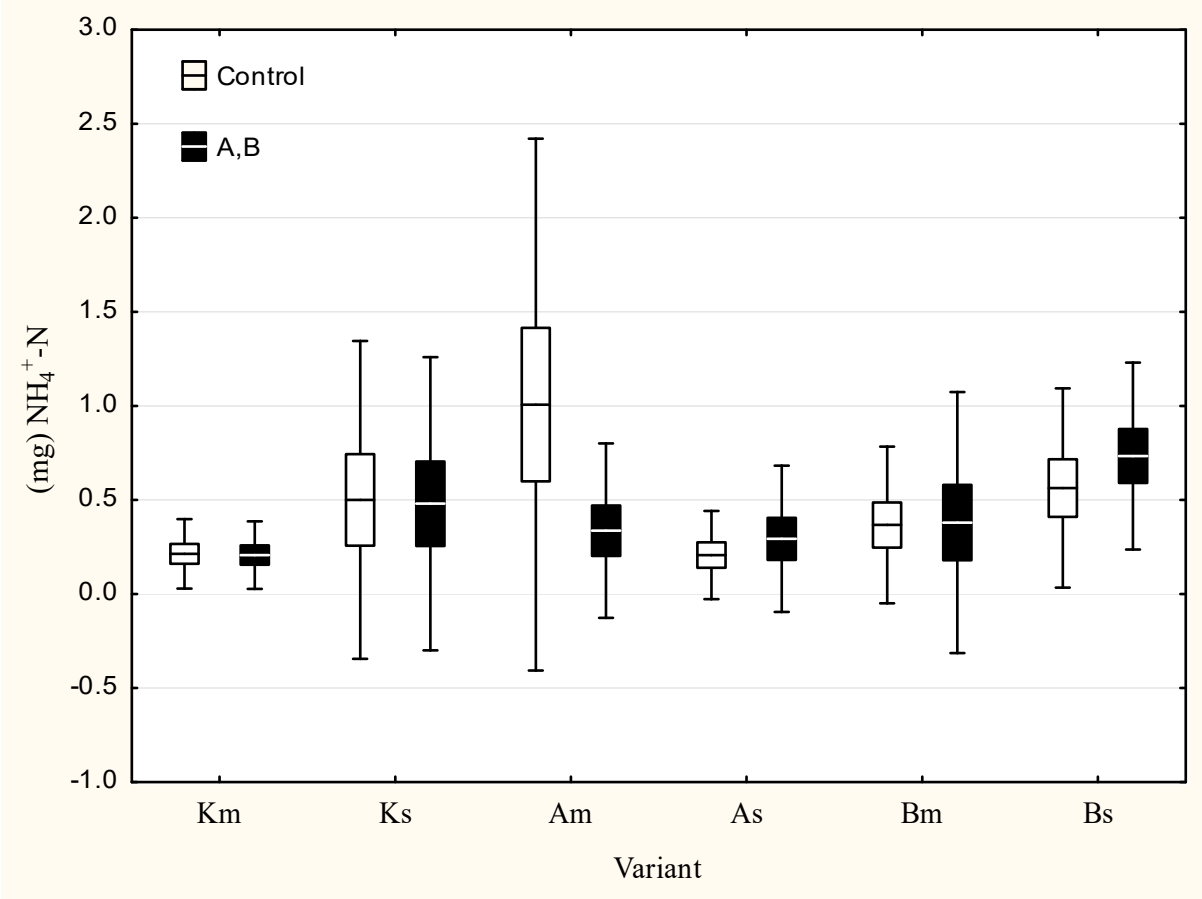
No demonstrable difference was found between variants Ks and As. The application of activated carbon thus had no effect on the production of aboveground biomass compared to the control variant. However, there is a statistically demonstrable difference between variants Ks, As and variant Bs. In variant Bs, the production of aboveground matter was more than doubled compared to variants As and Ks. The application of biochar therefore had demonstrable influence on the production of aboveground biomass in winter wheat in drought mode.

Figure 2 shows the graph of weight of dried underground biomass where A, B represent those halves of the experimental pots where activated carbon or biochar was applied; in variants Km and Ks, these only indicate the second half of the control variant In group A, B and in the control group, there are no statistically demonstrable differences between the individual variants. Demonstrable differences in the weight of total aboveground biomass were found only between variants Ks and Km, Bm and Ks, and As and Bm. However, the graph shows an increase in total aboveground biomass in variants As and Bs when compared to variant Ks. The graph indicates that the plants invested more into the development of root system in all variants with the addition of biochar and activated carbon compared to the other control half of the root system of the same plant. Significant differences were found primarily in variants As and Bs.

Figure 3 shows the graph of captured ammonium ions leaking from the system. The graph shows that the application of biochar and activated carbon had no statistically demonstrable effect on the leakage of ammonium ions from the system. In variant Am, the application of activated carbon reduced the loss of ammonium ions compared to the control half. The graph of nitrate nitrogen leakage is not

included here, since such leakage was minimal in all experimental variants, in the range of 0–0.07 mg NO_3^--N. In addition, there was no statistically demonstrable difference between the variants.

Figure 3 Graph of captured ammonium ions



Legend: Ks – Control in drought mode, Km – Control in irrigation mode, As – Activated carbon 50 t/ha in drought mode, Am – 50 t/ha in irrigation mode, Bs – application of biochar in an amount of 50 t/ha in drought mode, Bm – of 50 t/ha application of biochar in irrigation mode. A, B represent those halves of the experimental pots where activated carbon or biochar was applied; in variants Km and Ks, these only indicate the second half of the control variant

CONCLUSION

The data presented here shows that experimental variants in irrigation mode produced 5× more aboveground biomass than the control variant in drought mode. Activated carbon had no demonstrable effect on the production of biomass, but the application of biochar aided significantly in the production of aboveground biomass in winter wheat in the stressful conditions of simulated drought. The effect of biochar on the root system is not univocally demonstrable; it will thus be necessary to compare the results obtained with the results of further analyses, particularly with results of measured surface areas and lengths of root systems of experimental plants in the given variants. These and other results (mycorrhiza, microbiological analysis, emissions of gaseous microbial metabolites) will be presented in another article detailing the present experiment. However, from the data presented here, we can assert that the application of biochar had a positive effect on growing winter wheat in long-term drought conditions. The plants preferred to grow their root system in the half with added biochar and activated carbon.

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EFFECTS OF CEREAL/LEGUME INTERCROPPING ON NITROGEN LEACHING: LYSIMETRIC FIELD EXPERIMENT

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Abstract: The paper presents a year's summary (third year) of results of a long-term experiment which was established to examine the hypothesis that cultivation of a mixed culture of winter wheat (*Triticum aestivum* L.) and white clover (*Trifolium repens* L.) affects leaching of mineral nitrogen from the soil. The reason for the examination of this hypothesis is the increasing amount of mineral nitrogen in drinking water sources. The mixed culture or intercrop means the growing of two crops at the same time in the same place. In this case, the particular mixture consists of cereal/legume intercropping. A lysimetric field experiment was established to verify the hypothesis. Six variants were established in three repetitions. In two variants, only winter wheat with fertilisation (140 kg of N/ha/yr) and without fertilisation was grown. In the other four variants, winter wheat was grown alongside white clover (*Trifolium repens* L.) with the use of varying doses of mineral (DAM 390) and organic (Lignohumate B) fertilisers. Percolate leaching from the experimental lysimeters was collected and traps with ion exchange resin (IER) were placed in each container. The amount of ammonia (NH_4^+-N) and nitrate (NO_3^--N) nitrogen in the water was measured regularly. The amount of trapped mineral nitrogen into the structure of from IER was also measured. A statistically significant difference (ANOVA; $P < 0.05$) was found between variant CY and all other variants. Of particular importance is the difference between variants CY and A1, since both were fertilised with mineral fertiliser only. From this, we can deduce that intercrop/mixed culture reduced the leaching of ammoniacal nitrogen from the system. A statistically significant difference (ANOVA; $P < 0.05$) was found between variant CY and A2 in the capturing of ammoniacal nitrogen (NH_4^+-N) from traps.

Key Words: winter wheat, white clover, ground water, topsoil, subsoil

INTRODUCTION

Winter wheat is the second most important cereal in the world and plays an indispensable role in human nutrition. In recent years, modern agriculture's methods of cultivating winter wheat have resulted in high doses of nitrogen entering into the soil through mineral nitrogenous fertilisers. Long-term application of mineral fertilisers has negative impact which leads to the deterioration of the soil. One of the consequences of using mineral fertilisers is also the leaching of mineral forms of nitrogen into ground water and subsequent contamination of drinking water sources. Field ecosystems subjected to intensive agricultural processes leach 10–40% of nitrogen from fertilisers into the ground water on loamy soil and 25–80% of nitrogen on sandy soils (Howarth et al. 1996). One of the ever more frequently discussed options of limiting the leaching of nitrogen into ground water is the use of intercrops or mixed cultures. Mixed culture in the context of this paper means the growing of two crops in one area at the same time. Currently, the predominant intercrops crown are stubble crops for green manuring. Intercrops enrich the soil with easy-to-decompose organic matter which increases the microbial activity in the soil. Organic matter from roots and aboveground portion of plants improves the physical conditions in the soil (primarily the structure of the soil), contributes to protecting the soil against water and wind erosion and allows better utilisation of rainfall in the inter-vegetational period.

Cereal/legume intercropping can be an effective strategy to reduce N leaching losses and fertiliser inputs (Mariotti et al. 2015). Positive effects on the loss of nitrogen are also presented, for instance, by

(Szumigalski and van Acker 2006) or (Pappa et al. 2001). The use of legume–barley intercropping also stimulates microbial activity and as stated by (Scalise et al. 2015), increased mineral N made available in soil allows a comparable grain yield with a reduced N-fertiliser use. Aside from the positives listed, the use of mixed cultures also allows more effective use of nutrients, and the symbiotic bacteria of legumes fixate air N_2 and enrich the soil with it. The main aim of the present study was to verify the hypothesis that mixed cultures of winter wheat (*Triticum aestivum* L.) and white clover (*Trifolium repens* L.) affect the leaching of mineral nitrogen from the soil in the sense to reduce the leaching of mineral forms of nitrogen into ground water and prevent contamination of drinking water sources with nitrogen which results from intensive agricultural activity.

MATERIAL AND METHODS

To confirm the hypothesis, a lysimetric experiment was established. Eighteen cylindrical PVC (polyvinyl chloride) containers of the same size were built, 50 cm tall and 30 cm wide, which were then sunk into the ground. The containers were filled with 25 kg of subsoil and 25 kg of arable topsoil. The lysimeters and the area of interest were described, inter alia, in (Elbl et al. 2013b). The arable soil used to fill the containers was taken from fields surrounding the area of interest. Soil samples were sifted through a sieve with mesh size of 10 mm and subsequently homogenised. The topsoil and subsoil were sifted and homogenised separately. All seeping water from each lysimetric container was led through a drain into a plastic pipe into a collecting plastic container. The containers were located in an inspection shaft. The inspection shaft was regularly checked several times a week and if seeping water (percolate) was found in the collecting containers, the percolate was taken for evaluation. The entire site is located in a II. class water source protection zone in Březová nad Svitavou. All the sites of the water source protection zone fall within vulnerable areas (according to the so-called Nitrate directive, implementing regulation of government regulation 262/2012 Coll.) The total of average annual rainfall in the area is 600 mm with average annual temperature of over 7.6 °C. The local soil suffers from long-term lack of organic matter which is not supplied to the soil in sufficient amounts. Fertilisation and planting of individual variants is described in (Table 1).

Table 1 Overview of individual variants

Variant	Plant	Fertiliser
CY	Winter wheat	Application of 140 kg of N/ha/yr
CN	Winter wheat	No fertiliser
A1	Winter wheat + white clover	80% of the recommended dose of N for winter wheat.
A2	Winter wheat + white clover	50% of the recommended dose of N and 100% of the recommended dose of C_{org} for winter wheat.
A3	Winter wheat + white clover	50% of the recommended dose of N and 50% of the recommended dose of C_{org} for winter wheat.
A4	Winter wheat + white clover	No fertiliser

All variants were prepared in three repetitions. Variants CY and CN were thus only planted with winter wheat (*Triticum aestivum* L.). Variants A1–A4 were planted with a mixed culture of winter wheat (*Triticum aestivum* L.) and white clover (*Trifolium repens* L.). The experimental containers were planted and fertilised every year of this long-term experiment. The composition of the fertilisers used was already published in (Elbl et al. 2013b, Kintl et al. 2014): Nitrogen was applied as liquid fertiliser DAM 390. DAM 390 is a solution of ammoniacal nitrate and urea with an average content of 30% nitrogen (1/4 of nitrogen is in the form of ammonium, 1/4 is in the nitrate form and 1/2 is in the form of urea). One hundred litres of DAM 390 contain 39 kg of nitrogen. Organic carbon (C_{org}) was applied as organic fertiliser Lignohumate B (LG B). Lignohumate is a product of chemical transformation of lignosulfonate. This material is completely transformed into the final product: a solution containing 90% of humic salts (1 : 1 ratio of humic and fulvic acids). Two 15 cm long nylon stocking traps with ion exchange resin (IER) were installed in the soil in each experimental container. IER traps were prepared

as a nylon stockings from Uhelon filled with IER. Uhelon is a thick nylon mesh woven from polyamide fibres using technology which ensures the anchoring of the individual fibres in the mesh, thus providing constant mesh size. Both probes were filled with IER. One trap was filled with IER (CER type C100E), the other with IER (AER type A520E) made by company Purolite. The purpose of these was to collect the ammoniac (NH_4^+-N) and nitrate (NO_3^--N) nitrogen leaching from the system.

Determination of Mineral Nitrogen

In the event that the collecting containers located in the inspection shaft contained water seeping from the experimental containers, the water was taken for determining mineral nitrogen content (NH_4^+-N and NO_3^--N). The amount of percolate from each collecting container was measured separately, and a sample was taken. IER traps were dried at laboratory temperature. The IE resins were then transferred to plastic containers. Ammoniacal (NH_4^+-N) a nitrate (NO_3^--N) nitrogen was extracted from the structure of the IER using concentrated NaCl solution (10%). The containers with an exact amount of NaCl added were then shaken for one hour in a laboratory shaker so that the desired extraction occurs. The amount of mineral nitrogen in the individual extracts from the traps, as well as from the leaching water collected from the experimental containers was determined using a distillation-titration method in accordance with (Peoples et al. 1989). Distillation was performed on a Behr S3 device and titration on automatic burette Titronic 96.

Statistical Analysis

All results were analysed using Statistica 12 software. Potential differences in the results were analysed using single-factor ANOVA analysis in combination with the post-hoc Tukey's test.

RESULTS AND DISCUSSION

The study presents the results of the last year of measurements from an experiment initiated in 2012. The measurements are focused primarily on the ammoniacal and nitrate form of nitrogen, since these forms of mineral nitrogen have the greatest value for plants. Ammoniacal nitrogen is not very mobile in the soil profile, while nitrate nitrogen is very mobile. Nitrate nitrogen can therefore endanger the quality of the drinking water due to its leaching from the fields into the groundwater (Elbl et al. 2013).

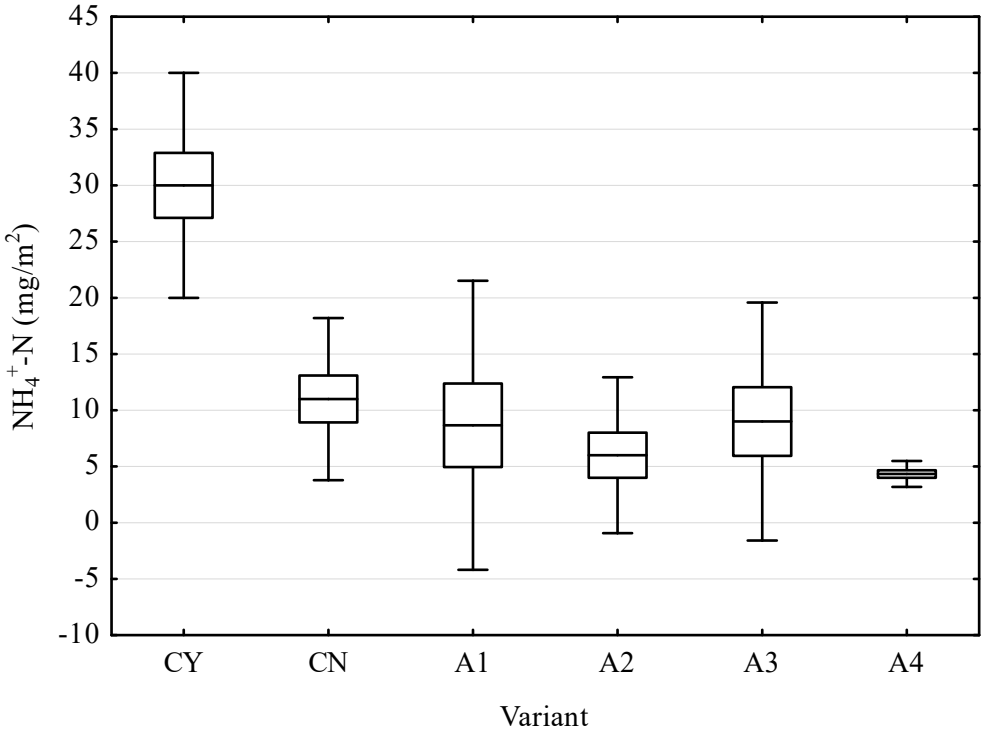
Figure 1 shows the amount of ammoniacal nitrogen recalculated for one m^2, as measured from seeping water captured in collecting containers located in the shaft. A statistically significant difference (ANOVA; $P < 0.05$) was found between variant CY and all other variants. No other statistically significant differences were found. Of particular importance is the difference between variants CY and A1, since both were fertilised with mineral fertiliser only. From this, we can deduce that intercrop/mixed culture reduced the leaching of ammoniacal nitrogen from the system.

Figure 2 shows the amount of nitrate nitrogen recalculated for one m^2, as measured from seeping water captured in collecting containers located in the shaft. A statistically significant difference (ANOVA; $P < 0.05$) was found between variants CY and A2, A2 and A1, A4 and A1. Especially important is the difference between variants A2 and CY. The application of organic carbon in the form of LG B in variant A2 along with intercrop/mixed culture cultivation likely encouraged microbial activity in the soil. The differences between variants A2 and A1 show that the difference in mineral nitrogen leaching was caused primarily by the application of LG B in variant A2. The positive effect of intercropping on leaching of mineral nitrogen was presented by (Szumigalski and Van Acker 2006, Pappa et al. 2011).

Figure 3 shows the graph of ammoniacal (NH_4^+-N) and nitrate (NO_3^--N) nitrogen captured from traps located in the soil. A statistically significant difference (ANOVA; $P < 0.05$) was found between variant CY and A2 in the capturing of ammoniacal nitrogen (NH_4^+-N). In variant CY, the amount of ammoniacal nitrogen captured was also higher compared to all other variants, but the difference is no longer statistically demonstrable, though it can be observed in the graph. Compared to other variants, the microorganisms as well as the cultivated plants were no longer capable of immobilising ammoniacal nitrogen efficiently in variant CY. The process of mineralisation was likely predominant. This may indicate that the microorganisms and plant communities present began to lose the ability to efficiently

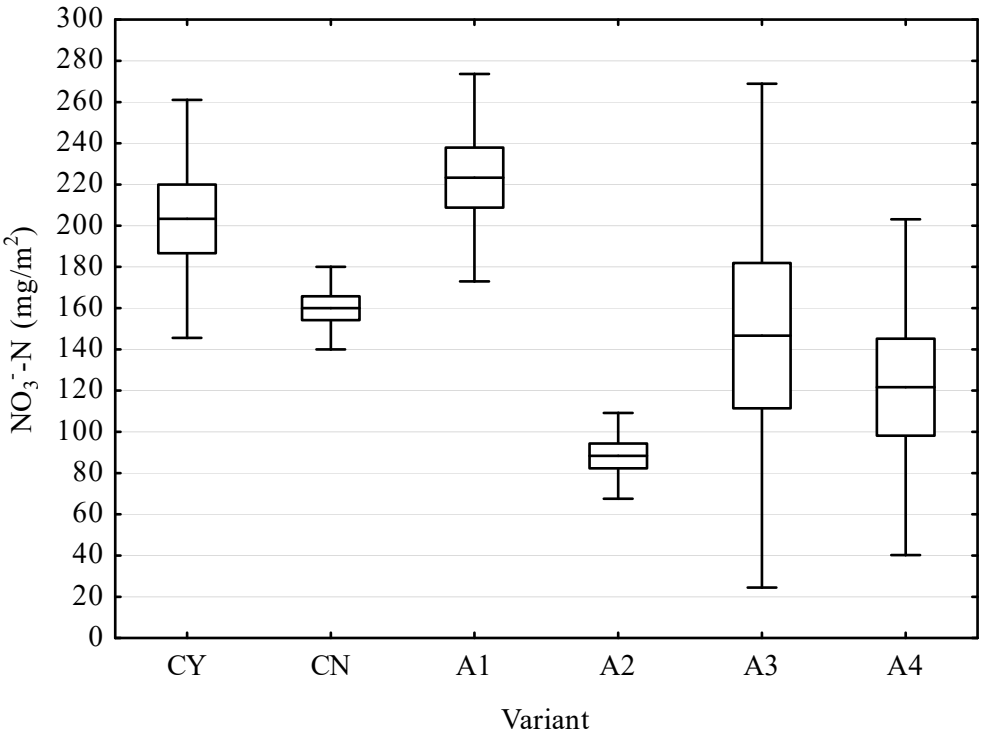
use the hard-to-access nitrogen. These results are in line with the first year of measurements (Kintl et al. 2014) and with other results which have yet to be published.

Figure 1 Graph of the amount of ammoniac nitrogen



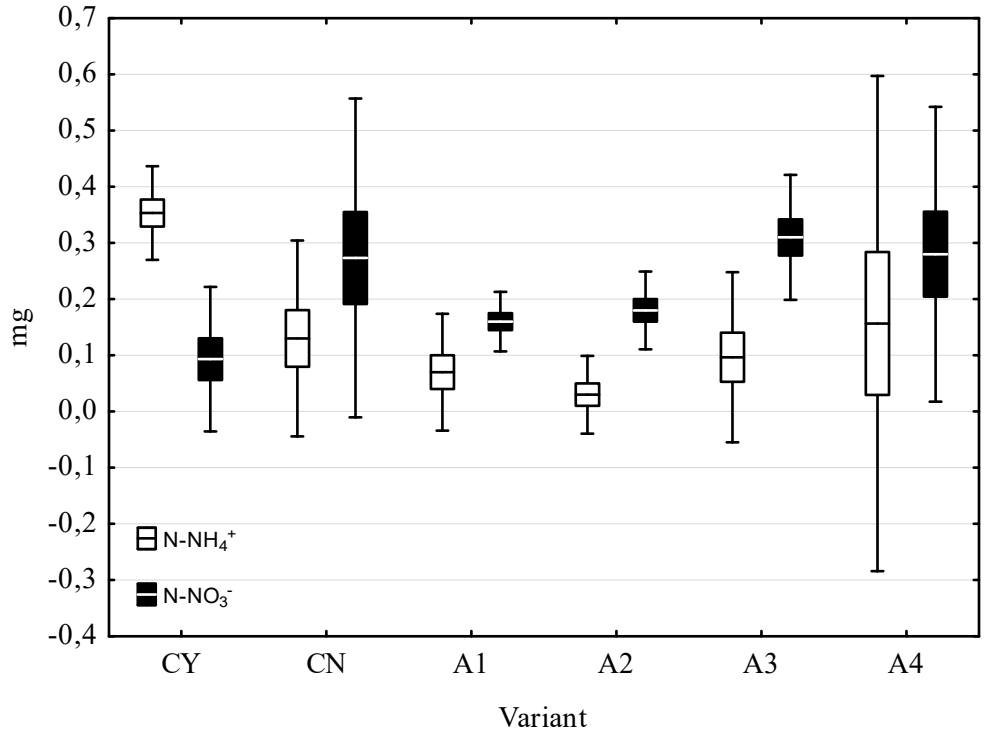
Legend: CY – wheat – 140 kg of N/ ha/yr, CN – wheat – no fertiliser, A1 – wheat and white clover – 80% of N, A2 – wheat and white clover – 50% of N and 100% of org. C, A3 – wheat and white clover – 50% of N and 50% of org. C, A1– wheat and white clover – no fertiliser.

Figure 2 Graph of the amount of nitrate nitrogen



Legend: CY – wheat – 140 kg of N/ ha/yr, CN – wheat – no fertiliser, A1 – wheat and white clover – 80% of N, A2 – wheat and white clover – 50% of N and 100% of org. C, A3 – wheat and white clover – 50% of N and 50% of org. C, A1– wheat and white clover – no fertiliser.

Figure 3 Graph of ammoniac (NH_4^+-N) and nitrate (NO_3^--N) nitrogen captured from traps



Legend: CY – wheat – 140 kg of N/ ha/yr, CN – wheat – no fertiliser, A1 – wheat and white clover – 80% of N, A2 – wheat and white clover – 50% of N and 100% of org. C, A3 – wheat and white clover – 50% of N and 50% of org. C, A1– wheat and white clover – no fertiliser.

CONCLUSION

The study presents the results from one year of measurements in a long-term experiment. However, alongside the already published results, the present study points to the possibility of using intrecropping/mixed culture cultivation as a promising method of reducing the leaching of mineral forms of nitrogen from the soil. Of particular importance is the difference between variants CY and A1, since both were fertilised with mineral fertiliser only. From this, we can deduce that intercrop/mixed culture reduced the leaching of ammoniacal nitrogen from the system. This is particularly important for areas where mineral nitrogen passes from the soil into the ground water and contaminates the drinking water supplies of the population, which currently presents a significant environmental problem. This also applies to the area of interest in Březová nad Svitavou, where the experiment was performed and which serves as a source of drinking water for the city of Brno.

ACKNOWLEDGEMENTS

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LAND FUND ANALYSIS AND PROPOSAL OF EROSION RISK REDUCTION MEASURES FOR AREA OF HUSTOPEČE

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Abstract: The article deals with the identification of risk areas in selected cadastral area Hustopeče in terms of soil conservation. Based on defined risks and their threat level, it is possible to design complex system of measures to protect soil and landscape. Gradually are defined and described various risk factors, which may effect on the landscape – water and wind erosion, size of land blocks, land use. The multi-criteria analysis was made to define the most threatened parts of land blocks in study area. The result is synthetic map showing the most vulnerable land blocks in the area. It was found, that the Hustopeče cadastre is highly vulnerable to soil degradation. Most of land blocks are in categories with high risk of soil erosion. The possible protective measures are described.

Key Words: soil, erosion, anti-erosion measures, land consolidation, Hustopeče

INTRODUCTION

The landscape is an open system shaped by the interaction of natural processes and human activities (Antrop 1998), which are changes in time and space. This leads to constant changes in the landscape of varying intensity and scope. The intensity of these changes depends on the position, the attractiveness of the area and degree of maturity or development company. One of the most visible manifestations are changes in land use, which are reflected changes in the relationship of natural and socio-economic sphere in a specific area (Jeleček et al. 1999). The largest and most significant changes of the landscape started in Industrial Revolution in the middle of 18th century. Furthermore these changes escalated the most in the second half of the 20th century.

Changes in the landscape can be divided into controlled or uncontrolled. Steered by changes such changes mean that their planned decisions and behavior affects the person. Directed changes are the changes, which affect the peoples by the planning decisions and and their behavior. It is for example a deliberate political decision making, planning and landscape management. Adverse changes may be caused by changes in natural conditions, whether it is a long term climate change or sudden natural disasters (Semančíková et al. 2008).

Soil erosion is in the Czech Republic the degradation process, which significantly affecting currently more than half of the arable land. Every year is threatened by erosion devastated more than 50% of arable land, which is about 1.5 million hectares, currently affected by water erosion is 40% of arable lands. Often in culture cropland shallow soils that are completely washed off, or which can be measured by one step shift soil depth (from 60 cm to 30 cm or less) (Dumbrovský 2009). Not only the high percentage of arable land, but especially the size of land parcels on sloping areas allowing extensive devastation of the land fund. In 1955, the average size of land was 10 hectare, now they are 50 and 100 or more per hectare blocks. Impair the physical and chemical properties of soils, biological degradation results in a reduction of organic matter in the soil and the quantitative and qualitative loss of soil microorganisms (Podhrázská and Karásek 2014).

Wind erosion is defined as the erosion of the soil surface by mechanical force winds (abrasion), soil particles being carried away by the wind (deflation) and their storage at another location (accumulation) (Pasák 1984). In Bohemia is punished or is it prone by the soil eolization 26% and in Moravia 45% of agricultural land. It is evident that the mainly southern Moravia belong to the territories threatened by the strong wind (Švehlík 1996).

Natural environmental factors causing wind erosion of soil by wind express vulnerability termed erodibility. Due to, that complex effect of all factors influencing wind erosion is relatively complicated, many authors are focused on the evaluation sectional factors. Various methods have been developed for ratings erosion processes (Holý 1994).

Current status of variable factors determining immediate erodibility (wind speed, soil moisture, surface roughness), it is important for retrospective detection, for example, to estimate the loss of soil erosion at a particular event. Determination erosion risks territory for wind erosion for design and engineering work for the protection of soil, water and land, must be based on methods other hand allowing the blanket, not a spot determination of endangerment territory and also provide guidance on the spatial and functional layout measures against wind erosion (Podhrázská and Karásek 2014).

In terms of protecting soil and water plays a vital role the way how we use farmland and their surface area. Negatively to the relational processes of soil–water–plant reflects, in particular the area of arable land, which are in terms of agricultural use of the most exploited. In addition to the supply of chemicals into the soil and agronomical measures adversely affects its low diversity and short term (seasonality) vegetation cover, because outside the growing season is arable land prone to degradation processes. These factors drastically affect all other risk processes, soil–water. In terms of protection of farmland and water quality can be regarded as the most effective permanent grassing. Evaluation of land use (Land use / Land cover) can be done to help Land Parcel Identification System (LPIS) database and orthophotos. Depending on the method you can use production blocks classified into 3 categories (arable land, special cultures like vineyards, orchards, hop fields, grassland). The highest risk is a production blocks as arable land, the lowest risk contrary to the culture of permanent grassland (Podhrázská and Karásek 2014).

From historical maps, historical photographs and aerial image shows that the 50s of the 20th century was characterized in our countryside grained mosaic facets of fields, meadows, pastures, supplemented by smaller islands of forests and rural settlements in the vicinity of roads and waterways. With the advent of modern agricultural technology have become habitat forming the natural boundaries of indigenous ownership of land an obstacle and they were removed. The average size of land has increased from 0.23 hectares in 1948 to approximately 20 hectares at present. Utilized agricultural land can be classified according to the surface area into 5 categories according to the size of the contiguous block production – very small (5 ha), small (5–20 ha), medium (20–40 hectares), large (40–60 hectares) and extreme (over 60 ha). The weights of the individual classes are determined by the impact of a flat stretch of land at risk of soil degradation and water quality 1 to 5 (Podhrázská and Karásek 2014).

MATERIAL AND METHODS

The method of multi-criterion analysis is based on the evaluation of four risk factors according to valid methodology of Research Institute of Soil and Water Conservation. Four information layers (factors) were selected as relevant for soil and water conservation in the landscape. These factors provided spatial information on the potential risk of the particular factor and were classified into categories 1 to 5 (the value of 5 means the highest risk – weight value of the particular factor).

The surface of agricultural land was identified in these localities using data from the database of the Ministry of Agriculture CR – Land Parcel Information System (LPIS). The land blocks were analysed for the particular risks and thematic layers were created for synthetic evaluation of these risks.

Input data for the first map (size of agricultural land blocks) are the orthophoto image and LPIS database. Land blocks was classify into five different categories (according to size of land blocks). The second map (map of land use) are input data LPIS database and orthophoto image. The land blocks was classify into three categories according to land use of arable land. The highest coefficient (arable land), vineyards and orchards – medium coefficient, grassland with the lowest coefficient. The result of these analyzes are maps that can identify potential dangers in terms of the method of use and the size of agricultural land.

The next stage was handled the analysis of the area in terms of water and wind erosion. Water erosion is creating as potential water erosion in study area. Analysis of the potential water erosion is based on two factors of equation USLE (Wischmeier and Smith 1978). We used a combination of K factor (soil erodibility) and LS factor (a combination of the length and slope). Input data for this

analysis are the database Estimated pedologic-ecological unit, LPIS database and a digital terrain model. Map of potential water erosion shows areas without risk, vulnerable, slightly endangered, endangered and highly or extremely vulnerable.

Analysis of potential wind erosion is based on a published methodology "Optimizing windbreak functions in agricultural landscapes" (Podhrázská et al. 2008), which uses risk assessment area, on the basis of soil and climatic characteristics. The final layer is divided into 5 categories classified as water erosion.

Location of model territory

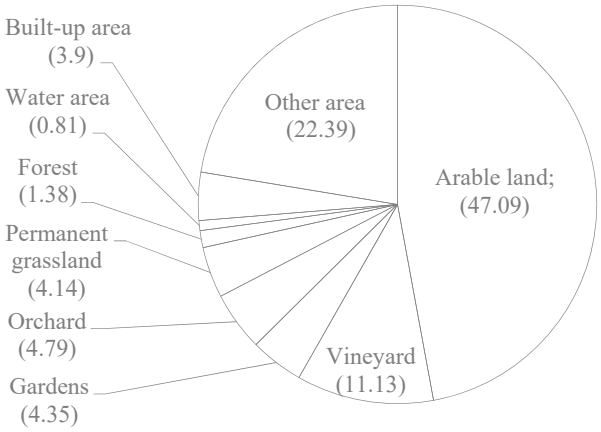
Hustopeče is located on the south-eastern Moravia, 28 km east from Brno and 25 km northwest from Breclav. The city lies on the northern edge of the Pannonian biogeographic province (Culek 1996) and the western edge of the vast Carpathian arc (Buček 2010).

Cadastre of Hustopeče consists of a relatively complex and spatially contrasting relief. They are created as distinctive flat depressions and gently rolling hills and higher ground ridges relief highlands with steeply sloping hillsides, articulated variety of dry valleys (Kirchner 2010). Study area (Hustopeče region) is mostly used as arable land or orchards/vineyards.

Table 1 and Figure 1 confirms the most abundant type of land – arable land, which reaches 47% of the acreage of the cadastral area.

Table 1 Total value of land types according to the land registry; Figure 1 Land use Hustopeče

Land use Hustopeče	he	%
Arable land	1 159	47.09
Gardens	107	4.35
Vineyard	274	11.13
Orchard	118	4.79
Permanent grassland	102	4.14
Forest	34	1.38
Water area	20	0.81
Built-up area	96	3.90
Other area	551	22.39
Total	2 461	100

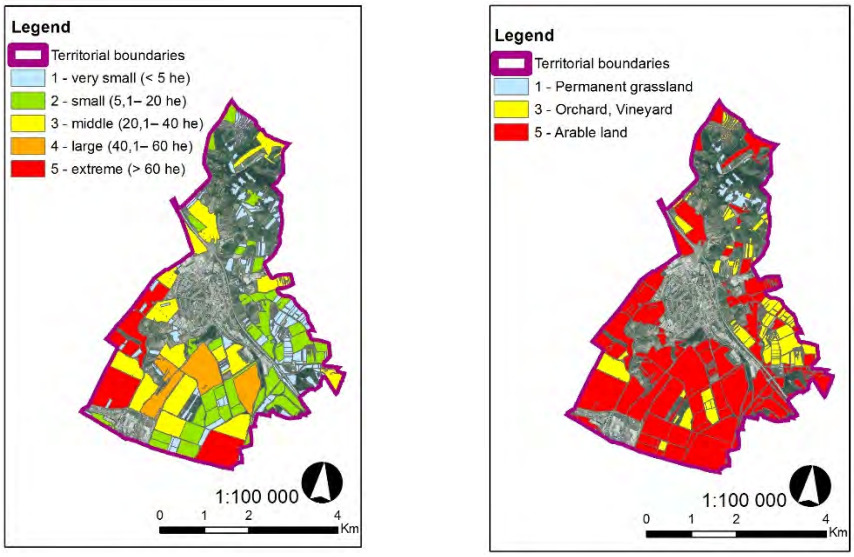


RESULTS AND DISCUSSION

Map of the size of agricultural land – Figure 2 (left) shows size of production blocks (in five categories according to size of blocks in hectares). The map shows that there occur many soil blocks in size 40 hectares and higher, also occur here also land blocks extreme sizes that are potentially susceptible to degradation and may require special protection.

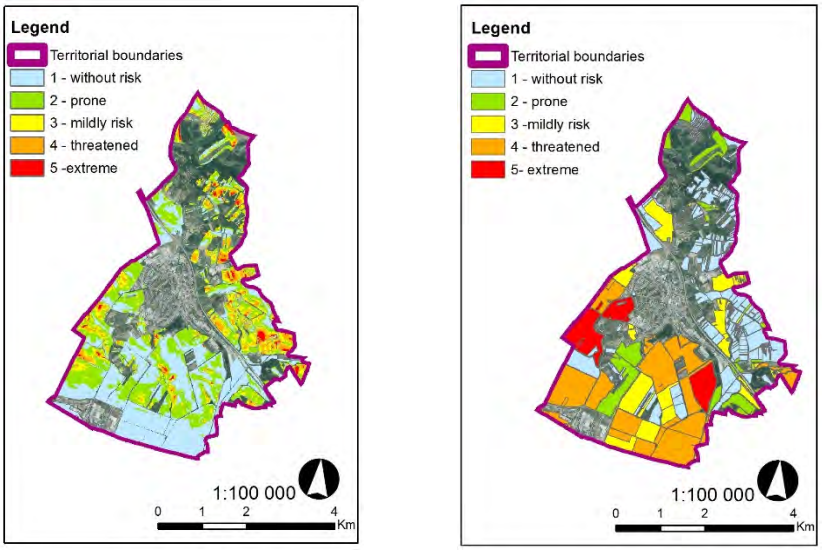
Figure 2 (right) shows map of land use in model territory. This map shows that there is the most abundant arable land, which is confirmed by the Czech statistical office data in Table 1 aggregate value of land species. It also confirmed the fact that the prevalence of permanent grassland is minimal. This phenomenon can be regarded as typical for the region of South Moravia

Figure 2 The map of land size (left) and the map of land use (right)



Map of the potential water (Figure 3 on the left) and wind erosion (Figure 3 on the right) shows the degree of potentially risks areas in the study area. A red areas are the most risked parts of the territory, the brightest displayed areas potentially without threat. Most of agricultural land is located on sloping land – with height erosion risk (red colour). Also, it can be stated that erosion can be greatly influenced by how the way land use and the size of land parcels, which could lead to land degradation.

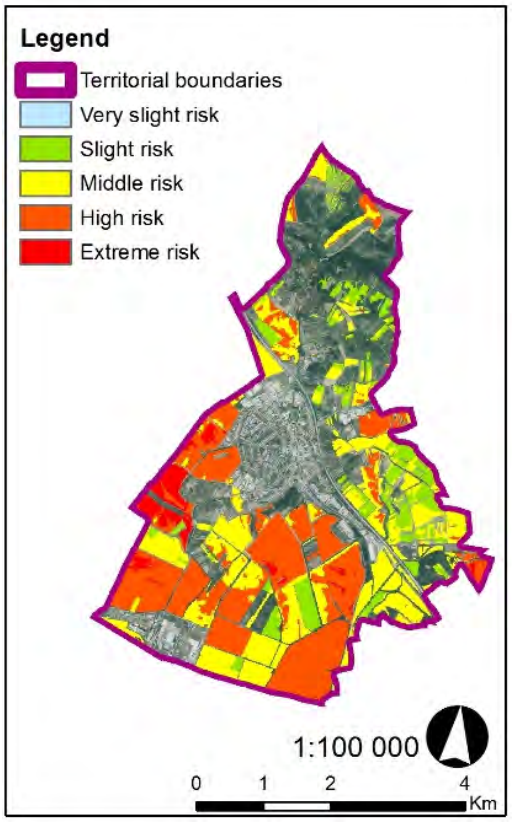
Figure 3 The map of potential susceptibility of land to water erosion (left) and map of potential vulnerability to wind erosion (right)



Then was made an identification of the most risky agricultural land in study area. It was made by multi-criteria evaluation of analytical input layers (four maps) and their synthesis. The "synthetic risk map of agricultural land in terms of land degradation and decreasing water quality" shows the most risked areas in study area. The red and orange areas are the most threatened. There should be processed some corrective measures. Also the effect of any measures will be hi than in other places.

Synthetic map (Figure 4) can be divided into individual data layers and the degree of risk factors can be chosen to focus on the most appropriate procedure and method of protection against these risk areas. For the process of making this synthetic maps were used layers of land size, layer of land use and the potential water and wind erosion. Synthetic map is useful for identify different threat levels of agricultural land in the study area. The map shows that the highest risk are arable land blocks with an area of 20 hectare and higher, mostly with high erosion risks. This map should be used as an input basis identify endangered land blocks / locations in the area of interest, which are suitable for implementation of protective measures to protect soil, water and landscape.

Figure 4 The synthetic map of riskiness of agricultural land in cadastral Hustopeče



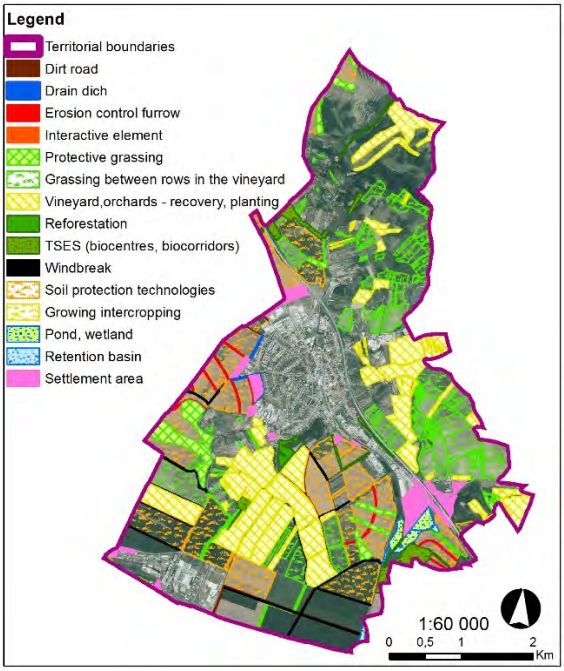
CONCLUSION

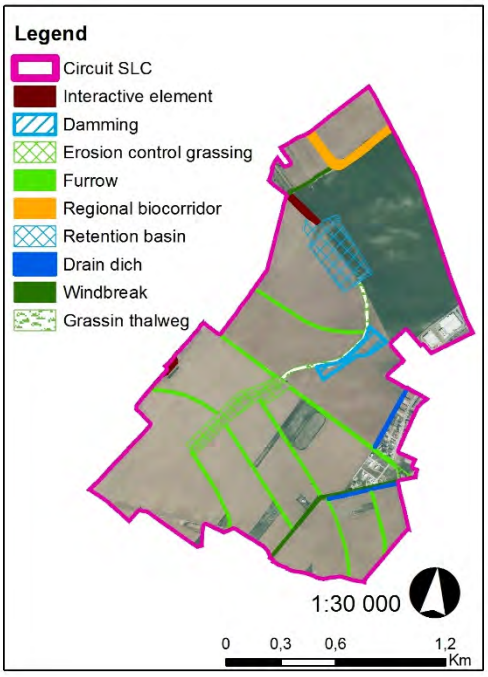
Through the identification of selected risk factors in the interest area Hustopeče was by a multi-criteria method processed synthetic map of riskiness of agricultural land. Through the identification of selected risk factors in the interest area Hustopeče was a multi-criteria method processed synthetic map riskiness of agricultural land. Synthetic map, these analytical maps, combining into one comprehensive map work.

The analysis showed that intensively farmed area of interest is threatened by water and wind erosion. Most of the land is used intensively – arable land, or as a special crops – vineyards, orchards. Permanent grasslands are here only in a small extent. After processing of the synthetic maps can be stated not so positive conclusions. Mainly the southern part cadastral area Hustopeče subject to a high or very high risk of degradation of soil / water. The landscape here is very poor for landscape elements, and this fact is reflected in the overall negative assessment. At the interface cadastral area of Hustopeče and neighboring cadastral area of Starovice was previously handled Land consolidation. Land consolidation was made in 2003 and the main reason was to protect the village built before the flood. The main element of this realization is retention reservoir. It was built to protect the urban area in extreme situations. Another important part is the grassed thalweg, which are designed to slow surface runoff and capture sediments.

For the whole study area was in 2014 prepared by the Research Institute for Soil and Water Conservation design of protective measures. It includes elements designed in a simple landscaping, elements of the proposed plan area, but also other newly proposed measures, which together form the skeleton of eco-stabilizing protective measures. The following maps displays the implemented and proposed measures to protect the territory. Map (Figure 5 left) shows the complex proposal of measures to protect soil and water in the landscape. This is a summation of all the measures which have been proposed by Research Institute of Soil and Water Conservation. On the riskiest areas is designed grassland and soil conservation agro-technologies. The Figure 5 (right side) shows the proposal of land consolidation. Protective measures was situated on the border of cadastral areas Hustopeče and Starovice.

Figure 5 The map of design measures





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DEVELOPMENT AND EVALUATION OF LANDSCAPE TRENDS ON A LOCAL LEVEL (NEVERICE VILLAGE, SLOVAKIA)

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Abstract: The paper presents an analysis of the development trends of landscape elements in the cadastral municipality of Neverice. The object of our study was to analyze the development of the secondary landscape structure over several time periods (1860, 1949 and 2015). Because of the limitation of the contribution, we present the evaluation of development trends concisely but sufficiently from our perspective.

Key Words: village Neverice, historical landscape structure, present landscape structure, landscape element, Slovakia

INTRODUCTION

The development of landscape structure is a continual process formed by natural phenomena and anthropogenic activities (Forman and Gordon 1993). In the study area, mostly anthropogenic activities prevail, which affect original ecosystems, thus changing the development of landscape structure (Kelle and Mariot 1983). Monitoring land-use changes over time is extremely important for the monitoring and evaluation of development trends. It is also important within financial investments in natural resource management (NLWRA 2006). The occupation of land is accompanied by contamination of soil, erosion, reduction of humus and also a reduction of agricultural production and crop yields (Zoppi and Lai 2014). Land use changes of agricultural land in rural areas are significantly affected by ownership relations (Muchova et al. 2015). This topic is part of our dissertation thesis. Our study area consists of several cadastral areas located in the foothill areas of Tribeč. The aim of this paper is to evaluate the development of a secondary landscape structure and to propose suitable land use of the Neverice village area based on historical landscape structures (1860, 1949, 1993), present landscape structure (2015) and evolutional trends arising from analysis.

MATERIAL AND METHODS

The first step in order to meet the aim of the paper was undertaking field research in the study area together with photo documentation. The second step was dedicated to graphical analysis of the study area, executed using ArcView software with landscape elements coding that was used in accordance with Petrovic et al. (2009). The authors divide landscape elements into 6 groups: 1. tree and shrubbery vegetation, 2. grassland, 3. agricultural cultures, 4. subbase baring and raw soils, 5. surface water and wetlands, 6. settlement and built-up areas. Each one of the group is further divided into four subgroups that create 4-digit coding.

Analysis of historical landscape structures were executed on the basis of:

- Year 1993 was evaluated on the basis of a topographical map 1:10 000.
- Year 1949 was mapped using aerial photos.
- Year 1860 was executed on the basis of secondary military mapping.

Analysis of the present landscape structure was carried out on the grounds of field research (2015) and aerial photo coverage carried out in 2007. Topographical maps from year 1993 were used for more precise documentation of roads.

The processes influencing creation or extinction of certain landscape structure groups that affect changes of landscape structures were compiled in accordance with Cebecauerova (2007). The code mark is stated in Table 1 and used in Figure 6.

Table 1 Evolutional trends legend

Unchanged	0	Soil waterlogging	7
Urbanization	1	Draining	8
Industrialization	2	Environment remediation	9
Exploitation of natural resources	3	Loss of agricultural land	10
Agricultural intensification	4	Deforestation	11
Agricultural extensification	5	Wetlands destruction	12
Afforestation	6		

In our study, we use differentiation of areal elements in four levels of code marking. This paper, according states a division in only the first two levels, which means that we can see 15 codes of areal elements – they are explained in Table 2. The code mark is used in Figure 2, 3, 4.

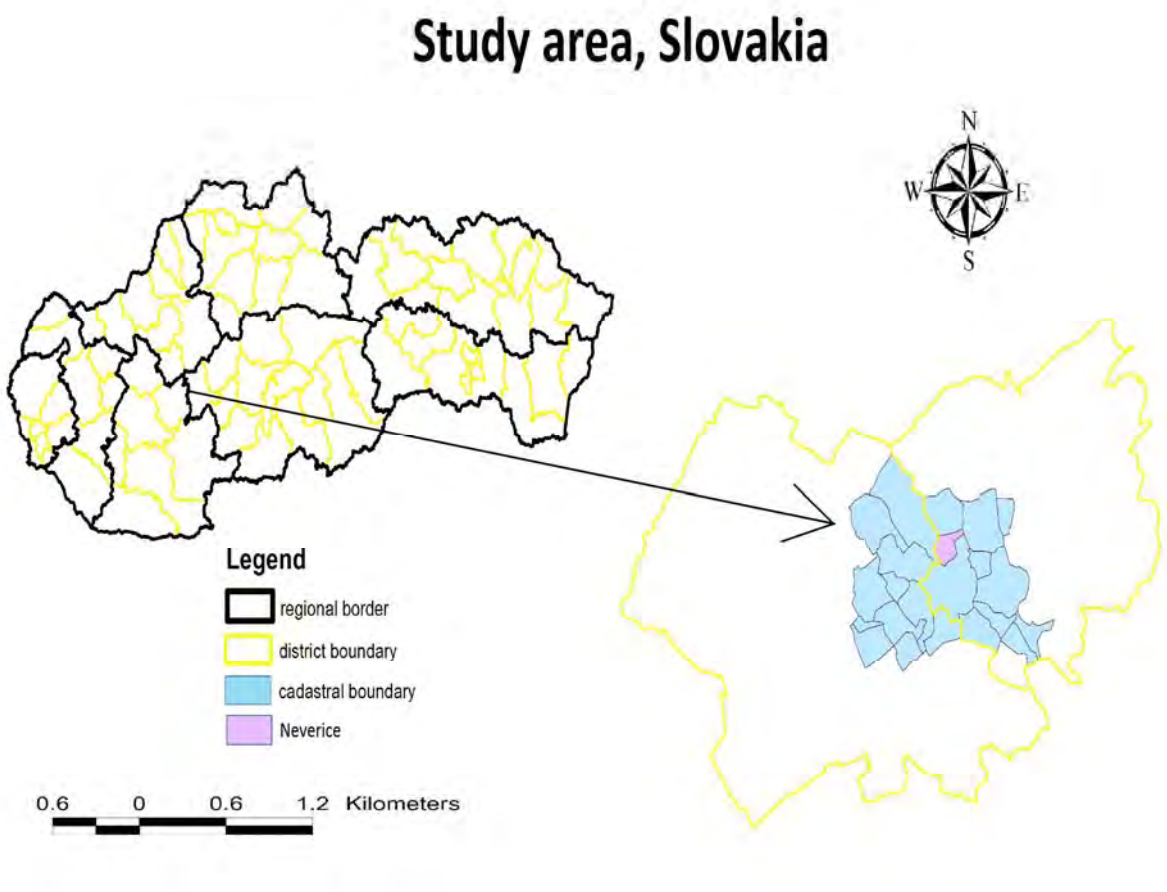
Table 2 Secondary landscape structures (SLS) legend

Sls1	Appellation 1	Sls2	Appellation 2
1	Tree and shrubbery vegetation	13	Non-forest timber vegetation
2	Grassland	22	Permanent grassland
		31	Arable land
		32	Gardens
		33	Multiannual cultures
4	Subbase baring and raw soils	42	Quarries
6	Settlement and built-up areas	61	Housing and complex amenities
		62	Urban and technical vegetation
		63	Sports facilities
		65	Industrial and technical objects
		66	Transport objects

STUDY AREA

Neverice village is 569 hectares large and with approximately 690 inhabitants. Through its territory flows the Drevenica stream, which meets the Jelenec stream in the south. Geographic coordinates of the study area are 48°22´02´´ North latitude and 18°16´31´´ East longitude. Neverice belongs to the Zlaté Moravce district in the Nitra region. The cadastral area of Neverice village is situated in the Žitava wold which belongs to Danube lowland, to the sub province Small Danubian plain, to the province West Pannonian basin, to the subsystem Pannonian basin and to the Alpine Himalayan system Mazur and Drdos (1980). In Figure 1 we can see the location of the study area within Slovakia.

Figure 1 The study area within Slovakia



Flora in the study area belongs to Pannonian flora, European xeroterm flora zone. This indicates that alder forests, poplar-willow wetlands, but also ash-elm-oak forests, oak-hornbeam forests and oak forests are in the area. However, intensive agriculture suppressed this potential. Nowadays, only non-forest shrubbery and tree foliage in the form of accompanying greenery of roads and streams and greenery in agricultural, educational and recreational areas occupy this area. Black locust has the greatest representation of this type of greenery. Flora is represented only by groups of plants close to settlements and by arable plant species.

Fauna is only represented by invertebrates that are immune to anthropogenic activities. The vertebrates represented are mostly rodents, birds-common song-birds, and mammals are represented mostly by the European hare (Havrancik and Siklenka 2013).

RESULTS

In the paper, we present the most important results. In Table 3 and Figure 4 we can see an overall comparison of the gathered data.

Historical landscape structure, year 1860 (Figure 2)

In this period the group of agricultural cultures elements was the most significant. In total it represented an area of approximately 545 hectares (ha) which represented 83%. The most widespread element was group of large fields.

The second group of elements was group of permanent grassland. In this period this group was formed only by elements of intensive grassland. They occupied 84 hectares (13%).

The third group was group of settlements and built-up elements, covering an area of 19 ha (3%).

The group of bared subbase and raw soils and group of water and wetland elements were not recorded.

Historical landscape structure, year 1949 (Figure 3)

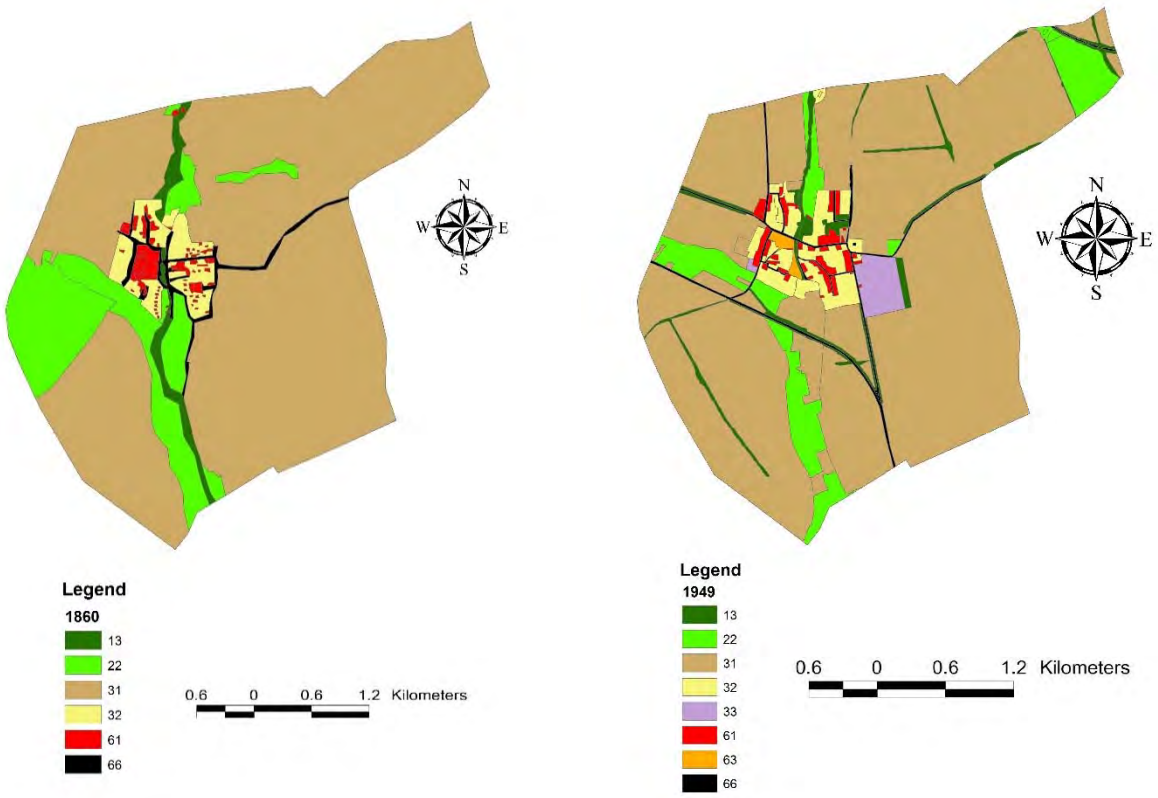
The prevailing group of elements was a group of agricultural cultures covering an area of 566 ha (86%). The most widespread element were large fields. Compared to the year 1860, this group of elements recorded a 21 ha increase.

The second group of elements remained the same as in 1860 (tree and shrubbery vegetation) that represented an area of approximately 51 ha (7.7%). The most represented element was intensive grassland. Compared to the year 1860, there was decrease of 33 ha to the area.

The third group of elements was forest and shrubbery vegetation that represented 22 ha (3%). There was an increase of approximately 10 ha.

The group of wetlands, bared subbase and raw soils was not recorded.

Figure 2 Landscape structure 1860, Neverice *Figure 3 Landscape structure 1949, Neverice*



Present landscape structure (2015, Figure 5)

Nowadays, in the Neverice area the most dominating group is a group of agricultural cultures elements with the largest element of fields. This group represents an area of approximately 602 ha (91%).

Group of settlements and built-up areas is the second biggest, with an area of approximately 34 ha (5%). The most widespread element of this group is continuous individual construction.

The third group with the greatest area is tree and shrubbery vegetation with an area of 21 ha (3%).

Just like historical landscape structures, group of bared sub base and raw soils and group of water and wetlands were not recorded.

Table 3 Comparison of results of the periods under review

SLS2	1860		1949		2015	
	Area (ha)	Percentage (%)	Area (ha)	Percentage (%)	Area (ha)	Percentage (%)
13	12	1.82	21.6	3.28	21.85	3.32
22	83.78	12.71	50.78	7.7	1.16	0.18
31	523.68	79.47	516.75	81.69	565.19	85.84
32	21.27	3.23	38.27	5.81	36.8	5.48
33	-	-	10.18	1.54	-	-
61	17.72	2.69	9.09	1.38	17.41	2.64
62	-	-	1.91	0.29	-	-
63	-	-	-	-	0.9	0.15
65	-	-	-	-	4.63	0.7
66	0.55	0.08	10.42	1.59	11.06	1.69
Total	659	100	659	100	659	100

Figure 4 Comparison of representation of each group of elements (ha) in Neverice

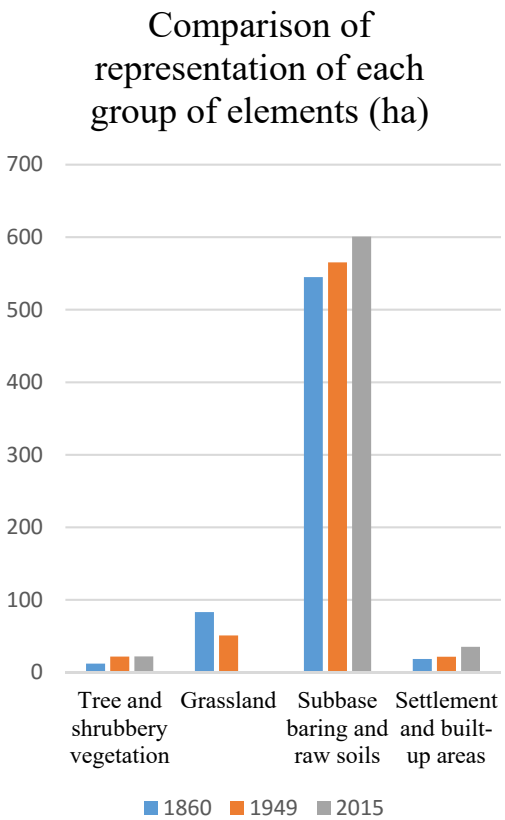


Figure 5 Present landscape structure (2015), Neverice

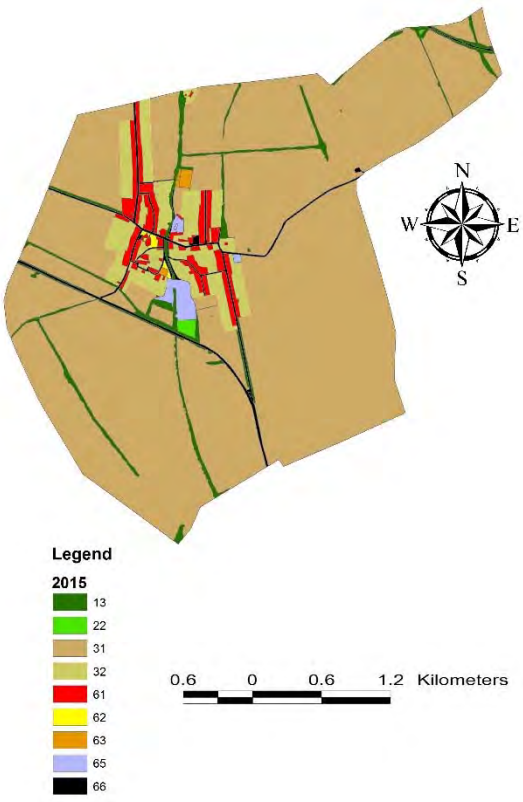


Figure 6 Landscape evolution trends 1949 2015, Neverice

Evaluation of evolutional trends

In the Figure 6 we can see the results of evolutional trends in the years 1949 and 2015. Spatial representation of the Figure is as follows:

0–598 ha,

1–12 ha,

4–45 ha,

6–4 ha.

Size changes of spatial elements within the published periods were summarized in Table 3.

DISCUSSION AND CONCLUSION

On the basis of this study we can define the landscape of Neverice village according to Ruzicka et al. (1978) as **cultural** (anthropogenic) landscape that was differentiated by natural and socio-economical influences.

According to research, in every period there prevails a group of agricultural cultures with a dominating element of large fields, we can therefore define this cultural landscape as **agricultural, which** would go extinct without anthropogenic activities. Landscape of this type was created secondary and has mainly economical character.

The frequency of land use puts our area of interest into **intensively cultivated landscapes**, where widely developed farming has persisted over several centuries (Gabris 1998).

Neverice village is situated approximately 10 km from the district city of Zlate Moravce and is approximately 23 kilometers from Nitra. Commuting to work, to health care facilities and other socio-economical services is very comfortable.

Nowadays, the village could be a very attractive place to live, since in Nitra a new industrial zone is being created that could create 7000 jobs by the year 2019. This major investment could lead to new investment toward study area. We propose that building smaller industrial zones in Neverice could have a positive impact on the economic growth of Neverice and the surrounding villages.

Labor migration of the population should have a positive impact on population increase. This phenomenon supports our proposal to create new construction opportunities for the element of continuous individual construction in the area as well as supporting the sale of existing properties.

Villages could support the idea of improving the attractiveness of an area by reconstructing public communications and sewerage. Similar results were published by Petrovic (2005).

Proposals were communicated to municipal authorities, in the view of municipal documentation and our vision of potential village development, such as in study of Mederly et al. (2008).

We propose the creation of several elements. In significantly agricultural landscape, we propose to complete the landscape with balks that would have a functional potential as anti-erosion barriers and a biocoridor that would connect surrounding areas with biocentres. Gardens and continuous individual constructions are some of the other proposed elements that are required in the village. Because of the location, we propose the construction of industrial zones and technical objects in the southern part of the area, close to the R1 highway.

The results of our study could be used for assessing the influences on the environment (E.I.A), for landscape-ecological planning and for the evaluation of biodiversity values (USES – The Local Territorial System of Ecological Stability) as well as serving as a great background for the creation of a new development plan of the village.

ACKNOWLEDGEMENTS

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Section – Food Technology

QUALITY PUFF PASTRY PRODUCTS AND THE QUANTITY OF FAT AND DIFFERENT EFFECTS ON LEAF PROCESSING

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Abstract: The aim of this study was to assess the impact of production technology on the technological and sensory value of puff pastry. The effect of recipe innovation has been validated by method of experimental baking and evaluated by objective measurements. We have monitored the influence of the percentage of fat content in the recipe on the number of produced layers. Sensory analysis and objective measurements revealed differences among the various kinds of processing. The recorded values were statistically processed using STATISTICA 8 software. Eighteen trained evaluators have conducted the sensory evaluation. Products that received the best sensory rating and were also the thickest (1.8 mm) were made in a combination of 60% fat for dough rolling and 243 layers in the classic recipe. Products with 60% fat in combination with 729 layers in the improved recipe used the Volumax enzyme product. The highest corpus (2.8 mm) was in the improved recipe with the fourth folding (2187 layers) containing 60% fat. The addition of the enzyme preparation significantly improved the sensory quality and height of the corpus.

Key Words: puff pastry, production technology, baker's attempt, sensory analysis introduction

INTRODUCTION

Puff pastry is gaining ever increasing popularity among producers and consumers alike. Over the last decade, this product line has had the greatest increase in volume of production and consumption of fine bakery products. The main contribution is the automated production on production lines, which facilitates production in very productive and rational manner. An important aspect is the possibility of cooling and freezing of basic laminated dough and finished raw products (Skoupil 2004). In the Czech Republic, the most widespread manufacturing process has three phases (Bláha et al. 2001): (1) treatment of fat and preparation of flour-water dough, (2) wrapping a block of fat with flour-water dough, and (3) folding of dough.

The structure of puff pastry consists of alternating of watery component (flour-water dough) and fat component. This structure must be maintained in the dough and there should be no mixing of layers even after multiple folding and thinning of the original layers. The very low adhesion also contributes to the uniform distribution of layers. The watery component must have a sufficient gluten content, which is flexible, ductile, and easily swelling in order to establish an appropriate ratio between the free water and colloidally bound water that limits the unwanted adhesion to fat (Newberry et al. 1996, Příhoda et al. 2003). The used fat should be characterized by high ductility and therefore its full emulsification is crucial. The fat component must, at a given temperature, have the same consistency as the watery component. This is because a stiffer fat during dough rolling disturbs the dough structure, while a softer fat is being absorbed into the watery component. In both cases, this adversely affects the volume and characteristics of the product (Morren et al. 2015). The specialty of manufacturing laminated dough is folding and rolling of layers with delays for maturing, which takes place in a cold environment. Folding of layers is carried out in a three-fold or four-fold system. The optimal number of layers is 144 while we usually count the fat layers. Today, the prevailing production method is commercial automatic production of laminated dough carried out on modern machinery by using a complex lamination and shock freezing at -30 °C and distributed to customers by freezer trucks (McGill 1975). When baking products from laminated dough, several important physicochemical and chemical processes gradually

take place in accordance with increasing temperature. It starts with gelatinization of starch and protein coagulation, which contributes to making the corpus skeleton. Then, it continues with converting the water from the watery layer to steam, which as an aerating medium contributes to the separation (lamination) of the individual layers of dough. This leads to aerating, increased volume of the products, and to the formation of its fragile structure resulting from regular alternation of horizontally laid layers of dough and fat.

The process is terminated by the formation of yellow dextrins, conducive to the creation of typical flavouring and aromatic substances (Macias 2016). The manufacturing technology was modified several times, but the final product always includes horizontal lamination of layers of fat and dough (Pidmit et al. 2008). The quality of products from laminated dough depends not only on the quality of used raw materials, but also on the production technology of laminated dough (Bláha et al. 2001). Pidmit et al. (2008) suggested an addition of fat during the process for rolling of the laminated dough in the range from 45% to 85%.

MATERIALS AND METHODS

The topic of this study was the influence of production technology, amount of used fat and different recipes in dough preparation on the quality of puff pastry. The experimental work, namely the preparation of laminated dough as well as its baking and evaluation were carried out in the technological and chemical laboratory of Mendel University. The Ireks-Enzyma Company has provided all the necessary ingredients and equipment. The laminated dough was prepared according to the classic recipe (see Table 1) and according to the recipe supplied by the Ireks-Enzyma Company (hereinafter referred to as "improved" recipe) (see Table 2). The Volumax additive with its enzyme component contributes to the improvement. The weight of the testing dough was given by used recipe (see Table 1 and 2).

Table 1 Classic recipe

Material	Amount
Fine flour T 530	100%
Egg melange	6.7%
Cooking salt	0.8%
Vinegar	1.7%
Water	41.7%
Fat	40 and 60%

Table 2 Improved recipe

Material	Amount
Fine flour T 530	100%
Volumax	1.4%
Cooking salt	1.2%
Sugar	2.9%
Gluten	1.6%
Water	50%
Fat	40 and 60%

For the preparation of the watery part of dough, we have used fine light wheat flour T530 of good quality, suitable for the preparation of laminated dough, as seen in the Table 3 summary.

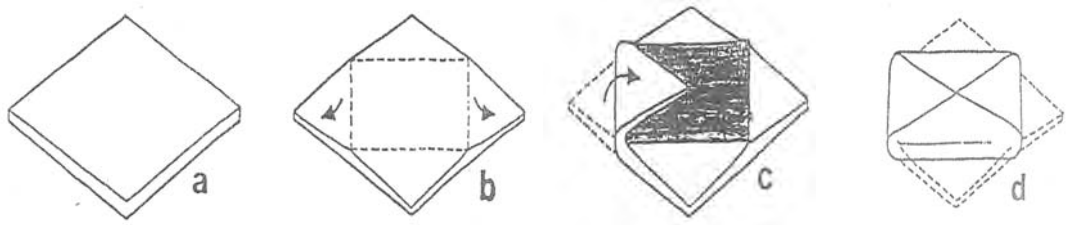
Table 3 Basic quality parameters for flour for laminated dough

Moistness [%]	14.2
Wet gluten in dry matter [%]	34.8
Gluten index	95
Falling number [s]	349
P/L(alveograph)	80/118
Zeleny test [ml]	51
W (alveograph) [$Jx10^{-4}$]	278
Flour valence at [14 %]	60.3

As fat, we have used the ductile Zich-Platte margarine, which has a suitable composition, and meets the manufacturing requirements in a wide range of temperatures. Its exceptional quality allows even fat dosage reduction while maintaining product quality (Skoupil 2004). A trimmed fat layer was wrapped with flour-water dough to form an envelope (see Figure 1) and rolled to a thickness of 8 mm.

The resulting semi-product was folded three times, which, after standing in an environment of about 10°C was repeated several times according to the methodology in 10 minute intervals. For rolling, we have used a Compass dough sheeter of the Rondo Doge Company. The flour-water dough layer under the fat prism and above it must be equally thick, so that the dough during the next folding and rolling would not tear (Příhoda et al. 2003, Morren et al. 2015).

Figure 1 Method of fat wrapping with flour-water dough layer, the so-called envelope



To assess the influence of the proportion of fat and number of folds on the quality of the final product, we have made dough with 40% and 60% fat on the proportion of flour. Each dough was folded always three times (total 7 times), thereby achieving 81, 243, 729, and 2187 layers. After the final rolling to a thickness of 2 mm, we have cut the tested slab using a cutter to form a square 100 x100 mm. To stabilize the height of the baked product and to refine its assessment, before baking we have pierced the top layer of dough with a roller docker to prevent blistering, and put a load at the centre of the product with 10 g of poppy seed filling. After treatment, the sheets with products were placed in a hot air oven MIWE Aeromat that due to the heating principle permits to reduce the temperature and baking time. Laminated dough was baked at 200 °C for 10 minutes.

Evaluation of finished products was carried out after cooling and always after two hours. We have measured the height of samples in the centre of the product with a caliper and expressed it in millimetres. For the sensory evaluation, we have selected samples with fat content of 40% and 60% and the number of 4, 5, 6, and 7 folds, which corresponds to 81, 243, 729, and 2187 layers. All samples underwent sensory evaluation using several parameters. They included crust colour, height and shape, lamination, fragility, mouthfeel and overall impression using an ordinal sensory scale, where 1 was the best quality while 5 was the least acceptable sample (Renzetti et al. 2016, Dukic et al. 2009). Eighteen trained evaluators conducted the evaluation.

RESULTS AND DISCUSSION

The results of measuring the product height with a different fat content ratio and number of folds have demonstrated that in the classic recipe, as evident from Figure 2, the maximum was achieved by the second folding (234 layers), for the products containing 60% fat it was also the double folding, where the product height was by 0.5 mm higher. Wickramarachchi et al. (2015) reported quadruple folding for the classic recipe to be optimal.

For the improved recipe in products containing 40% fat, the maximum height at the fourth folding was 2.4 mm. When using 60% fat, the achieved maximum height was 2.9 mm, as seen in Figure 3. Often, the fat is rolled into the flour-water dough layer thus undermining the aerating effect while the height of the product is reduced.

The number of folds depends on the quality of flour, baker's skills and the type of product. The dough made from flour with strong gluten can tolerate a larger number of folds and requires a longer period of maturing between repeated rolling. For dough with weaker gluten the effect is opposite (Moran et al. 2015).

Laminated dough made according to the improved recipe exhibits more stable values. McGill (1975) carried out measurements at 75% fat to flour and English method of wrapping. The form of the curve was the same as in our work. The highest increase of the product was at the level of fourth repetition of folding. As indicated by Newberry et al. (1996), it is likely that the area of this maximum will depend on the perfection and uniformity of rolling and folding, in order to avoid harming the integrity of the layers.

Shaped corpuses are brushed with egg yolks diluted by milk before baking. Through brushing, products receive better colour and the escape of vapour during baking through the surface is slower (Haegens 2014, Simovic et al. 2015). According to the results of our observation, brushing has no demonstrable effect on the height of the product.

Figure 2 Dependence of the height of the product on the amount of fat in the classic recipe

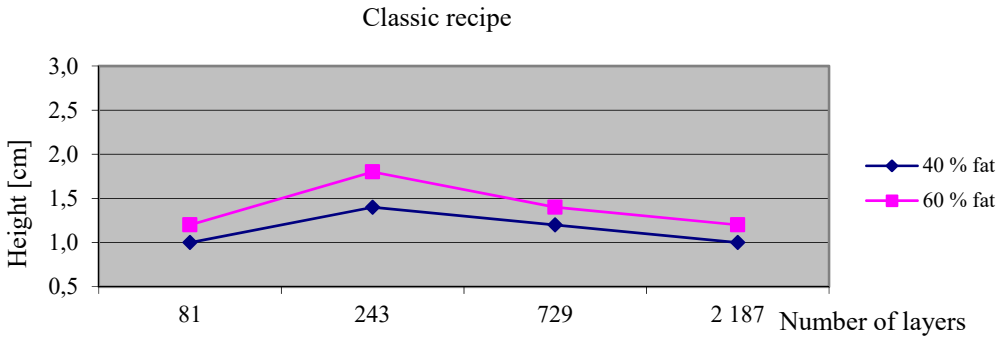
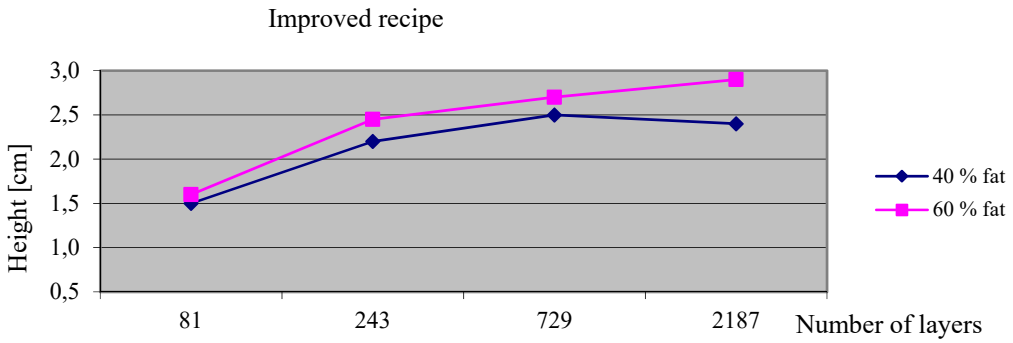


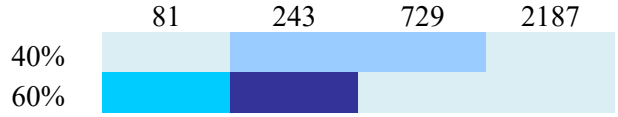
Figure 3 Dependence of the height of the product on the amount of fat in the improved recipe



The resulting sensory protocol was compiled from scoring average values of a given parameter from eighteen trained evaluators who assessed the intensity of sensory perception using an ordinal scale (1 – best quality, 5 – least acceptable sample). We have evaluated recipes with ratio of fat 40% and 60% for rolling. Each recipe in combination had 81, 243, 729 or 2187 layers in the dough**.**

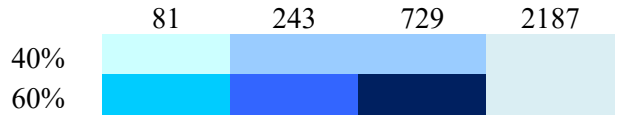
Samples were sorted in descending order according to the proportion of fat in two rows and four columns with rising tendency of layers from left to right. The colour intensity represents the intensity of sensory perception in so arranged samples (see Tables 4 and 5). The more intense the colour, the better is the positive sensory evaluation of the sample. In our observation of the classic recipe, the best sensory evaluation received the variant with 60% fat at 234 layers. The improved recipe achieved the best sensory evaluation at 60% fat content and 729 layers.

Table 4 Diagram of intensity of overall sensory perception for the classic recipe



Legend: the darkest color - the best sensory quality (graded 1), the lightest color - the worst sensory value (graded 5)

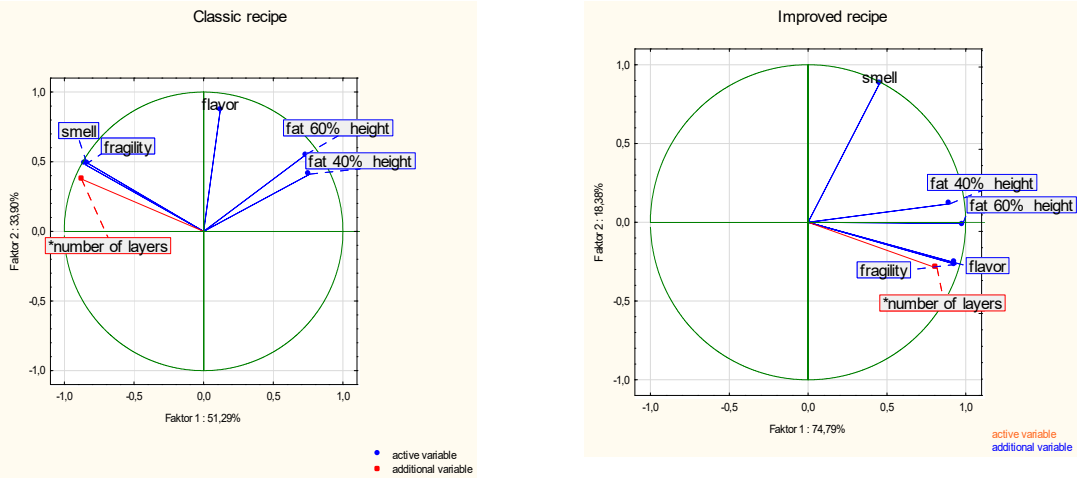
Table 5 Diagram of intensity of overall sensory perception for the improved recipe



Legend: the darkest color - the best sensory quality (graded 1), the lightest color - the worst sensory value (graded 5)

Figure 4 illustrates an output of data processing in Statistica 8 software as a projection of the variables on the factor plane. The degree of dependence of individual variables is evaluated here according to the size of the angle between them. The smaller the angle, the stronger is their dependency. Concurrently, the cosine of the angle indicates the approximate value of the correlation coefficient. If the arrows are horizontal and point in the same direction than it means that the variables are dependent on each other positively. If the arrows are horizontal but point in the opposite direction than it means that the variables are dependent on each other negatively. If the arrows are perpendicular to each other, variables can be considered independent. The length of an arrow indicates the variability of the measured data.

Figure 4 Projection of the variables on the factor plane



In Figure 4, we can see that the recipe closely positively correlates with smell and fragility as well as dependence of the height on fat content in the classic recipe. Fragility and flavour have a strong correlation with the recipe and the dependency of the height of the baked corpus depends on the amount of the used fat. Smell in the improved recipe is perpendicular to the number of folds, which means that the variables are independent of each other. The greatest variability of measured values (the longest arrow) was recorded in the smell.

CONCLUSIONS

According to past experience, we could expect that, due to the influence of different amounts of fat on rolling and due sheeting technology, there will be change in the character of the dough. The addition of fat above 60% and removing of fat for rolling below the threshold of 40% has caused structural damage of lamination, even a loss of the typical character of the laminated dough. In practice, the optimal number of layers in laminated dough is 144. This work has shown that the products are sensorically acceptable in the range between 81 and 729 layers. However, it is necessary to draw attention to the fact that all assessments have been carried out in laboratory conditions with the utmost care. We can therefore assume that in the operating conditions, results may be different.

Sensory properties, evaluated by the customer, were evaluated by a complex evaluation of pastries using the method of sensory analysis. The best sensory evaluation of products in classic recipes, have been made in the combination of 60% fat for rolling and 243 layers. The improved recipe received the best sensory evaluation with 60% of fat content in combination with 729 layers. The addition of the enzyme preparation significantly improved the sensory quality and height of the layered corpus.

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THE NEW PACKAGING MATERIAL WITH A PROTECTIVE EFFECT AND ITS INFLUENCE ON THE MICROFLORA AND COLOR OF MEAT

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Abstract: The aim of this study was to test the effect of packaging foil with essential oils on meat samples. Slices of pork roast were packed and stored in a refrigerator for 7 days. Different groups of microorganisms were determined. Significantly lower amounts of aerobic plate counts were observed in a prototype compared to the control after 7 days of storage. For the other groups of microorganisms was not found significant differences. This study describes the importance of testing of the antimicrobial agents effect directly on foodstuffs. The study was also focused on monitoring of the packaging foil effect on colour of meat.

Key Words: aerobic plate count, lactic acid bacteria, psychrotrophs, coliforms, enterococci

INTRODUCTION

The main purpose of the packaging material is to protect food from spoilage. According to the Nařízení Evropského parlamentu a Rady ES č. 1935/2004 (2004) exist the active food contact materials and articles. These are intended to extend the shelf-life or to maintain or improve the condition of packaged food and are designed to deliberately incorporate components that would release or absorb substances into or from the packaged food or the environment surrounding the food. The active food packaging is already successfully used in the US, Australia and Japan. However, in the European Union, these systems are regulated by strict legislative criteria. Active packaging cannot exceed the overall migration limit of 10 mg/dm^2 or 60 mg/kg of food (Vědecký výbor pro potraviny 2008, Nařízení Komise EU č.10/2011).

Active food packaging includes innovative and promising antimicrobial packaging, in which the packaging container, product and environment mutually interact in order to extend the lag phase or inhibit the growth of microorganisms. This concerns both pathogenic microorganisms, that can cause foodborne illnesses and endanger the health of consumers, and microorganisms that cause food spoilage, resulting in considerable economic loss. Suppression of these microorganisms increases the products shelf life and improves its quality and safety (Suppakul et al. 2008, Ghabraie et al. 2016).

Increasingly longer shelf life and higher level of protection against contamination is required by the sellers for the products to be placed on the market, especially to avoid the waste of food. A variety of chemical additives are used to extend the expiration date, but lately alternative natural products are given a great interest (Marino et al. 2001). Plants contain many biologically active substances, essential oils are considered as one of the most popular among them. Essential oils are prepared by steam distillation, and generally are consisted of a mixture of phenolic compounds, terpenes, terpenoids, aldehydes and alcohols. It is generally known that essential oils of some plants possess antimicrobial, antiviral and antifungal activity (Burt 2004, Jay et al. 2005, Laird and Phillips 2012, Bučková et al. 2016). Although most essential oils are Generally Regarded as Safe (GRAS), their use in food is often restricted from a sensory point of view, given that the effective dose to reduce microorganisms may exceed organoleptically accepted level (Zinoviadou et al. 2009).

During the past decades, many tests have been carried out in order to evaluate the antimicrobial activity of natural compounds against undesirable microorganisms. However, most of them have been made in synthetic growth media using diffusion disk method (Bučková et al. 2016). It is preferable to test the antimicrobial properties directly on real food, where various compositions of the product, various microflora, and the availability of nutrients can influence the antimicrobial effect of essential oils (Ha et al. 2001, Gutierrez et al. 2009, Laird and Phillips 2012).

A several studies of antimicrobial foils were carried out. Significantly lower aerobic plate count (APC) was observed during the entire period of storage (12 days) of the beef that was tightly packed into the foil with oregano oil in a study by Zinoviadou et al. (2009). In a study by Suppakul et al. (2008), where cheddar cheese was tightly packed into the antimicrobial film containing the active substance of basil, occurred a significant reduction in the APC, but no difference was observed for coliform bacteria and yeasts and molds. In study by Ha et al. (2001) the film coated with grapefruit seeds extract significantly suppressed growth of APC and coliforms on the ground beef.

The effect of essential oils in the vapour phase can be completely different from the effect in direct contact. Lipophilic substances in the water phase form micelles which reduce attachment of essential oils on microorganisms, while in the vapour phase can be realized direct attachment. Bacteria can be therefore inhibited by some essential oils even more effectively in the vapour phase than in the liquid phase (Laird and Phillips 2012, Bučková et al. 2016). Another advantage is that substances are better dispersed in the vapour phase and therefore the organoleptic properties of food are not much affected in comparison with the liquid phase (Laird and Phillips 2012).

Colour of meat is one of the most important criteria affecting the choice and preferences of consumers (Pathare et al. 2013, Quevedo et al. 2013). It is the interaction of light upon reflection or passing of the food, and it may decide on an overall assessment and acceptance of the product itself (Saláková 2012). For meat, the assessment of colour, along with texture, aroma and taste, is of the most important descriptor of subjective sensory evaluation and in case of measurement is desirable to compare results, and determine the colour difference between the samples using a mathematical method of deviation ΔE^*_{ab} (Jůzl 2014).

The aim of this study was to test the effect of the packaging foil with essential oils on selected groups of microorganisms occurring on the surface of the meat stored at refrigerator for 7 days. The study is also focused on monitoring the effect of this foil on colour of meat.

MATERIAL AND METHODS

Samples of boneless pork roast were used to test the antimicrobial activity of packaging foil with a mixture of essential oils (clove, cinnamon, thyme). Samples were purchased in stores in Czech Republic. Experiment was carried out twice with an interval of 5 months. The meat was cut into slices of about 100 g. Slices were packed one by one in polystyrene trays and sealed with packaging foil without coating of protective agents (control) and packaging foil coated with a protective material (prototype). During the storage (0, 2, and 7 days) at 4 to 6 °C following groups of microorganisms were monitored: aerobic plate counts (APC) on PCA (Biokar Diagnostics, France) at 30 °C for 72 h, lactic acid bacteria (LAB) on MRS (Biokar Diagnostic, France) at 30 °C for 72 h, bacteria of genus *Pseudomonas* on *Pseudomonas* CN agar at 37 °C for 48 hours), psychrotrophic microorganisms on PCA at 6 °C for 240 h, enterococci on SB (Merck, Germany) at 37 °C for 72 h, coliforms on VRBL (Biokar Diagnostics, France) at 37 °C for 24 h. Swab method was selected for the surface microflora of meat monitoring. A smear was taken from the area of 10 cm^2. Data were statistically analyzed using the software Statistica 12. Shapiro-Wilk normality test was selected in order to test the normality of the data. Two-sample t-test was chosen for data with normal distribution to determine the difference between the control and the prototype. Nonparametric Mann-Whitney test was chosen for data without normal distribution.

The meat colour was measured on a spectrophotometer CM-3500d (Konica Minolta, Japan) in reflectance mode on the upper and lower surfaces of the cut of meat. The colour was evaluated in the CIELAB system, where L* represents luminants of lightness component, and takes values from 0 to 100 (black to white), and the other points are determined by the chromatic coordinates, a* (-a* green to +a* red) and b* (-b* blue to yellow +b*), allowing to express the colour in this three-dimensional system,

and then its changed by objective deviation ΔE^*_{ab} (CIE 1976, Třešňák 1999, Quevedo et al. 2013), or the total colour change. Total colour change ΔE^*_{ab} is an important, widely accepted method of colour difference evaluating. According to its size, it can be evaluated by the degree of disagreement of two colours, when $\Delta E^*_{ab} < 1.5$ is slight disagreement, $\Delta E^*_{ab} = 1.5$ to 3.0 clearly recognizable, $\Delta E^*_{ab} > 3.0$ moderate to significant disagreement (Třešňák 1999). Statistical evaluation of differences between groups was made through t-test in UNISTAT 3.5 software.

RESULTS AND DISCUSSION

Coating with antimicrobial effect for printing on polymer film (Utility model no. 27586) was developed during project TA03010799. Porous filler with the active agent is dispersed in an aqueous solution of the carrier coating. The active agent is gradually released from the dry coating film, sprayed onto the foil, due to the moisture from the packaged food. Based on testing of these samples of the film with sprayed oils or their mixtures (Kalhotka et al. 2015), packaging foil with suitable parameters for food packaging (which was tested in this study) was produced.

Samples of pork roast were chosen for testing the antimicrobial activity of the film directly on the foods. Gutierrez et al. (2009) found that high protein content and low pH increased the antimicrobial activity of oregano and thyme. Therefore, it should be further investigated application of essential oils on foods rich in proteins and/or foods containing simple sugars with low pH which can support the antimicrobial effect of essential oils. The average numbers of different groups of microorganisms on pork slices are shown in the following table (Table 1).

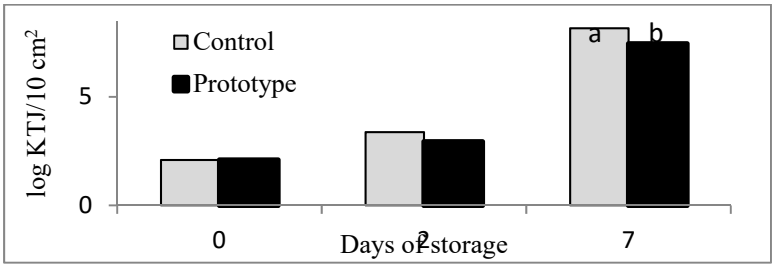
Table 1 Average values of microorganisms (CFU/10cm^2) on meat during storage

Days of storage	Treatment	Coliforms	Enterococci	APC	Psychrotrophs	LAB
0	-	ND	ND	1.2×10^2	3.4×10^1	ND
2	Control	$<10^1$	ND	2.4×10^3	1.2×10^3	3.3×10^1
	Prototype	ND	ND	8.5×10^2	6.4×10^2	2.1×10^1
7	Control	6.9×10^2	$<10^1$	1.4×10^8	1.9×10^8	1.6×10^4
	Prototype	ND	ND	2.8×10^7	2.9×10^7	3.2×10^3

Legend: APC – aerobic plate count, LAB – lactic acid bacteria

Idea of overall contamination of meat is provided by aerobic plate count (Figure 1). Aerobic plate count was immediate before packaging 1.2×10^2 CFU/10 cm^2 of meat. APC increased the second day after the packing in the control of one order of magnitude (2.4×10^3 CFU/10 cm^2). APC of meat packed in prototype foil was kept at 8.5×10^2 CFU/10 cm^2. Further increase was observed after 7 days of storage; up to 1.4×10^8 CFU/10 cm^2 for control, the prototype however increased only to 2.8×10^7 CFU/10 cm^2 of meat. This difference in aerobic plate count between the control and prototype after 7 days of storage was statistically significant ($p<0.05$). According to Feiner (2006), characteristic changes of spoilage (sliminess, souring or discoloration) are fully exhibited when the numbers of bacteria reach around 10^7/cm^2 of meat, but a moderate signs of spoilage can be observed much earlier, between 10^5 and 10^6 bacteria per 1 cm^2. This was confirmed as the seventh day after packing was possible to observe off-odor and visible colour changes. Visible colonies of molds on the samples of meat at the end of storage were not observed.

Figure 1 Average values of aerobic plate counts (CFU/10cm^2) on meat during storage



Legend: different letters indicate significant differences ($p<0.05$).

Psychrotrophic microorganisms were one of the most represented groups of microorganisms on meats surface. Psychrotrophs are known for their ability to grow at low temperatures. Optimum temperature for their growth and reproduction is between 20 and 30 °C, but they grow well even below 7 °C. Number of psychrotrophic microorganisms was immediately before packaging 3.4×10^1 CFU/10 cm^2 of meat. On second day counts of psychrotrophs increased by one order of magnitude (at 6.4×10^2 CFU/10 cm^2) in the prototype, and by two orders of magnitude (at 1.2×10^3 CFU/10 cm^2) in control. After 7 days we observed a further increase up to 1.9×10^8 CFU/10 cm^2 in the control and 2.9×10^7 CFU/10 cm^2 in the prototype. Despite the fact that the average number of psychrotrophic microorganisms was lower by one order of magnitude at seventh day of storage in the prototype, this difference was not statistically significant ($p>0.05$).

Bacteria of the genus *Pseudomonas* are described as the most important representatives of psychrotrophic microorganisms. These were not detected in the first repetition of experiment in any of the samples. Pseudomonads therefore were not determined in the second repetition of microbiological analyse, although it is stated that pseudomonads are the most important bacteria causing spoilage of aerobically stored meat (Jay et al. 2005, Zinoviadou et al. 2009). *Pseudomonas aeruginosa* is known as a saprophytic bacterium that can cause off-odors and discoloration of meat stored at low temperatures. Pseudomonads exhibit resistance against the action of antimicrobial agents from plants usually (Ghabraie et al. 2016). However, in study by Zinoviadou et al. (2009), in which the beef was tightly packed in packaging foil with oregano oil, a significant reduction in the number of bacteria of the genus *Pseudomonas* was observed during the whole period of storage (12 days), when compared to the control.

Enterococci and coliforms may contribute to spoilage of meat, but may also produce biogenic amines (Kalhotka et al. 2012). The correctness and accuracy of technological processes and good sanitation of tools and equipment is indicated by the number of coliforms (Gorner and Valík 2004). In our study, counts of enterococci for the entire storage period did not exceeded 10^1 CFU/10 cm^2 of meat and counts of coliform bacteria did not exceed 4.5×10^3 CFU/10 cm^2 of meat. Numbers of these bacteria were therefore kept relatively low. On the basis of the first repetition of microbiological analysis, when coliform bacteria were detected, a significant difference between control and prototype at the seventh day after packing was observed. These results suggested, that prototype could have decreased counts of coliforms in meat samples. But in the second repetition of microbiological analysis coliforms were not detected in control neither prototype, so statistical analysis was not performed. By evaluating all the results combined, no significant difference was found.

Lactic acid bacteria (LAB) include several genera of Gram-positive bacteria. These bacteria ferment sugars to form lactic acid, which lowers the pH of the meat. In large amounts, LAB can cause a number of negative changes such as sliminess or discoloration (Jay et al. 2005). Lactic acid bacteria were not detected immediately before packing, the second day after packing their numbers increased to the order of magnitude 10^1 CFU/10 cm^2 in control and the prototype both. The seventh day after packing the counts of LAB in prototype were increased by two orders of magnitude (3.2×10^3 CFU/10 cm^2), whereas in the control by the three orders of magnitude (1.6×10^4 CFU/10 cm^2). A statistically significant difference, however, was not found. In a study by Zinoviadou et al. (2009) beef tightly wrapped in packaging foil with oregano oil, almost complete inhibition of gram-positive lactic acid bacteria was observed. Gram-negative bacteria are generally more resistant to the plant extracts in comparison with Gram-positive, due to lipopolysaccharide outer membrane of the cell wall. However, the hydrophobic components of essential oils are able to penetrate into the periplasmatic space of gram-negative bacteria via the outer membrane proteins (Zinoviadou et al. 2009).

The following tables (Table 2 and Table 3) show the parameters of colour measurement. With time, the specific lightness L* was increased. In case of control was lightness significantly ($p<0.05$) greater in the upper part of the slice compared to the experimental group. Chromatic coordinates a* and b* in the experimental group also showed lower increase of green and yellow colour than the control, especially at the lower parts of a slice. In a study by Quevedo et al. (2013) was observed increase of redness intensity (a* value) for a short time (from 0 to nearly 5 hours), followed by decrease up to 2 days and nearly 5 hours of storage, indicating that discoloration in the samples started after nearly 5 hours of storage.

Table 2 Parameters of meat colour (CIELAB) and its changes over time of storage at upper part of slices

Days of monitoring	Treatment	Upper part of slices			ΔE^*_{ab} (D65)
		L*(D65)	a*(D65)	b*(D65)	
0	-	60.75 ±1.45^{a}	0.88 ± 0.88^{a}	11.43 ± 0.2^{ab}	0
2	Control	63.32 ± 0.71^{b}	0.00 ± 0.89^{a}	10.41 ± 0.35^{a}	2.90
	Prototype	60.74 ± 1.64^{a}	0.14 ± 0.24^{a}	10.62 ± 0.55^{a}	1.10
7	Control	62.85 ± 1.67^{b}	-1.14 ± 0.83^{b}	10.95 ± 0.94^{ab}	2.95
	Prototype	62.04 ± 1.88^{ab}	-1.48 ± 1.08^{b}	11.85 ± 0.89^{b}	2.72

Legend: a,b,c,d - the indexes indicate a statistically significant difference between the lines at a significance level (p<0.05).

Table 3 Parameters of meat colour (CIELAB) and its changes over time of storage at lower part of slices

Days of monitoring	Treatment	Lower part of slices			ΔE^*_{ab} (D65)
		L*(D65)	a*(D65)	b*(D65)	
0	-	60.68 ± 2.06^{a}	-0.18 ± 0.34^{b}	11.05 ± 0.75^{ab}	0
2	Control	65.01 ± 0.75^{b}	0.52 ± 1.18^{ab}	11.46 ± 0.90^{a}	4.47
	Prototype	63.98 ± 1.73^{ab}	-1.91 ± 1.45^{b}	12.68 ± 0.46^{c}	3.31
7	Control	62.66 ± 2.81^{ab}	-0.46 ± 1.22^{ab}	10.40 ± 1.02^{b}	3.57
	Prototype	62.04 ± 2.76^{ab}	-0.18 ± 0.34^{b}	11.05 ± 0.75^{ab}	2.19

Legend: a,b,c,d - the indexes indicate a statistically significant difference between the lines at a significance level (p<0.05).

Total colour change ΔE^*_{ab} (D65) during storage at the lower part of slices were higher than the upper side. Colour of meat changed, compared to control, on second day after packing, especially at the lower part of slices on which the meat in the package laid. At both lower and the upper part of experimental samples was found lower change of colour compared to the control, which entails a positive effect on the sensory indicators of meat during storage (Pathare et al. 2013). Packaging foil with essential oils reduced undesirable change of colour from 3.57 to 2.19 at the lower part of slice and from 2.95 to 2.72 at the upper side, which means in the area of moderate changes a slight disagreement (Třešňák 1999).

CONCLUSION

Plants with their biologically active constituents are in the food industry mainly used for flavouring of food. Many of essential oils possess also antimicrobial and antifungal activity. In our study, meat samples were used to determine the effect of packaging foil with essential oils on major groups of microorganisms on surface of roast pork. A significantly lower aerobic plate count was observed 7 days after the packing. Statistically significant difference was not found for the other groups of microorganisms. This study shows the importance of testing the effect of antimicrobial agents directly on foodstuffs. Antimicrobial food packaging should not serve as a substitute for good manufacturing and hygienic practices, but it should enriched food safety as an additional hurdle. For meat, the assessment of colour, along with texture, aroma and taste is one of the most important descriptor of subjective sensory evaluation. Packaging foil with essential oils slightly reduced undesirable colour changes.

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COMPARISION OF SENSORY AND ANALYTICAL CHARACTERISTICS OF RED WINES INFECTED BY *BRETTANOMYCES/DEKKERA*

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Abstract: The *Brettanomyces/Dekkera* yeast can infect wine, and, thanks to its metabolism, it can produce volatile substances that affect the aroma of wine. Volatile phenols, sensorily active substances formed due to the yeast metabolism, can deliver desirable spicy tones or, on the contrary, very unpleasant wet animal-like odour to the wine. It depends on the concentration of mainly 4-ethylphenol and 4-ethylguaiacol in wine as well as on wine consumers´ ability to perceive these tones. Twenty-four samples of red wines suspected of having increased content of volatile phenols, produced by *Dekkera/Brettanomyces* yeasts, were gathered for this experiment. The content of ethylphenols (4-ethylphenol and 4-ethylguaiacol) in the samples was analysed by GC-MS and ranged from 10 to 3035 µg/l. The samples were then evaluated via sensory analysis carried out by professional tasters. The results were compared and correlated between the concentration of volatile substances and sensory perception of 'Brett' characteristics in wine. Highly probative correlation of the average evaluation of 'Brett' character in analysed wines and the real content of ethylphenols measured by GC-MS is an important outcome of the research. Weak negative dependence was calculated for parameters of sensory evaluation, which confirms generally decreasing evaluation (on the 100-point scale) of samples with a growing 'Brett' character.

Key Words: Brettanomyces, *Dekkera*, red wine, ethylphenols, yeasts, GC-MS, sensory evaluation

INTRODUCTION

The *Brettanomyces/Dekkera* yeast belongs to a group of wild or apiculate yeast. This yeast comes mainly from the surface of the skin of grapes. The yeast does not prevail on the grapes, but it plays an important role in the development of a wine´s aroma (Renouf et al. 2006). Hydroxycinnamic acids are metabolically transformed thanks to this yeast, and enzymes and so-called volatile phenols are formed. These very substances are responsible for the 'Brett' character in red wine. The aroma of such wine is reminiscent of a barnyard, a horse stable, sweaty horse saddle, leather, earth or smoked meat (Eder et al. 2006, Curiel et al. 2010).

The presence of *Brettanomyces* yeast was attributed to inadequate hygiene in cellars, where there is a greater risk of contamination. It was found out later that, despite increased efforts to maintain adequate hygienic conditions, contamination by this specific yeast was still very common (Eder et al. 2006, Oelofse 2008).

Contamination by the *Brettanomyces* yeast may already come from the grape. A large increase in *Brettanomyces* yeast population can be found during maceration or during the very fermentation process. *Brettanomyces bruxellensis* can adapt very well to the beginning of alcohol fermentation. The yeast has also proved to be far more resistant to the conditions of increased alcohol and sugar loss near the end of fermentation than *Saccharomyces cerevisiae* (Suarez et al. 2007, Oelofse 2008). The presence of the *Brettanomyces* yeast during malolactic fermentation is associated with low content of free sulphur dioxide, certain concentrations of residual sugar and the yeast autolysis associated with mild microbial instability. Such technological procedures as storing wine in oak barrels of barrique type also complies with the multiplication of yeast of this species (Renouf et al. 2006, Wedral et al. 2010).

Brettanomyces bruxellensis belongs to the species of yeast which adapts very well to conditions of dry red wine. In certain concentrations, it is resistant to SO_2, to high alcohol content, lack of oxygen and low amount of fermentable sugars (Romano et al. 2008, Coulon et al. 2010).

Increased content of volatile phenols causes the 'Brett' character in red wines. All strains of *Brettanomyces* genus which have so far been discovered are capable of producing volatile phenols. However, *Brettanomyces bruxellensis/Dekkera bruxellensis* is the only species discovered in wine (Agnolucci et al. 2010).

Volatile phenols make a large group of substances that may have a very positive effect in small concentrations due to the production of certain substances, such as vanillin, eugenol, syringaldehyd etc. In this case, very desirable smoky or spicy tones may come into existence in red wine. Also, substances that are not evaluated very positively if they exceed the threshold value of their concentration belong to this group. Such phenols include mainly ethylphenols (4-ethylphenol and 4-ethylguaiacol), but also vinylphenols (4-vinylphenol, 4-vinylguaiacol), which precede ethylphenols formation.

The formation of volatile phenols (ethylphenols) via *Brettanomyces* yeast is a result of the enzymatic transformation of hydroxycinnamic acids, ranking among phenolic substances (Eder et al. 2006, Harris 2011).

The results of a previous experiment focused on the sensory perception of ethylphenols, i.e. substances responsible for the aroma of sweaty horse saddle and barnyard, which show that experts are much more sensitive to the perception of ethyphenols than common wine drinkers.

It has been demonstrated that certain other substances present in wine are able to mask high concentrations of ethylphenols, in particular, isobutyric and isovaleric acid (Romano et al. 2008). If the content of 4-ethylguaiacol exceeds the value of 70–100 μg/l, wine aroma is negatively affected. This is manifested, for example, by the aroma reminiscent of burnt wood. Moreover, concentrations of 4-ethylphenol over 400–600 μg/l are referred to as phenolic aroma, i.e. aroma described as smoked bacon or reminiscent of horse-stable or barnyard (Eder et al. 2006).

MATERIAL AND METHODS

Samples and process

This study gathered a total of 24 samples of red wine suspected of having symptoms related to the change of aroma caused by the activity of *Dekkera/Brettanomyces* yeast and of subsequent formation of volatile phenols. The range of samples comprised nineteen moravian and five foreign wines. A total of five samples from the evaluated collection were labelled as cuvée; the rest were varietal wines. The selection of the wines was based on a questionnaire distributed to wine producers.

To evaluate the samples, two basic methods were used: a sensory analysis and gas chromatography with mass spectrometry(GC-MS) for measuring volatile substances.

Sensory evaluation

The evaluation was carried out using a computerized evaluation system. The samples were evaluated on a scale of 100 points, and the aroma and body profiles of the wines were assessed. As agreed with the tasters, the last part of the aroma profile evaluation included the extent of a 'Brett' character of the wine in question. All the parameters were evaluated on a scale of 1 to 10 based on the level it reached in the particular category.

Samples preparation

Prior to the tasting itself, the samples had been kept at a stable temperature of 12 °C. The bottles were opened about 150 minutes before the tasting to let the wine 'breathe', as it is called. Twenty minutes before serving, the individual samples were decanted in a glass pitcher. When served, the temperature of the wines was about 16 °C.

The jury

The tasters' jury consisted of ten members. The tasters included experts specialising primarily in Moravian wines as well as those with considerable experience in evaluating foreign wines. All of the tasters also own the Certificate of Passing the Selection Process of Specialized Evaluation Experts for Sensory Analysis of Wines in Compliance with the Czech Technical Standard ISO 8586-2.

Analytical evaluation using GC-MS

During the tasting, an amount of wine was withdrawn from each sample. Using a funnel, this amount was poured into 250 ml glass bottles with a screw cap. Samples prepared in this way were, immediately after the tasting, transported to the laboratory of the Department of Viticulture and Enology in Lednice where they were analysed by means of GC-MS.

Table 1 GC-MS Analysis

Instrument:	Shimadzu GC-17A
Autosampler:	AOC-5000
Detektor:	QP-5050A
Software:	Gcsolution

Table 2 Conditions of Separation

Column:	DB-WAX 30 m x 0.25 mm; 0.25 μm of stationary phase (polyethylenglycol)
Injection volume:	1 μl split 1:5
Gas flow (He):	1 ml/min (linear speed of gas 36 cm/s)
Temperature of injection port:	180 °C

RESULTS AND DISCUSSION

Results of the sensory evaluation

The average score of the samples ranged within a span of 11 points, where the sample with the lowest score reached 74.13 points while the top sample score was 85.25 points. Moreover, each sample could be subjectively commented on by the evaluators. In individual evaluations, the score ranged from 67 to 90 points (Table 3). The evaluation of the respective samples varied a great deal among the jury members.

Taking into account the fact that the ‘Brett’’ character in red wines, caused by the activity of the yeast *Brettanomyces/Dekkera*, is described as a wine fault in expert literature, it can be assumed that there would be a certain relation between the score on the 100-point scale and the described extent of its ‘Brett’’ character. The underlying assumption is that the wine that was specifically evaluated as having stronger ‘Brett’’ characteristics would be accordingly lower in score on the 100-point scale system. Its score would then correspond to that of faulty wine. All wines, ranked as being ‘Bretty’ to a greater extent and reaching 6 points or more on the 10-point scale evaluation of ‘Brett’ characteristics, have not obtained more than 80 points out of 100.

Evaluations of certain samples by individual experts very often varied to a great extent. Personal notes that evaluators put down about each sample were important for evaluating the individual perception of ‘Bretty’ characteristics.

Result of the GC-MS analysis

The professional literature provides a limit of volatile phenol perceptibility as 400–600 μg/l for 4-ethylphenol and 33–100 μg/l for 4-ethylguaiacol. Eder (2006) asserts that the ‘Brett’’ character in red wine is perceptible provided that the total of the 4-ethylphenol and 4-ethylguaiacol content is greater than 425 μg/l. There were 11 such samples out of the 24 evaluated ones (Table 3).

Figure 1 below shows which samples exceeded the limit given for the sensory perception threshold. If the lower limit for 4-ethyl phenol is 400 μg/l, there was a total of 10 samples surpassing it. The greatest amount of 4-ethylphenol was measured in sample No. 7, where the perception threshold was exceeded by nearly six times. In sample No. 14, the value was about five times above the limit.

Table 3 Summary Results of Sensory and Analytical Analysis

Number of sample	Sensory evaluation (100 points scale)	Sensory evaluation of Brett character	4-ethylphenol (µg/l)	4-ethylguaiacol (µg/l)	4-ethylphenol + 4-ethylguaiacol (µg/l)	Number of sample	Sensory evaluation (100 points scale)	Sensory evaluation of Brett character	4-ethylphenol (µg/l)	4-ethylguaiacol (µg/l)	4-ethylphenol + 4-ethylguaiacol (µg/l)
1	74.13	4.30	14	0	14	13	81.63	5.00	122	20	142
2	80.88	6.20	542	70	612	14	78.75	7.70	2038	367	2405
3	79.13	3.00	430	45	475	15	81.00	4.50	25	3	28
4	84.13	4.90	593	67	660	16	76.50	7.20	985	269	1254
5	79.13	4.60	1184	129	1313	17	81.50	4.80	42	11	53
6	79.63	4.80	288	35	323	18	80.38	4.70	192	44	236
7	78.00	6.40	2355	680	3035	19	78.63	5.10	10	0	10
8	78.38	5.50	32	12	44	20	83.50	4.30	75	12	87
9	77.38	4.20	469	94	563	21	85.00	3.50	35	8	43
10	81.50	4.20	142	25	167	22	85.25	4.70	9	9	18
11	82.25	5.10	1138	141	1279	23	78.88	6.90	343	312	655
12	76.13	6.50	1096	312	1408	24	81.13	3.70	205	33	238

Figure 1 Values of 4-ethylphenol Content

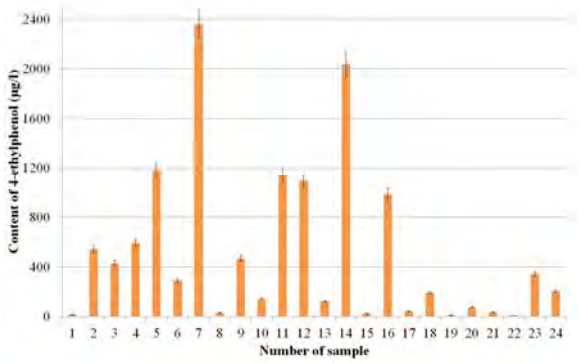


Figure 2 Values of 4-ethylguaiacol Content

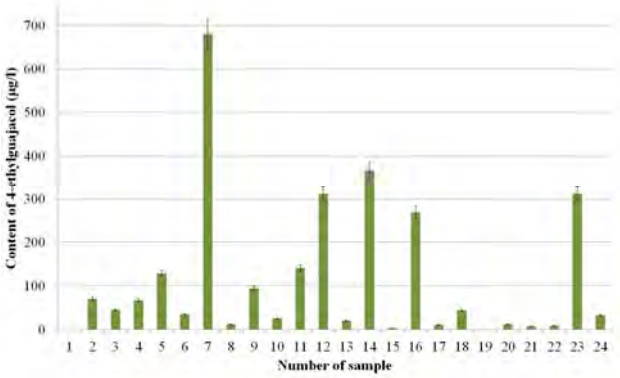


Figure 2 shows the values of 4-ethylguaiacol measured in the individual samples. The minimum perceptibility limit is ascertained to be 33 µg/l. Among the analysed samples, this number was surpassed in a total of 14 cases. In sample No. 7, the content of 4-ethylguaiacol was about twenty times higher than its perception threshold limit.

Highly probative correlation of average evaluation of the 'Brett' character in analysed wines and real content of ethylphenols measured by GC-MS is an important outcome of the research. Weak negative correlation was calculated for parameters of sensory evaluation, which confirms a generally decreasing evaluation (on the 100-point scale) of samples with growing 'Brett' character (Table 3). The connecting lines in figures 3 and 4 confirm the linear dependence which clearly indicates that the more 'Brett'' character is detected in a wine, the greater content of 4-ethylphenol and 4-ethylguaiacol.

Figure 3 Dependence of 'Brett'' Character Perception Perception on the Measured 4-ethylphenol Content

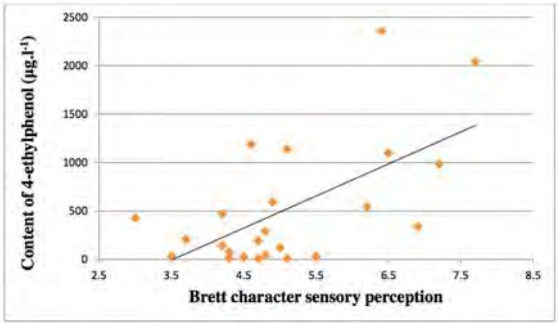
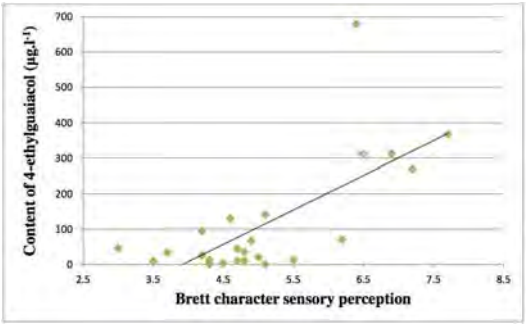


Figure 4 Dependence of 'Brett'' Character on the Measured 4-ethylguaiacol Content



DISCUSSION

After sorting all analysed samples from those with the most intense 'Brett' character to the samples manifesting it the least as well as sorting them from samples with the highest content of ethylphenols to the lowest content, we have found out that samples numbers 7, 12 and 14 were in the top five. Sample number 7, which contains up to seven times greater concentration of ethylphenols than the value stated by Eder (2006) as threshold content of perception, was not evaluated as the most sensorily 'Bretty', but took 5th place. Sample number 14 was regarded as the most 'Bretty'. It has the second-biggest content of ethylphenols 2 405 µg/l.

Bende, et al. (1997) determined that different perceptions of these specific substances in wine can be caused by many factors: personal experience, increased or reduced sensitivity, as well as by the presence of other substances that may affect perception of ethylphenols. Romano, et al. (2008) observed that the perception of ethylphenols in wine can be affected by isobutyric and isovaleric acids, and, by their presence in wine, the 'Brett' character may be masked. For this reason, the analysis of these carboxylic acids in all samples was conducted within our research as well. The correlation of sensory perceptions of ethylphenols and increased concentration of isobutyric and isovaleric acids has not been proved. However, their increased concentration in some samples was recorded.

CONCLUSION

Our research proves that there is a highly probative correlation between the perception of the 'Brett' character in wine and the real content of alcoholic ethylphenols, i.e. substances which are responsible for the 'Brett' aromas in wine. We can thus claim that experts are certainly able to identify manifestations of 'Brett' character in wine. However, experts are far from having the same opinion on the degree of positive or negative perceptions of such tones. The perception threshold of a substance´s presence varies with every person. Also, the border of tolerability of its manifestation differs with every person. A hundred wine tasters may perceive this threshold value in a hundred different ways. This has also been confirmed through sensorial evaluation. Variations in range in total wine quality evaluation of nine samples equalled 15 and more points. Based on the evaluations of 10 tasters who analysed 24 samples with a 'Brett' character, we can claim that the 'Brett' character was sensorily identified in all samples that had an increased content of ethylphenols. The extent to which the influence of these microorganisms is positive cannot be determined on the basis of this research.

According to less number of international samples there was no significant difference between the assessment of domestic and international wines.

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EATING BEHAVIORS OF UNIVERSITY STUDENTS

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Abstract: The nutritional intake during young adulthood supports the maintenance of physical health, impacts risk for future disease, and plays a role in the prevention of excess weight gain. According to the Food and Agricultural Organization of the United Nations (2013), Czech Republic ranks as the fattest country in Europe, based on the prevalence of obesity among adults. Around 28.7% of the adult population is considered obese, and this number is projected to rise according with the European Association for the Study of Obesity (EASO) in 2030. Currently young people's health is receiving more attention because of alarming data regarding risk factor such obesity prevalence. Identifying people at greater risk of developing obesity is important for the development of effective public health strategies to prevent and treat excess weight gain and its associated co-morbidities. The presented study was conducted to describe food-preparation behaviors, cooking skills, resources for preparing food, and associations with diet quality among young adults from Mendel University in Brno. Finally, a number of recommendations, according to the results of this study, were formulated to foment new eating habits among the experimental sample.

Key Words: food habits, eating behaviors, young adults, buying choices, obesity, prices

INTRODUCTION

According to Osorio et al. (2002) the eating behavior is a "normal behavior related to eating habits, selecting foods that you eat; culinary preparations and quantities of ingestion". Eating well can become a habit and so can eating poorly. Eating poorly might help develop serious diseases as obesity, malnourishment, among others. Food habits and obesity can barely be separated. The rational for that is, that the way an individual eats determines her health status. Precise food behaviors or habits, regime factors, and surrounding mechanisms may be accountable for variances in global diseases (Azevedo et al. 2016). The study further showed that an average number of deaths were preventable i.e. the deaths were the consequence of avoidable sources, comprised of suboptimal food habits with low intake of fruits and vegetable, pulses, whole grains and nuts and high quantities intake of sugar and salt.

According with the World and Health Organization 2015, the variations in worldwide food market generate opportunities for change people's eating behaviors or habits to decrease the risk of developing various health problems as obesity. Obesity is nonstandard or disproportionate fat growth that grants a danger to one's well-being. It is measured by the Body Mass Index (BMI). An individual with a BMI of 30 and above is normally reflected obese. Equivalent to or above 25 is measured overweight respectively. Also, overweight can be defined as an excess of weight that is more than the allowed.

Neumark-Sztainer et al. (1995), noted that eating habits and mealtime arrangements which are unhealthy in diet regularly led consistently to overweight and obese students. Their research findings suggested that school-based agendas still had the potential to contribute to main anticipation dietary challenges. The report of the Food and Agriculture Organization (2013), mentions that the Czech Republic is considered as the country with the highest percentage of obese or overweight people in Europe, 28.7%. But this number contains not just students but adults of all ages.

The hypothesis of this study is that the students of Mendel University have good understanding of eating habits and adequate knowledge about nutrition. This research assumes that they care about their eating habits and their health, so they can perform better their daily activities now and in the future.

MATERIAL AND METHODS

This research was conducted with students from Mendel University in Brno, located in the region Moravia, Czech Republic. To be selected to be part of the study, the participants had to be regular undergraduate students of any degree, at least in their second year, over 18 years old. The students voluntarily participate in the study by signing an informed consent.

Their eating behaviors were determined and evaluated by a questionnaire. This questionnaire, which consisted of 37 questions, was divided into 4 blocks: overall overview, health, food habits and sugar consumption. It assessed the subjects' frequency of eating meals and other different factors that influencing their food choices at different places.

The fulfilled questionnaires then were analyzed with Microsoft Excel.

Research Sample

This study was carried out between February to April 2016. During this period 107 students signed up, which represents around 1% of all students of Mendel University. The subjects were between 18 to 26 years old. This was from the target population who were university students at Mendel University in Brno.

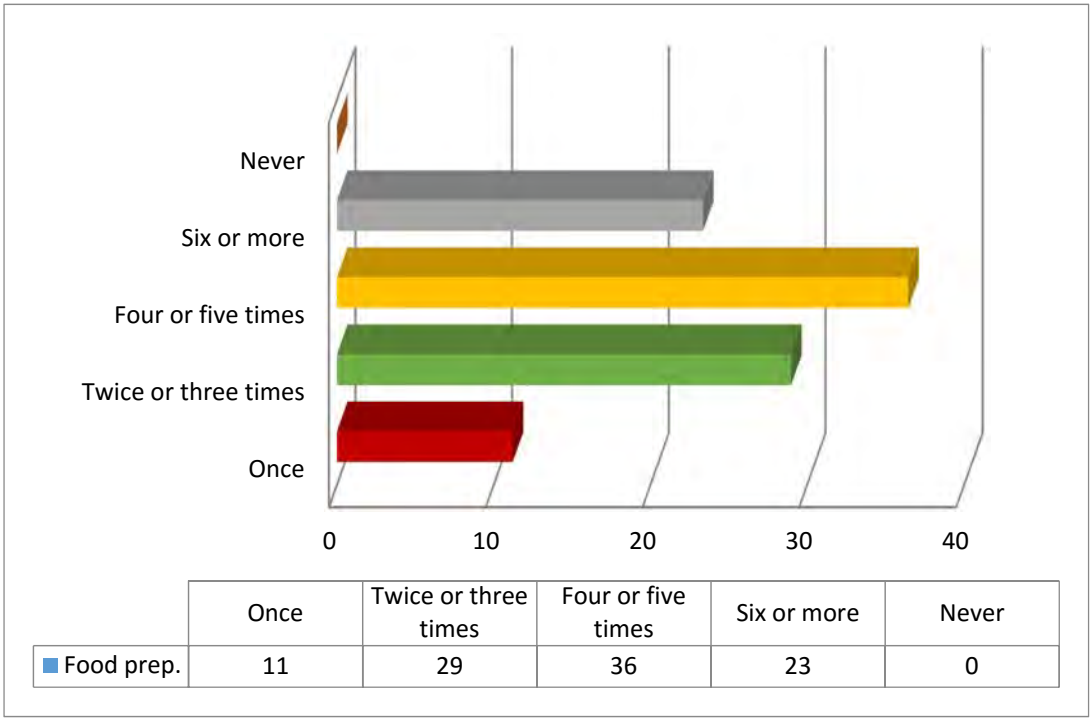
RESULTS AND DISCUSSION

This study strived to get the demographic background information of the respondents in order to classify the individuals who engaged in the study. Most of the participants were women (67%) and the rest (33%) were men.

Most of the interviewed students were people between 18 and 21 years old (70%). Also, the study counted with the participation of other students from 22 to 25, and 26 to 29 and the numbers were 35, and 2 of the total number respectively.

Relative frequency of places where the students had their meals indicated Canteen 8%, home 89%, Restaurant 1%, and others 2%.

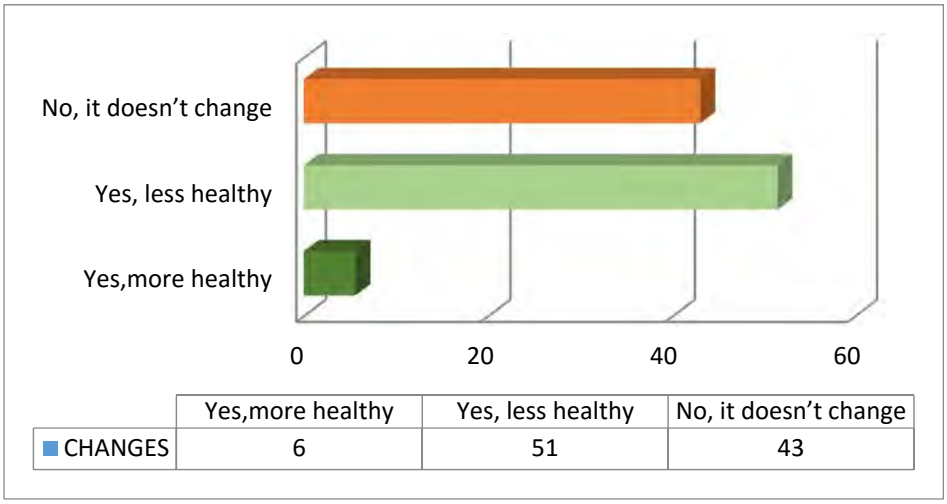
Figure 1 How often do the students prepare their own food per week



	Once	Twice or three times	Four or five times	Six or more	Never
Food prep.	11	29	36	23	0

Figure 1 above shows the frequency how often students prepared their own food at home. 11% was cooking once in a week, 29% cooking two or three times, 36% for four or five times per week, and 23% for six and more times.

Figure 2 Change of the eating habits of the students during the exam period



	Yes,more healthy	Yes, less healthy	No, it doesn't change
CHANGES	6	51	43

Figure 2 above shows the frequency the students change their eating habits during the exam period, 6% responded yes to healthier eating, 51% answered yes to less healthy eating, 43% answered no to change in their eating habits or behaviors.

Preference for low-fat products indicated that only 20% of the students prefer low fat foods. Moreover, the subjects said that they are used to read the label of the products (78%).

Figure 3 Do the students make their buying choices based on how healthy the product is or based on its price?

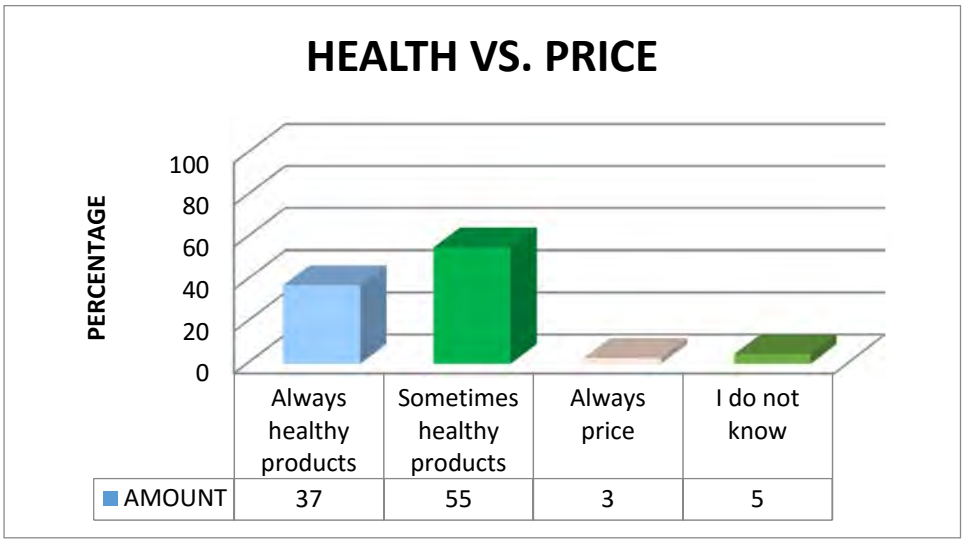


Figure 3 shows whether the students care about eating healthy or they prefer to eat cheap. The results showed that 37% of the students always buy healthy products, 55% of them sometimes chooses healthy products but this decision depends on the price of the product, whereas 3% of sample responded that considered price vital, and 5% answered that they do not think that this is an important factor to make their decision.

The responses on the consumption of sweet beverages whose percentage frequencies are 34% for once and never, 21% for two or three times, 2% for four or five and 9% for six or more times. The investigation results further indicate the favorite alcoholic drinks.

The results also show that only 6% of the responses does not drink alcohol while the other 94% does.

Heather and Theresa (2013) mentioned that features such as social surroundings, plus various socioeconomic and sociocultural aspects such as parents' training, time restraints, and culture influence peoples' eating habits. This research found out that the significant percentage of students eat and prepare their meals at home. These results are positive because the subjects know the ingredients as its quantities and their quality and plan what they eat, as well as save money.

The report of the National Obesity forum (2016) mentioned that the people's preference for low/fat and low cholesterol foods is originating in a health crisis. Basically, this report mentioned that the sugar and packages labeled as "low-fat", "light" and "low cholesterol" removed the fat from the food and often is replaced with sugar or salt to make up the lost flavor which makes the people gain weight. This investigation demonstrates that the subjects are not used to consume low-fat products (80%) and they might know the consequences of eating high fat products/foods, which is equally illustrated by the high number of subjects (78%) who constantly read the labels on food products.

Kurubaran et al. (2012) established that university students ate healthy foods but they however argued that such eating habits can change due to stressful events. This same situation can as well be confirmed in this research results as the frequency of the students that changed their eating habits because of stressful events such as examination periods made students to eat less healthy food is quite high (more than 50%).

Students responded on purchasing choices showed that 63% of them not always choose the healthy product option. Several studies (Pitt and Rosenzweig 1984, Magnusson et al. 2003, Taylor et al. 2005), in their various investigations show that price affects the choice of food consumed by the people in their different environments.

Later, Andreyeva et al. (2010), further stress that approximating price/value affects the substitutions from healthy to unhealthy foods and price sensitivity amongst risky populations. They equally suggested that fluctuating food prices hence create changes in diets. It is significant to comprehend how price variations affect demand for several foods. This could also be supported by the numerous studies (Stiglitz 2013). He contended that diverse commodities in the marketplace are subjective to prices than any other aspects such as availability of substitutes amongst other.

The responses on the consuming of sweet drinks whose proportion incidences is 66% for once or more times a week. These results are alarming because they not only consume per week sugary drinks, which are rich in free sugar; but also, other sugary products as chocolates, cookies, etc. and these just increased they daily sugar intake. Also, 94% of the responders consume alcohol regularly. According with the World Health Organization (WHO), the daily intake of free sugars should be less than 10% of the total energy intake. The same report mentioned a further reduction to below 5% or roughly 25 grams (6 teaspoons) per day would provide additional health benefits.

The consumption of too many sugary products which compose most sweet drinks, and alcoholic beverages, is likely to cause obesity or overweight. According with the European Health report in 2015, Czech Republic is the fifth country with prevalence for overweight and obesity in Europe. Also, it is mentioned in the report that the high amount of deaths were the result of preventable causes, which included suboptimal food habits with low eating of fruits and vegetables, pulses, whole grains and nuts rather high quantities intake of sugar and salt.

CONCLUSION

The aim of this research was to know which food habits the university students have. This research concludes that although they are used to read the labels of the products which they buy, everything is pointing that they do not have enough of information about what they should eat: they drink sweetened soft drinks containing more sugar than their daily need is. Also, they are used to drink alcohol as part of their diet. A representative part of the participants does not always choose the healthy option or they are not concerned about what they eat.

Also, they have the tendency to change their food habits with academic stress situations, to even less healthy, especially during the exam period. This irregular food habit should be avoided, because could bring consequences related with malnutrition, either deficit or excess, in this very critical period.

The study also concludes that price has a great impact on the choice of healthy foods although other factors such as social and environmental factors were vital in the determinants of eating habits amongst students. It can also be concluded that the economic situation may be a condition to carry out a convenient feed, which might limit the selection of certain types of food and consequently in the impossibility to maintain healthy eating behaviors.

Even in this small sample of students, roughly 60% of the participants are not interested about what they eat. And although they might have an adequate knowledge about nutrition they do not use it in practice. Because of this, they are at risk of becoming overweight and obese in the future. This situation is alarming and this study recommends to conduct a future research with broadened scope and larger representative sample size, which should include not only the students but also the teachers. Moreover, this research recommends that the university should prepare some action plan to educate its students about nutrition and eating habits and teach them how to use this knowledge in practice. Because a healthier population brings many advantages to the country. People are more motivated, more productive and the state can spare some money on health care.

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THE USE OF COLOR WHEAT SPENT GRAIN AS AN INGREDIENT FOR THE PRODUCTION OF BAKERY PRODUCTS

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Abstract: The cereals grains are excellent sources of digestible carbohydrates, dietary fiber, and proteins in addition to providing vitamins (group B and vitamin E) and minerals (zinc, phosphorus, selenium, and iron). The whole grain cereals contain high levels of bioactive phytochemicals, such as antioxidants that could provide protective effects against human chronic diseases. Its enrichment with more amount of anthocyanins, allows a better use of its potential bioactive property. For this reason, the state authorities are seeking functional foods to mitigate health problems as cancer, diabetes and heart diseases. They would like to create functional foods that satisfy the function to nurture and to prevent. This research focuses on the use of the brewer spent grain (BSG) varieties of colored wheat, which are enriched genetically with anthocyanins, to its use in bakery products.

Key Words: colours wheat, spent grain, bread, sensory evaluation

INTRODUCTION

The effort to increase the nutritional value of the commercial bakery products is considered a worldwide trend. As is well known the wheat per se, is a rich source of dietary fiber in particular the arabinoxylans and hemicelluloses. The consumption of whole wheat leads to a better absorption in the small intestine (increased fecal volume) and decreased bulk pH, which enhances the elimination of cholesterol and potential toxins or carcinogens (Rotimi 2012).

But there are more genotypes (*Triticum aestivum*) that have different colour than common caryopses (Dostalova et al. 2015). The colored grain wheat is one kind of new germoplasm resource in cereal crops, which are rich in beneficial anthocyanins (Zifeng et al. 2011). These dyes are present in different parts of the caryopsis. The main varieties are the purple pericarp and blue aleurone, which contain larger amounts of dyes (Vaculova et al. 2010).

The purple pericarp or purple wheat grain contains mainly 3-glucoside of cyaniding and peonidin 3-glucoside anthocyanins (Knievel et al. 2009). The cyaniding has various effects on the cells, most of which can be described as being anti-diabetic and possibly slightly benefit other parameters associated with 'metabolic syndrome' (anti-inflammatory, anti-oxidant, etc.); while Peonidin 3-glucoside is important for its relation with the inhibition of tumor cell growth and reduction of metastasis of lung cancer cells.

Also the blue aleurone or blue wheat grain, the aleurone layer contents 3-glucoside of delphinidin and 3- rutinoside of delphinidin (Knievel et al. 2009). The 3-glucoside of delphinidin or Myrtillin tends

to stabilize the blood sugar, which otherwise fluctuates widely, and that it spares insulin; while 3-rutinoside of delphinidin has anti-inflammatory, antioxidant and antimicrobial effects. Furthermore, the blue wheat grains tend to have higher anthocyanin contents, compared with the wheat with purple pericarp (Martinek et al. 2012).

Due to the antioxidant activity of the anthocyanins on the caryopsis of the wheat, its inclusion could thus provide in the long-term beneficial effects on human health (Kienevel et al. 2009). The use of wheat with purple or blue aleurone, is mainly used in whole meal flours or by the addition of bran (Vyhnanek et al. 2015). In this study the brewer spent grain (BSG) is used as an adjunct for the production of commercial bread (Hernández et al. 2016).

Along this research barley spent grain variety Malz was used, which has favorable protein content and excellent extract content during the malting (Hernández et al. 2016). But its use was not limited to the technical characteristics of the product itself, but also to its beneficial effects. Having a diet rich in barley or β-glucan extracts is known to have beneficial physiological effects, because of the soluble state and high molecular weight of this polysaccharide. β-glucan has been shown to increase daily fecal bile acids output, which leads to lower blood cholesterol and lipoprotein concentrations in human subjects (Rotimi 2012).

The main aim of this research is to combine the positive effects of the barley spent grain and the blue aleurone containing purple wheat in a bakery product. This new bakery product should content more anthocyanins and fiber.

MATERIALS AND METHODS

Characteristics of the use spent grain

For the production of the bakery products BSG from 100 liters of brewer beer was used BSG from the microbrewery located at Mendel University in Brno. The brewer spent grain (BSG) from barley, variety Malz was used; and wheat varieties with purple pericarp and blue aleurone; individually and jointly in a proportion of 50 : 50 were used.

Baking experiment

The recipes were prepared using four varieties of color wheat spent grain: Rosso with purple pericarp; also Skorpion with blue aleurone; and barley. For comparison, for the control sample a special wheat flour, T530, was used, which contains 0.53% of ash in dry matter. This flour also is known as 00.

To prepare 500 g of dough, 500 g (for variant 1) and 450 g (for the rest of the variants) of flour was used. Also 7.5 g of salt, 5 g of sugar, 5 g of oil, 25 g of fresh yeast and 330 ml of water was used. The development of all the experiment the Rapid mix testwas followed. To control of the volume, in all the variants, baking powder was used. It was required due to the presence of fiber that could affect the development of the gluten network. The baking powder had a composition of diastase, malted wheat flour, sugar E332. E472e, guar gum, ascorbic acid, dextrose and E450.

Table 1 Dough formulation for each sample

Variety	BSG content and its modifications
1	0% (without brewer spent grain)
2	10% whole BSG from purple wheat
3	10% cut BSG from purple wheat
4	10% whole BSG from blue wheat
5	10% cut BSG from blue wheat
6	10% whole BSG from purple wheat and barley (50 : 50)
7	10% cut BSG from purple wheat and barley (50 : 50)
8	10% whole BSG from blue wheat and barley (50 : 50)
9	10% cut BSG from blue wheat and barley (50 : 50)

BSG- brewer spent grain

Later, BSG was used in a proportion of 10% of baker's percentage, because previous studies carried out in this department showed this is the most favorable proportion. According to Huige (1994), the addition of 10% of spent grain increase the amount of protein and amino acids and also double the fiber content in comparison with the traditional bread. In addition, the bread made with spent grain

contains around 7% less calories than the standard one. The caloric value of the spent grain is about 50% lower than the caloric value of the cereals.

The variant 1 was the control sample for this baking experiment (baked without spent grain). The rest of variants used moist BSG (whole grain and coarsely cut). A summary of the tested formulations are shown in the following table (Table 1).

The dough was prepared by mixing all raw materials at once. The dough was kneaded in a dough-kneader for about one minute. It was raised in a proofer at 32 ± 1 °C and humidity of 80 ± 5% for 20 minutes. After the removal from the proofer, the dough was rested for 10 minutes and weighted. Then it was shaped into the desired pieces weighing 80 g and it was allowed to rise again at 32 ± 1°C and humidity of 80 ± 5%, for 25 minutes. Before loading the pieces into the oven, they were sprinkled it with water, and baked at 230 °C to 240 °C in a laboratory oven with a proofer. At the beginning of the baking, the oven was steamed with 50 ml of water. The baking time was 20 minutes.

Sensory analysis: Evaluation of the product

The baked products were subject to sensory evaluation of the influence of the ingredients over the physical characteristics of the bread. The sensory evaluation was carried out by a team of trained tasters and the results were evaluated using a sensory analysis test (n = 10). The sensory analysis provides values of the following characteristics of the product: shape, color of the crust, aroma, flexibility of the crumb, color of the crumb, easiness of biting, sensation after chewing, consistency, moisture of the crumb, taste and overall impression. The sensory evaluation was made by unstructured graphic scales, which had a range of 10 cm, 10 cm which meant 10 points (100%), i.e. the best ratings.

Evaluation of the results

The statistical evaluation of the identified data was performed using Microsoft Excel and Statistica 12. The one-way ANOVA method was used, which is used for the evaluation of the analysis of variance.

RESULTS AND DISCUSSION

The quality parameters of the bread rolls

The results of this baking experiment are shown below in the Table 2. The highest loss (14.59%) was observed in the variant 1 (control), while the lowest loss (11.36%) was in the variant 7 (with cut BSG from purple wheat and barley 50 : 50). Hampl and Příhoda (1985) mentioned that the losses during baking of common pastries are ranged between 10 and 13%, depending on the shape and weight of the product, as well as baking time and temperature, dough moisture, or the type of flour. In this research, the loss could because of the effect of the dough moisture and the content of spent grain in the dough, because all the breadrolls were made under the same conditions.

Later, Müllerova and Skoupil (1988), mentioned that higher specific volume of pastry, the more suitable is the wheat variety for bakery production. In this study, the highest specific volume was achieved by the variety 2 which 10% whole BSG from purple wheat (324.9 ml/100 g) and by the variety 4 with around 300 ml. These samples also had the highest losses during baking, up to 14%. This loss could be caused by the enormous water evaporation, because the samples contained whole fresh spent grain. On the other hand the lowest losses, around 11.3%, were at sample 7, which had grind spent grain from red wheat and barley. As optimal shapes of the bread rolls were identified the samples 4, 6 and 8. These samples contained whole spent grain, so it could be said that the whole spent grain has a positive influence on the shape of the product. The most arched sourdough bread was the sample 4, with the addition of whole fresh spent grain of purple wheat.

This experiment also showed a great homogeneity. As it was mentioned before, for the preparation of the samples baking powder was used. The shape and the appearance of the bread were not significantly affected by the addition of various types of spent grain. All the bread rolls had a regular shape and color of the crust, without big differences between them. Also, the addition of different amounts of spent grain was barely noticeable during the cutting of the products. Some participants stated that the difference was not noticed at all, for the average consumer, these differences cannot be considered as significant.

Table 2 Quality parameters of the breadrolls

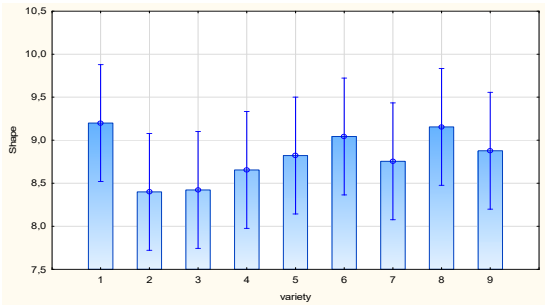
Variety	Specific pastry volume/100 g of dough (ml)	Baking losses (%)
1	303.75	14.59
2	324.90	14.07
3	292.24	12.52
4	302.05	13.89
5	292.94	13.53
6	292.13	13.01
7	280.58	11.36
8	287.82	11.99
9	292.07	13.13

The sensory evaluation of the products

The analyzed characteristics of the bread rolls were: shape, color of the crust, aroma, flexibility of the crumb, color of the crumb, easiness of biting, sensation after chewing, consistency, moisture of the crumb, taste and overall impression.

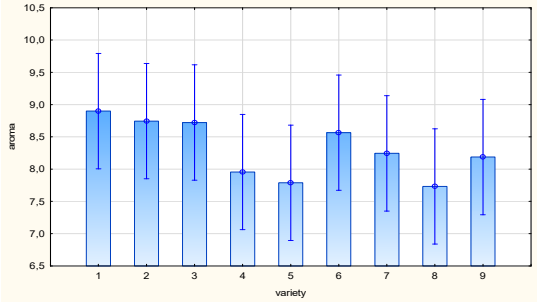
Figure 1 shows that the shape of products that contains only color wheat spent grain (samples 2 to 4) were less regular in comparison than those which contained equal percentage of spent grain of wheat and barley (samples 6 to 9). The samples 2 and 3, which contained spent grain of purple wheat, were rated as the worst.

Figure 1 The shape of the bread rolls (vertical column indicates a confidence interval of 0.95)



Also, it was perceptible that the best aroma was founded on those samples which contained purple wheat spent grain, samples 2 and 3 (Figure 2). Further, as good samples were evaluated those products which contained purple wheat spent grain and barley. Also some evaluators mentioned that the sample 8 had no sharp smell. Ktenioudki et al. (2013) in his study writes that the addition of 10% of spent grain strongly changes the smell of the product, because some roving materials are created and released.

Figure 2 The aroma of the bread rolls (vertical column indicates a confidence interval of 0.95)



As it is shown in Figure 3, the effects of the different types of spent grain on the flexibility of the crumb doesn't have a unique effect. The values ranged from 8 to 8.8 points. In general, it could be said that, the crumb was compared with the control sample which has a tougher crumb.

The tastiest samples (Figure 4) were the samples 4 and 5, which contained only spent grain of purple wheat and were evaluated to more than 8 points. The samples which contained some amount of spent grain from barley and from blue wheat, samples 8 and 9, were also evaluated better than the samples which contain spent grain only from purple wheat. The taste is significantly affected whether the samples contained whole or cut spent grain as it is demonstrated in this study.

Figure 3 The flexibility of the crumb (vertical column indicates a confidence interval of 0.95)

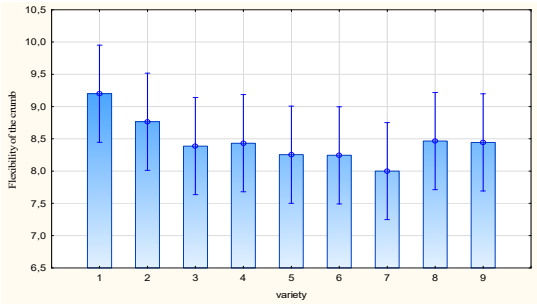


Figure 4 The taste of the bread rolls (vertical column indicates a confidence interval of 0.95)

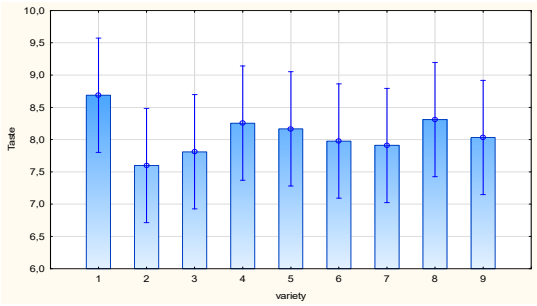
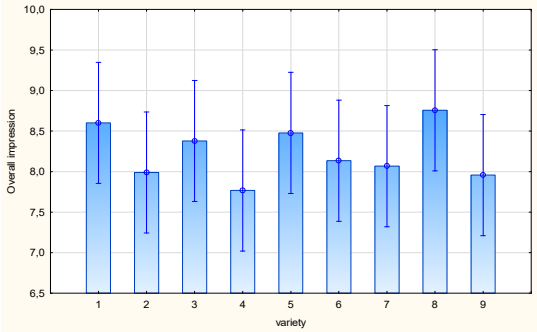


Figure 5 shows the overall impression. The best score (8.8 points) was received by sample 8 that contains whole purple wheat spent grain and barley in a proportion 50 : 50.The second best rated sample was the sample 5, which contained only cut spent grain from blue wheat. The worst sample was number 4, where the value of the overall impression reached only 7.8 points.

Figure 5 The overall impression of the bread rolls (vertical column indicates a confidence interval of 0.95)



Only small differences should be noticed between all the samples. The values related with the overall impression were ranged from 7.8 to 8.8. For this reason, it is not clear if exist a significant difference between the type of spent grain (barley or wheat) and the size (whole or coarsely cut) of spent grain on the overall impression. Moreover, this experiment shows that the addition of wheat color spent grain is perceived positively by the consumer. It could be said that this addition improves the ethical value of the bread but it is necessary to choose the appropriate type and amount of spent grain.

CONCLUSION

The aim of this research was to evaluate the use of various types and proportions of color wheat spent grain for use in bakery products. Subsequently, these products have been evaluated by sensory evaluation by their form, and were studied their quality characteristics. The trial was conducted in the laboratory of the Institute of Food technology of Mendel University in Brno.

It can be noted that, the participants rated the products which contained fresh spent grain, very pleasant. The best samples were those which contained whole or cut fresh spent grain. The samples were enriched with baking powder. Their overall impression was rated from 7.8 to 8.8. Their total sensory profile was balanced.

The experiment was conducted by the addition of fresh spent grain to the samples, where different types of spent grain were used, which contained various colors of wheat, genetically modified, and barley, used separately or in proportion of 50 : 50.There was no difference in the evaluation of the individual parameters that were considerable. The biggest difference could be found in the evaluation of the color of the crust, but this could be caused by irregular baking.

As the best and the most consistent sample was that one which contained whole spent grain from blue wheat and barley in proportion 50 : 50. It was rated with 8.8 points. As the second best sample was rated the one which contained fresh cut spent grain from blue wheat. The worst sample was considered the one which contained whole spent grain of blue wheat. It is overall impression was evaluated to 7.8 points.

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HYDROXYMETHYLFURFURAL IN SYRUPS, DOUGHS AND IN SYRUP´S BISCUITS

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Abstract: Hydroxymethylfurfural (HMF) is formed by dehydration of sugar and Maillard reaction during storage and food processing. The aim was to determine the amount of HMF in selected syrups, honey, cane molasses and sucrose, and then in doughs and biscuits made from these sweeteners. Biscuits were baked at 175 °C, 200 °C and 225 °C. In doughs was measured pH. The lowest specified value of HMF was in biscuits with sucrose, the dough with sucrose had the highest value of pH.

Key Words: HMF, bakery products, corn syrup, wheat syrup, date syrup

INTRODUCTION

HMF is highly reactive crystalline colorless solid, in the air immediately browns, causes brown or yellow-brown color in the product. HMF respectively its reaction products exhibit slightly "fruity odor" (Bacílek and Kamler 2009). HMF is well soluble in water, ethanol, methanol and ethyl acetate, and less soluble in petroleum ether. Absorption maxima of HMF are at 284 nm and 230 nm (Gökmen and Morales 2014).

HMF is formed in Maillard reaction and dehydration of sugars during caramelization. Maillard reaction is non-enzymatic browning, which leads to a chemical reaction between a reducing sugar and an amino acid at high temperature. HMF occurs in processed foods. HMF molecule consists of furan ring, a hydroxymethyl group and a carbonyl group (Gökmen and Morales 2014).

The main precursors for the formation of HMF are amino acids and sugars, particularly hexoses. HMF is formed in carbohydrate´s foods such as jams, fruit juice concentrates and honeys. Increasing temperature during storage or processing is leading to the faster formation of HMF. It is therefore appropriate to reduce the temperature e.g. using a vacuum residue or biscuits. HMF quantity is increased at lower pH. Therefore, temperature should be optimized during the storage and processing of acidic foods, to reduce the amount of HMF. The amount of HMF is increased with the length of storage and HMF formation is accelerated with low or average moisture (Gökmen and Morales 2014).

HMF is formed more in bread, which contains glucose, than sucrose. Also the formation of HMF is higher in more acidic dough and leading to intensive surface browning (Gökmen et al. 2007). Fructose is degrading (to HMF) more rapidly in comparison to sucrose and glucose (Přidal 2013).

Hydroxymethylfurfural is probably toxic and mutagenic substance (Velíšek and Hajšlová 2009) In living organisms. HMF is converted to 5-sulfoxymethylfurfural (SMF) which is genotoxic (Capuano and Fogliano 2011). HMF short study on the creation of cancer in the intestinal tract can not be clearly labeled as carcinogenic HMF. Daily dietary intake of HMF is in the hundreds mg/kg, which is much higher than the income of other toxic food substances. Intake of HMF is estimated 4 to 30 mg/person/day. Intake above 350 mg/person /day is possible, e.g. consumption of prune beverages. In experiments it was found that the amount of 80–100 mg of HMF/kg body weight/day showed no adverse effect in experimental animals. Of the mentioned knowledge seems to be the current exposure hydroxymethylfurfural for humans safe (Abraham et al. 2011).

HMF is included in U.S. Public Health Service's National Toxicology Program on sheet for Toxicological studies (Rupp 2003).

The amount of HMF is recorded only in honeys. According to the Czech Decree no. 76/2003 Coll. the current limit of HMF with floral and honeydew honeys is 40 mg/kg, and for honeys from areas with

tropical climates and their mixtures is 80 mg/kg. The amount of HMF in honey is an indicator of the age of honey, honey heating and poor storage conditions. The requirements for the amount of HMF in honey are incorporated in the legislation of the European Union, and in the Codex Alimentarius (Zappala et al. 2005).

The quantity of HMF occurring in honey, is monitored for quality reasons. HMF is highly toxic to bees, wherefore bees must be supplementary feeding sugar of good quality and no overheated honey, in which is higher levels of HMF (Přidal 2013). Bee feeding of hydrolyzed sucrose catalyzed by acid is danger to bees, because it contains HMF. In contrast, the enzymatic hydrolysis of sucrose doesn´t arise HMF (Titěra 2009).

HMF quantities in some foods: biscuits from 0.5 to 74.5 mg/kg, white bread 3.4 to 68.8 mg/kg, breakfast cereals from 6.9 to 240.5 mg/kg, honey 10.4–58.8 mg/kg, dried fruit from 25 to 2900 mg/kg, marmalade from 5.5 to 37.7 mg/kg, malt 100 to 6300 mg/kg, instant coffee from 400 to 4100 mg/kg, coffee 100–1900 mg/kg, chicory 200 to 22500 mg/kg, beer from 3.0 to 9.2 mg/l, red wine from 1.0 to 1.3 g/l, balsamic vinegar 316.4 to 35251.3 mg/l (Capuano and Fogliano 2011).

The aim was determining the amount of HMF in selected sweeteners, doughs and biscuits. pH test to determine the dependence on the amount of HMF in dough and biscuits and baking temperature dependence of different formation HMF.

MATERIAL AND METHODS

Sweeteners used: syrups: date, wheat and corn syrup, as well as honey, cane molasses and sucrose. All sweeteners were purchased in the Czech Republic. Dough and biscuits were made at the Department of Food Technology. Used recipe was 40 g of sweetening agent per 100 g of flour. Biscuits were baked at 175 °C, 200 °C and 225 °C.

The preparation of sample for the determination of HMF (Zhang et al. 2012): First, solutions Carrez I and Carrez II were prepared. Solution Carrez I: to 15 g $K_4[Fe(CN)_6] \cdot 3H_2O$ (from Lach-Ner, Ltd.) was dissolved in water in 100 mL volumetric flask, and the solution was thoroughly mixed. Carrez II solution: 30 g of $ZnSO_4 \cdot 7H_2O$ (from Lach-Ner, Ltd.) was dissolved in water in 100 mL volumetric flask.

A sample of 1 g was put into a 20 mL centrifuge tube with a cap and 250 μL Carrez I below 250 μL Carrez solution II and 9.5 mL of demineralized water. Centrifuge tube was shaken vigorously on a shaker (450 revolutions/min.) for 5 minutes and subsequently centrifuged for 15 min. at 4000 revolutions/min. and 4 °C. The solution was filtered through a 0.45 μm filter disk, loaded in Eppendorf microtube (1.5 ml) which were sealed and stored in a freezer at -18 °C until determination of HMF by HPLC. The values measured by HPLC were computed in Microsoft Excel 2010

HMF has been measured on high pressure liquid chromatography, HPLC (Agilent 1100 Series) with UV/VIS detection. The column used was an Agilent ZORBAX Eclipse XDB-C18 column size 4.6 x 150 mm, particle size 5 μm, manufactured in the USA.

Detection was carried out at a wavelength of 284 nm and 25 °C. Mobile phase of methanol-water (5 : 95; V : V) was used isocratic elution. The mobile phase flow was 1 mL/min, the volume of sample was 10 μL. Data were obtained and processed utilizing Agilent ChemStation software. Standard 5-hydroxymethyl-2-furancarbaldehyd (Merck spol. s r.o.) was used for calibration (0.1–100 μg/mL).

Each sample was measured twice, in the case of different results of three to four times with the exclusion of outliers.

The pH of doughs was measured by table pH meter HANNA Instruments pH 212. Electrode of pH meter was put in the dough and measured three times.

RESULTS AND DISCUSSION

The lowest average value of the amount of HMF (Table 1) was found in the sweetener sucrose and the highest measured value was in cane molasses. HMF quantity of the doughs varied from 0–8.06 mg/kg in biscuits from 0.48 to 13.99 mg/kg.

Table 1 Average concentration of HMF in sweeteners, doughs and biscuits and pH of doughs

Sweetener	pH of dough	Concentration of HMF [mg/kg]				
		Sweetener	Dough	Biscuits 175 °C	Biscuits 200 °C	Biscuits 225 °C
Sucrose	7.19	0.12	0	0.68	0.81	0.48
Honey	6.60	20.24	2.72	9.44	8.96	20.43
Wheat syrup	6.47	4.64	0.68	2.30	2.60	1.90
Corn syrup	6.31	0.47	0	1.67	0.99	3.08
Date syrup	5.79	8.36	1.29	3.50	4.16	6.69
Cane molasses	5.66	41.38	8.06	13.99	10.83	10.39

The amount of HMF and pH were correllated in doughs (R = -0.62), in biscuits (baked at 175 °C R = -0.54; at 200 °C R = -0.53; at 225 °C R = -0.23). Further, the correlation of the HMF and baking temperature for each type of biscuit was estimated separately (with sucrose R = -0.60; honey R = 0.85; wheat syrup R = -0.57, corn syrup R = 0.66, date syrup R = 0.95, cane molasses R = -0.92). Statistical significance has not been found for any of the above mentioned correlation coefficients (R, alpha 0.05).

However in the study Vorlová et al. (2006) the amount of HMF in fruit syrups, syrups for preparation of beverages was determined. Average value of HMF's concentration was in fruit syrups 6.6 mg/kg, which is higher than average value for wheat and corn syrup, which we measured.

In the study by Ruiz-Matute et al. (2010) HMF quantity 23.48 mg/kg was found in High-Fructose Corn Syrups, produced by enzymatic hydrolysis of manufacturers in cornstarch. This value of HMF is several times higher than in our corn syrup.

Study of Jafarnia et al. (2016) compared the amount of HMF in date syrups produced by the industrial and traditional methods. Traditional methods: Date with water are heated, filtered and again heated until needed concentration. Industrial method: the filtrate is concentrated under vacuum, the temperatur set to 70 °C. Amount of HMF was higher in traditional production: the fresh product 1000–2675 mg/kg, old product of 2580–6450 mg/kg. The industrial method fresh from 12 to 456 mg/kg, old 611–943 mg/kg. From the values of the steam as the growth of HMF during storage. Our value determined in data syrup was lower, maybe was produced by industrial method.

In study of Ramirez-Jimenez et al. (2000), it was found that there wasn't linear correlation between concentration of HMF and color in bakery products, while the linear correlation between methylfurfural and HMF in bakery products was obtained. HMF quantity was 4.1 to 151.2 mg/kg and the color index (100-L*) was from 23.1 to 42.9.

Gökmen et al. (2007) reported that decreasing the pH of the dough will increase the amount of HMF formed during the baking, and increase the surface browning. Our lowest specified amount of HMF was actually measured in dough and biscuits with the highest pH (products with sucrose), while the highest amount of HMF and biscuits dough showed the lowest pH (product cane molasses). However Purlis (2010) reports that pH>7 leads to cleavage and dehydration of sugar, degradation of amino acid (Stecker degradation), the polymerization and production of melanoidins.

Ameur et al. (2006) reported that the formation of HMF is dependent on water activity, temperature of baking (200 °C, 250 °C and 300 °C) and used sugars (fructose, glucose, sucrose). Biscuits baked at higher temperatures had 10 to 100 times more HMF (167.4 to 1100.1 mg/kg), than baked at 200 °C (9.9 to 39.6 mg/kg). The amount of HMF was higher at higher baking temperature. Biscuits with sucrose had the least HMF at 200 °C (9.9 mg/kg) than fructose and glucose (39.6–34.2 mg/kg). The amount of HMF in biscuits with sucrose was rapidly risen when baking at 300 °C, which was caused by thermal degradation of sucrose (HMF quantity 1100.1 mg/kg).

In this study had only the biscuits with data syrup higher value of HMF with higher baked temperature.

CONCLUSION

The lowest measured value of HMF was in biscuits with sucrose, respectively using non-reducing sugar. Among them, the lowest amount was in biscuits baked at 225 °C. From the measured values it is obvious that the amount of HMF depends not only on the sweetener used, but also on the temperature of baking.

The amount of HMF in biscuits from syrups isn´t high, it did not constitute an increased risk to human health.

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PROPORTION OF IMPORTANT FATTY ACIDS IN COW AND GOAT MILK FAT

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Abstract: Selected fatty acid (FA) contents and their groups were determined in milk fat of two cattle (Czech Fleckvieh, Holstein) and two goats' (Brown Shorthair, Anglo-Nubian) breeds. Goat fat in comparison with cow fat showed higher proportion of volatile FA (14.9–15.6% and 8.4% from total FA, respectively), and lower proportion of C16:0 (25.6–26.8% and 30.7–31.5%, respectively). The differences in milk FA between imported breeds and breeds of Czech origin have been also tested. The statistically significant differences were confirmed only in goat fats for C18:3*n-3*, *cis*-9, *trans*-11 C18:2 ($p<0.01$, 0.001), and in groups of saturated and unsaturated FA ($p<0.05$, 0.01).

Key Words: cow, goat, milk, fatty acids, breed

INTRODUCTION

Milk belongs among well balanced basic food group in human diet. Its annual production reached 802 million tons, of which cow milk represented 83.3% and goat milk 2.2% (FAO Statistical Databases 2010). An important part of milk solids is milk fat. Its content is the highest in ovine milk (6.09%–6.80%), the fat content in cow and goat milk is similar (3.16%–4.96%) – Mahmood and Usman (2010). Milk fat consists predominantly of esters of glycerol and fatty acids (FA), which affect the property of milk and dairy products (Hillbrick and Augustin 2002). FA are divided into various groups most often by the presence of double bonds and the number of those (Velíšek and Hajšlová 2009), eventually by the number of carbons in FA chain. Proportion of FA groups or indices between the groups is important for its nutritional value (Barłowska et al. 2011) as well as from technological view (Michalski et al. 2004), or even for its sensory properties (Chilliard and Ferlay 2004). For example, ratio between palmitic acid (C16:0) and oleic acid (*cis*-9 C18:1) is an important criteria of textural properties of butter. This "spreadability index" relates to melting point of milk fat and thus determines the hardness of butter (Couvreur et al. 2006).

Ruminant milk fats are characterized by proportion of volatile FA (VFA; C4–C10), which is higher in goat milk (15 to 18%, Raynal-Ljutovac et al. 2008) than in cow milk (7.9 to 9.2%, Hanuš et al. 2010). Typical for milk fat is also high proportion of saturated FA (SFA), low proportion of polyunsaturated FA, and occurrence of *trans* isomers of unsaturated FA. The latter group contains unique isomer *cis*-9, *trans*-11 C18:2 (RA, rumenic acid), one of the isomers of conjugated linoleic acid (CLA). RA is known for its biological effects – anticarcinogenic, antiatherogenic, or immunomodulatory (Parodi 2004).

Milk FA composition depends on many factors, such as: genetic predisposition, stage of lactation, nutrition etc. (Stoop et al. 2008). Several studies have discussed the effects of breed on FA composition. This effect is relatively high in SFA (Dewhurst et al. 2007), while nutrition is the main factor in proportion of unsaturated FA (UFA) – AlZahal et al. (2009). Samková et al. (2012) reported that milk fat produced by indigenous breeds, dual-purpose breeds and crossbreeds appears to have a more desirable profile of FA than milk fat produced by imported dairy breeds (mostly Holstein).

The aim of this work was to evaluate FA proportion in milk fat of dairy cows and goats depending on breed (indigenous vs. imported). And further, the differences between cows' and goats' milk fat were assessed.

MATERIAL AND METHODS

Experimental design

The study was carried out in the Czech Republic. The Czech Fleckvieh (CF) and Holstein (H) cattle, Brown Shorthair (BSH) and Anglo-Nubian (AN) goats were assessed – Table 1. The cows were housed together in one tie-stall barn. The goats were from two goat farms.

Individual milk samples were collected during lactation within the regular testing of milk efficiency. The samples were immediately cooled (to 6 °C) and transported to the laboratory in a cool box.

Table 1 General characteristic of sampling and cattle and goat breeds

Breeds[1]	Sea level (m)	Management	Origin of breed	Number of		Number of (%)	
				sampling[2]	animals	primiparous	multiparous
CF	420	conventional	Czech	4	32	27	73
H	420	conventional	imported	4	37	33	67
BSH	445	organic	Czech	4	10	40	60
AN	516	organic	imported	3	12	39	61

Legend: [1]CF – Czech Fleckvieh, H – Holstein, BSH – Brown Shorthair, AN – Anglo-Nubian; [2]March, June, September and December (for CF and H), May, June, July, October (for BSH), May, September, October (for AN);

All cows were fed under the same conditions. Total mixed rations consisted of maize and grass silages, mashed oats, meadow hay (60.6, and 4% of dry matter, respectively). Production feed mixture (30% of dry matter) composed of barley, wheat, extracted soybean meal, and salt, minerals, vitamins in proportion 32, 32, 32, and 4%, respectively.

Both breeds of goat were grazed on natural pasture. And further, BSH goats were fed mixture (1.4 kg/day) of mashed oats and barley (1:1), AN goats were fed mixture (1.6 kg/day) of oats, barley, and sugar beet (1:1:0.5).

Chemical and statistical analysis

Fat, protein and lactose contents were determined spectrophotometrically using a MilkoScan 4000 apparatus (Foss Electric, Hillerød, Denmark) – Table 2.

Table 2 Milk composition for the breeds of cattle and goats

	Fat (%)			Protein (%)			Lactose (%)		
	Mean	SD	*P*	Mean	SD	*P*	Mean	SD	*P*
Czech Fleckvieh	4.21	0.89		3.62	0.41		4.78	0.38	
Holstein	4.04	0.83	0.2448	3.49	0.38	0.0506	4.77	0.27	0.7597
Total	4.12	0.86		3.55	0.40		4.77	0.33	
Brown Shorthair	4.46	2.51		3.25	0.35		4.56	0.27	
Anglo-Nubian	5.21	1.84	0.2032	4.28	0.78	0.0000	4.06	0.30	0.0000
Total	4.94	2.11		3.91	0.83		4.24	0.38	

Legend: SD – standard deviation; P – probability;

Milk fat was extracted with petroleum ether from freeze-dried milk samples. FA in isolated fat were re-esterified to their methyl esters with a methanolic solution of potassium hydroxide. Methyl esters of FA were determined by a gas chromatographic method (GLC) using a Varian 3300 apparatus (Varian Techtron, USA) under conditions described by Frelich et al. (2012). The identification of FA was carried out using analytical standards (Supelco, USA). In total, 64 FA were observed, 50 of which

were identified. The proportions of individual FA were calculated from the ratio of their peak area to the total area of all the observed acids.

Data were statistically analyzed by the program Statistica CZ 12 (Statsoft CR). Student´s *t*-test for comparison between groups was used.

RESULTS AND DISCUSSION

Fatty acid composition of cow milk fat

The difference in composition of milk fat of two breeds was assessed in this study, the CF (dual-purpose breed of Czech origin) and H (imported dairy breed).

The proportions of SFA (67.94% for CF and 67.98% for H) and UFA (29.34% and 29.37%, respectively) were in both breeds almost identical (Table 3). Also the group of VFA, essential FA (C18:2*n-6*, C18:3*n-3*) and RA were similar. Higher differences were found in the proportions of C16:0 and *cis*-9 C18:1. The proportion of C16:0 in milk fat of CF was lower in comparison to H (30.66% and 31.50%, respectively), whereas the proportion of *cis*-9 C18:1 was higher (19.16% and 18.53%, respectively). However, there were no statistically significant differences. Similar results were found by Pešek et al. (2008) or Adamska et al. (2014).

The spreadability index (depending on C16:0/*cis*-9 C18:1) seemed to be more desirable in milk fat of CF dairy cows. The oxidative stability of milk fat set by the SFA/UFA index is almost identical in both breeds.

Table 3 Proportion of fatty acids (FA) and groups of FA (% of total FA) in cow and goat milk fat depending on breed

Item	Cow		*P*		Goat		*P*
	Czech Fleckvieh	Holstein			Brown Shorthair	Anglo-Nubian	
	Mean ± SD	Mean ± SD			Mean ± SD	Mean ± SD	
C16:0	30.66 ± 3.64	31.50 ± 3.85	0.1819		25.59 ± 3.93	26.79 ± 2.28	0.1131
cis-9 C18:1	19.16 ± 3.40	18.53 ± 3.94	0.3188		17.79 ± 2.41	18.47 ± 3.29	0.3046
C18:2*n-6*	1.69 ± 0.35	1.67 ± 0.34	0.6737		1.99 ± 0.39	1.96 ± 0.27	0.7272
C18:3*n-3*	0.44 ± 0.13	0.44 ± 0.14	0.8955		0.76 ± 0.21	0.62 ± 0.16	0.0020
RA	0.42 ± 0.14	0.38 ± 0.13	0.1455		1.21 ± 0.54	0.53 ± 0.19	0.0000
VFA	8.41 ± 1.44	8.44 ± 1.61	0.9099		14.91 ± 1.91	15.64 ± 2.14	0.1197
SFA	67.94 ± 4.41	67.98 ± 4.83	0.9585		67.98 ± 4.37	70.11 ± 4.13	0.0324
UFA	29.34 ± 4.34	29.37 ± 4.90	0.9704		30.29 ± 4.18	27.83 ± 3.89	0.0099
C16/C18	1.67 ± 0.42	1.79 ± 0.50	0.1015		1.49 ± 0.42	1.51 ± 0.36	0.8048
SFA/UFA	2.38 ± 0.45	2.40 ± 0.51	0.8314		2.31 ± 0.48	2.59 ± 0.51	0.0162

Legend: SD – standard deviation; P – probability; RA – rumenic acid (cis-9, trans-11 C18:2); VFA – volatile fatty acids (C4–C10); SFA – saturated fatty acids (including branched-chain fatty acids); UFA – unsaturated fatty acids (including CLA); C16/C18 – C16:0/cis-9 C18:1;

Fatty acid composition of goat milk fat

Also in goat milk, the differences between the breeds of Czech origin (BSH) and imported breed (AN) were assessed. The proportion of SFA in milk fat of BSH breed was lower than in AN breed (67.98% and 70.11% for AN, respectively; $p<0.05$), whereas the proportion of UFA was higher (30.29% and 27.83%, respectively; $p<0.01$) – Table 3. High statistical significant differences were found in the proportion of C18:3*n-3* (0.76% and 0.62%, respectively; $p<0.01$) and RA (1.21% and 0.53%, respectively; $p<0.001$). It leads to conclusion that nutritionally desirable profile of milk fat is from the milk of indigenous breed rather than from imported breed. On the bases of gathered information, it seems that the significant differences in the proportion of listed FA were mainly caused by the variety of keeping standards and conditions for particular breeds and also by the difference in food composition, which was not identical. According to several authors (stated by Kalač and Samková 2010), the botanical

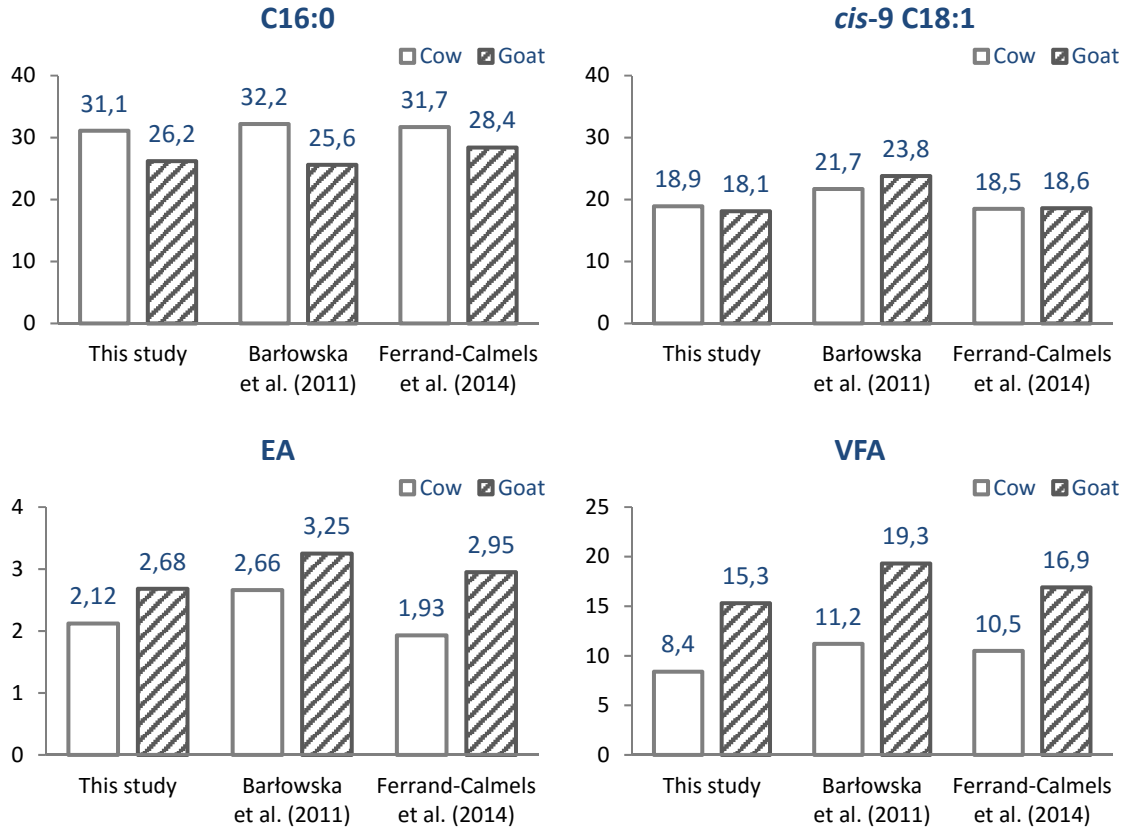
composition of pasture could have a significant effect on the composition of milk fat. However, even animals kept under same conditions and fed same feed ration can show significant differences among breeds as reported in sheep breeds by Tsiplakou et al. (2006).

Spreadability index was almost identical in both breeds. The SFA/UFA index in BSH breed was statistically significantly lower than in AN breed (2.31 and 2.59, respectively; $p<0.05$). Thus, the oxidative stability was more suitable in imported breed.

Comparison of cow and goat milk fat

Whilst comparing the composition of milk fat, it became apparent that goats had higher proportion of VFA (14.91–15.64% and 8.41–8.44%, respectively) and lower proportion of C16:0 (25.59–26.79% and 30.66–31.50%, respectively) than cows. Furthermore, goat milk fat contained higher levels of essential FA – C18:2*n-6* a C18:3*n-3*. Our results correspond to similar studies, see Figure 1. High proportion of VFA in goats´ milk fat can highly affect sensory properties of goat milk and its products as reported by Soryal et al. (2005).

Figure 1 Proportion of selected fatty acids (FA) and groups of FA (% of total FA) in cows´ and goats´ milk fat

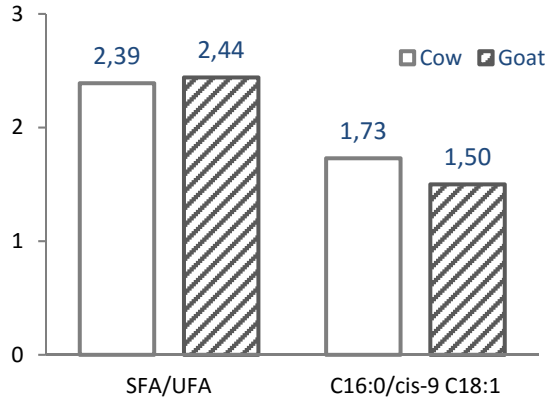


Legend: EA – essential fatty acids (C18:2n-6+C18:3n-3), VFA – volatile fatty acids (C4–C10)

Even though, the proportion of SFA in cow milk fat and goat milk fat were similar; the SFA/UFA index slightly varied (2.39 and 2.44, respectively; Figure 2).

The spreadability index was in cows (1.73) higher than in goats (1.50). This composition of milk fat results in better spreadability of butter made of goat milk. This ratio was also confirmed by Hurtaud et al. (2001) as the most accurate indicator of the hardness of butter.

Figure 2 Proportion of index SFA/UFA and spreadability index (C16:0/cis-9 C18:1) in cows´ and goats´ milk fat



Legend: SFA/UFA – saturated fatty acids/unsaturated fatty acids

CONCLUSION

Milk fat is predominantly assessed for its nutritional values, while the lower proportion of saturated fatty acids is preferred. However, required higher proportion of unsaturated fatty acids leads to decrease in the oxidative stability of milk fat.

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COLOUR CHANGES IN TWO KINDS OF SEMI-HARD CHEESE DURING RIPENING

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Abstract: The aim of the paper was to compare the colour changes of semi-hard cheeses, depending on days of ripening. The surface of cheese samples were treated of two techniques i.e. oiling and waxing. CIELAB Colour parameters (L* a* b* and ΔE^*_{ab}) were measured for cheese samples (oiled and waxed) and measuring points were located either near edge or in middle of cheese samples sliced in half. Samples were subjected to measurement after manufacture and then every 10, 20, 30, 40, 60, 85, and 115 days of ripening, respectively, there were observed statistically significant differences in all colour parameters during ripening and between waxy and oiled cheese samples. Lightness (L*) declined and yellow tone (b*) was more appreciable for oiled cheese during ripening. Wax protective layer prevented moisture loss and that caused that value of L* and a* parameters were not so variable. Total colour difference was (ΔE^*_{ab}) clearly perceptible for all cheese samples.

Key Words: cow´s semi-hard cheese, colour, surface treatment of cheese, cheese ripening

INTRODUCTION

One way how to judge foodstuffs is their appearance. In that respect, colour is a clue for many qualities of food such as flavour, sanity, naturality or maturity, and consumers may accordingly decide which product is most acceptable for them (Dufossé et al. 2005). Perception of taste by consumers can actually be influenced by colour, which is overlooked sensory attribute. On this basis, the taste of cheese affects acceptance and marketing of products (Young et al. 2004, Yates and Drake 2007).

Ripening of cheese is complex of biochemical processes which takes take place under physical, microbial, and enzymatic conditions. Under the influence of these conditions, blank curd is converted into a mature cheese having the flavour, texture, and aroma characteristic of intended type of cheese. During the ripening, the structure, composition and organoleptic properties are modified (Fox 1993, Spree 1998, Walstra 2006). Young et al. (2004) observed the effect of Cheddar cheese in various stages of maturity on the preference of consumers was observed.

Dufossé et.al. (2005) monitored the ripening process and quality of PDO (Protected Designation of Origin) red-smear soft cheeses (Epoisses, Munster, Maroilles, Livarot, etc.). Spectrocolorimetry analysis in the CIE L*a*b* colour space was used to detect connections, how colour affects the perception of taste and consumer acceptability. For food producers is important that the colour of the product was appropriate to the characteristics of the product because consumers associate certain colours with certain flavours (Wadhwani 2012). Colour can be evaluated visually (sensory evaluation) or instrumentally. Both methods have their advantages and disadvantages, but in fact complement each other. The advantage of using instrumental methods to quantify colour is that these are repeatable and objective. Its disadvantage is that, often, are difficult to interpret by people unfamiliar with them. Colorimeter is the name of the equipment that quantify colour and are more sensitive that the human eye, whose measures are reproducible and well correlated with perception human (Ramírez-Navas 2010).

The aim of this paper was a comparison the colour changes of semi-hard cheeses during ripening, where were used different surface treatment of cheese.

MATERIAL AND METHODS

Cheese samples

Cheese samples were manufactured in division of cheese production of Mendel University in Brno at Department of Food Technology. Cheese samples were manufactured by the following flowchart (Figure 1). Milk for manufacture of cheeses samples came from Holstein dairy cows from South Moravia region. Milk composition analysis and measurement methods are shown in Table 1.

Figure 1 Flowchart of manufacture process of cheese

Heat treatment

(raw milk was heated at 72 °C for 30 sec → cooled at 33 °C)

↓

Acidification

Addition of cheese culture TM1 and MC1 (Bulgaricus, CZ)

(milk was held at 32 °C during 40 min, adding 10 ml 36% CaCl)

↓

Coagulation

Addition of commercial chymosin rennet Naturen 145 IMCU (Chr. Hansen, Denmark)

(for 70 l of milk was used 40 ml chymosin rennet, time: 40 min)

↓

Cutting of curd

(size: small cubes 15 x 15 mm, time: 15 min)

↓

Draining of the whey

Whey was removed (18 l) and 15 l of warm water (40 °C) was added.

↓

Heating and agitation

(heated to 40 °C and stirred for 30 min)

↓

Moulding and formation

Curd was filled into forms, whey was separated

(formation and pressing: 45 min → the curd was turned and pressed next 20 min)

↓

Fermentation

(time: 20 hours)

↓

Pasta Filata treatment

(temperature of water: 85 °C, time: 2 min)

↓

Salting

Cheeses were placed into salt brine (18% NaCl) for 20 hours at 10 °C

↓

Drying and resting

(time: 2 days, temperature: 12 °C)

Table 1 Milk composition analysis and measurement methods

Parameter	Value	Method	Standard
Dry matter	12.69%	gravimetry	ISO 6731:2010
Fat content	3.6%	Gerber	ISO 2446:2008
Protein content	3.19%	Kjeldahl	EN ISO 8968-1:2002
Lactose content	4.92%	polarimetry	ČSN 570530
Titratable acidity	6.5SH	Soxhlet-Henkel	ČSN 570530

Cheese protecting and ripening

After two days of drying and resting, cheese loaves were divided in half. First part of cheeses was covered by cheese wax (Driml, Czech Republic, BB: 1/2018) and the second half was oiled (rape seed oil, Czech Republic). Maturation of threated cheeses took place in ripening chamber at 12 ± 1 °C and relative humidity 85% for 115 days. Applying next oil films on cheeses as well as removing surface moulds was performed as needed.

Due to study of chemical parameters, colour, and sensory evaluation during ripening were produce 40 cheeses. After 10, 20, 30, 40, 60, 85, and 115 days of ripening, part of cheeses was subjected to physicochemical analysis and sensory evaluation. The cheeses had cylindrical shape, average width 71.8 mm and average height 33.7 mm. Weight of cheese loaves ranged between 112 g to 140 g.

Colour measurement

Colour measurements were performed in a spectrophotometer Konica Minolta CM-3500d (Japan). Data was analysed with the SpectraMagic software version 2.0. Reference illuminant was D65 (standard daylight). The colour parameters of L* (lightness), a* (green-red value), and b* (blue-yellow value) of samples were determined. Cheese samples were measured in reflectance mode and 8 mm aperture was used. Cheese loaves were cut in half. Colour measurements always took place on the inner surfaces of each half of cheeses. Measurements were made in triplicate for part following the edge of cheese and for middle part of sample as well. There were observed L* a* b* parameters depending on the length of maturation.

The differences between the samples were evaluated either in individual parameters (L * a * b*), or by using the total colour difference (ΔE^*_{ab}) that is dependent on given parameters and calculated using following formula:

$$\Delta E^*_{ab} = [(\Delta L^*)^2+(\Delta a^*)^2+(\Delta b^*)^2]^{1/2} ,$$

where the resulting difference in colours are by Zmeškal et al. (2002) from undetectable to very pronounced or interfering. This change is an accepted method of evaluating colour difference. Final values of ΔE^*_{ab} were compared with a range by Zmeškal (Table 2).

*Table 2 Colour difference based on the total difference (*Zmeškal *et al.* 2002)

ΔE^*_{ab}	Colour difference	ΔE^*_{ab}	Colour difference
0.0–0.2	Imperceptible	3.0–6.0	Middle
0.2–0.5	Very light	6.0–12.0	Significant
0.5–1.5	Light	12.0–16.0	Very significant
1.5–3.0	Clearly perceptible	More then 16.0	Interference

Statistical analysis

For determining statistically significant difference in ripening on individual parameters was used program STATISTICA 12.

RESULTS

During cheese ripening was statistically conclusive change in the colour of the edge part of oiled cheese – the edge part of cheese turned dark till 60 days of ripening. In next 45 days (60–115 day of ripening) cheeses become lighten. The same trend was obvious in a* parameter, when value of this parameter declined till 60 day of ripening. According to the criteria Zmeškal et al. (2002) is a colour change during storage most noticeable in first 10 days of ripening. Changing in the parameters of edge part of oiled cheese during storage is given in Table 3.

Table 3 Colour parameters of edge part of oiled cheeses during ripening

Days of ripening	L* (D65)	a* (D65)	b* (D65)	ΔE^*_{ab}
0	89.56 ± 0.26^{d}	0.52 ± 0.12^{b}	15.83 ± 0.42^{c}	---
10	86.69 ± 0.47^{a}	0.49 ± 0.14^{b}	17.36 ± 0.59^{b}	4.29
20	85.89 ± 0.82^{a}	0.14 ± 0.05^{a}	18.53 ± 0.48^{a}	3.18
30	84.04 ± 0.59^{c}	0.12 ± 0.05^{a}	18.54 ± 0.22^{a}	1.58
40	82.80 ± 0.58^{b}	0.04 ± 0.12^{a}	17.39 ± 0.08^{b}	2.08
60	82.30 ± 0.13^{b}	0.19 ± 0.06^{ac}	18.78 ± 0.64^{a}	1.06
85	85.71 ± 0.83^{a}	0.51 ± 0.13^{b}	18.62 ± 0.15^{a}	2.93
115	85.94 ± 0.30^{a}	0.35 ± 0.05^{bc}	17.02 ± 0.11^{b}	3.58

$^{a, b, c, d}$ *– different superscripts in a column indicate a statistically significant difference at P < 0.05*

The values measured in the middle part of oiled cheese had a similar trend to the values measured in the edge part. Parameter L* (lightness) decreased during period from 0 to 60 day of ripening. In compare with samples from the edge part, higher values were measured for parameter b* (yellow axis). The values were from 16.26 (0 day) up to 20.32 (60 day). Consequently, yellow tone was most saturated between 40 and 60 days of ripening, therefore, it was statistically difference from the other days. Changing in the parameters of middle part of oiled cheese during storage is shown in Table 4.

Table 4 Colour parameters of middle part of oiled cheeses during ripening

Days of ripening	L* (D65)	a* (D65)	b* (D65)	ΔE^*_{ab}
0	87.93 ± 0.05^{e}	0.61 ± 0.01^{ab}	16.26 ± 0.02^{e}	---
10	85.14 ± 0.45^{c}	0.65 ± 0.07^{b}	18.44 ± 0.40^{ab}	2.44
20	84.09 ± 0.43^{bc}	0.54 ± 0.17^{ab}	18.71 ± 0.90^{ab}	1.56
30	81.45 ± 0.74^{a}	0.54 ± 0.05^{ab}	19.28 ± 0.30^{ac}	1.49
40	81.87 ± 0.44^{a}	0.47 ± 0.02^{ac}	20.05 ± 0.33^{cd}	1.32
60	80.29 ± 1.03^{d}	0.36 ± 0.07^{c}	20.32 ± 0.68^{d}	2.85
85	81.58 ± 0.92^{a}	0.54 ± 0.08^{ab}	19.02 ± 0.38^{a}	1.42
115	83.62 ± 0.32^{b}	0.61 ± 0.09^{ab}	18.10 ± 0.31^{b}	1.42

$^{a, b, c, d, e}$ *– different superscripts in a column indicate a statistically significant difference at P < 0.05*

Parameter L* for edge part of waxed cheeses did not have so enormous variation in values. There was no statistically significant difference among 0 and 40 day of ripening. After 40 days of ripening, cheeses samples started to darken. The most noticeable change of b* parameter was observed in first

10 days of ripening. The value 15.72 is the lowest value of yellow tone from all measured results. Changing in the parameters of edge part of oiled cheese during storage is plotted in Table 5.

Table 5 Colour parameters of edge part of waxed cheeses during ripening

Days of ripening	L* (D65)	a* (D65)	b* (D65)	ΔE^*_{ab}
0	87.81 ± 0.08^{a}	0.38 ± 0.02^{ab}	18.02 ± 0.28^{abcd}	---
10	88.43 ± 0.66^{a}	0.40 ± 0.21^{ab}	15.72 ± 0.60^{e}	3.25
20	87.58 ± 0.27^{a}	0.36 ± 0.10^{ab}	17.06 ± 0.28^{ab}	2.08
30	87.11 ± 0.12^{a}	0.36 ± 0.80^{ab}	17.33 ± 0.71^{abc}	1.70
40	87.10 ± 0.70^{a}	0.13 ± 0.18^{c}	16.88 ± 1.07^{ae}	1.91
60	84.17 ± 0.61^{b}	0.21 ± 0.11^{ac}	18.87 ± 1.00^{d}	2.38
85	85.67 ± 0.94^{b}	0.41 ± 0.06^{b}	18.52 ± 0.90^{cd}	1.63
115	83.96 ± 0.64^{b}	0.13 ± 0.06^{c}	18.24 ± 0.62^{bcd}	2.50

$^{a, b, c, d, e}$ *– different superscripts in a column indicate a statistically significant difference at $P < 0.05$*

Statistically significant difference wasn´t detected among L* values of 10, 30, 40, and 85 days of ripening, respectively; and between 40, 60, and 85 days of ripening as well. Statistically significant differences in parameter b* were calculated from 30 to 115 day of ripening. However, no statistically significant differences were observed for 0, 10, and 20 day of ripening. Changing in the parameters of edge part of oiled cheese during storage is given in Table 6.

Table 6 Colour parameters of middle part of waxed cheeses during ripening

Days of ripening	L*(D65)	a*(D65)	b*(D65)	ΔE^*_{ab}
0	88.61 ± 1.30^{e}	0.49 ± 0.04^{ab}	16.43 ± 0.47^{b}	---
10	86.39 ± 1.38^{ac}	0.72 ± 0.08^{c}	17.17 ± 0.68^{ab}	1.07
20	87.80 ± 0.37^{e}	0.58 ± 0.04^{a}	16.83 ± 0.13^{ab}	2.27
30	86.81 ± 0.91^{ac}	0.47 ± 0.11^{ab}	17.47 ± 0.40^{a}	1.39
40	86.14 ± 0.84^{ab}	0.34 ± 0.07^{b}	17.63 ± 0.56^{ac}	1.15
60	84.71 ± 0.11^{bd}	0.45 ± 0.11^{ab}	18.57 ± 0.69^{d}	1.66
85	86.16 ± 0.29^{ab}	0.50 ± 0.11^{a}	17.57 ± 0.11^{ac}	0.78
115	84.13 ± 0.17^{d}	0.54 ± 0.03^{a}	18.35 ± 0.15^{cd}	1.85

$^{a, b, c, d, e}$ *– different superscripts in a column indicate a statistically significant difference at $P < 0.05$*

At the end, all samples include edge and middle part of oiled and waxed cheese were statically compared to each other. Most pronounced changes in parameters ΔE^*_{ab} were observed in colour of edge part of oiled cheese. Statistically the least perceptible difference in colour change was evident for colour measurement of middle part of waxed cheeses. When comparing the colour changes at the beginning (0 day) and at the end of ripening (115 day), the highest colour change was observed again in colour of edge part of oiled cheese (3.58). The slight change during maturation showed values ΔE^*_{ab} for colour measurement of middle part of oiled cheese (1.42). The total colour change of all samples was noticeable by the naked eye of the consumer. When comparing all measured values, values of parameter L* and b* for colour of middle part of oiled cheese samples, edge and middle part of waxed cheese samples were homogenous. Values of parameter a* were similar for measurements of edge part of oiled and waxed cheese as well. Statistically significant difference wasn´t demonstrable for a* value of middle parts of oiled and waxes cheese as well. Values of parameters L* and b* for colour of edge part of oiled cheese were different in compare with values of parameters L* and b* for the other measure points. There was water evaporation from the surface of the cheese and darken the edges.

CONCLUSION

This paper observed the changes in colour of semi-hard cheese, treated by oil and wax, during ripening. Colour of cheeses was monitored in the period of 115 days of ripening. Four parameters were

determined, L* (lightness), a* (green-red value), b* (blue-yellow value) and ΔE^*_{ab}. Each parameter was measured for edge part and middle part of oiled and waxed cheese as well. Lightness (L*) of oiled cheese were changed more in waxed cheese. Oiled cheeses slightly turned dark during ripening on the edge and similar tendencies were observed in middle part of cheeses. The significant changes in colour perception were perceived for edge part of oiled cheese, on the other hand, slight change during maturation were showed for middle part of waxed cheese. Using wax protection technique could give stable results in compare with oiled techniques.

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DETERMINATION OF THE CONTENT OF SELECTED PHENOLIC COMPOUNDS IN EIGHT KINDS OF SPICES BY USING LIQUID CHROMATOGRAPHY WITH MASS SPECTROMETRY

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Abstract: The aim of the experiment was to determine the content of selected phenolic compounds (3,4-dihydroxybenzaldehyde, caffeic acid, chlorogenic acid, cryptochlorogenic acid, ferulic acid, gallic acid, neochlorogenic acid, *p*-coumaric acid, *p*-hydroxybenzaldehyde , *p*-hydroxybenzoic acid, protocatechuic acid, salicylic acid, sinapic acid, syringic acid, vanilic acid, vanilin, cinnamic acid) in eight kinds of spices (marjoram, sweet pepper, black pepper, caraway seeds, anise, thyme, cinnamon and oregano) by using high performance liquid chromatography Agilent 1200 Series with diode array detector and a triple quadrupole mass detector 6460 Triple Quad (LC / MS). The extraction with methanol at various concentrations (60%, 80% and/or 100%) was done prior to determination of the mentioned phenolic compounds. The content of phenolic compounds was determined by using of method of HPLC-MS. The highest content of caffeic acid was determined in oregano (82.6 ± 4.0 μg/g), marjoram (93.7 ± 3.8 μg/g) and thyme (115.8 ± 3.6 μg/g). In anise and caraway seeds, the highest content was found at cryptochlorogenic acid (anise = 314.7 ± 14.0 μg/g, caraway seeds = 290.5 ± 14.0 μg/g). Ferulic acid (26.5 ± 1.3 μg/g) was the most abundant in sweet pepper. Cinnamic acid (278.0 ± 15.3 μg/g) was mostly occured in cinnamon. 3,4-dihydroxybenzaldehyde (53.0 ± 2.6 μg/g) was determined as of the highest content in black pepper.

Key Words: spices; phenolic compounds; methanol; high performance liquid chromatography.

INTRODUCTION

Constantly changing eating habits of humans use more spices (Delbeke et al. 2015). Nowadays, the spices are used especially for the high content of phenolic and flavonoid compounds (Moghaddam and Mehdizadeh 2015). These organic compounds produce and also affect the characteristic properties of spices (e.g. color, taste and smell) having antioxidative properties (Mayer 2005, Zacker 2006). These antioxidants are present in almost all plant parts, which are then utilized for the production of spices.

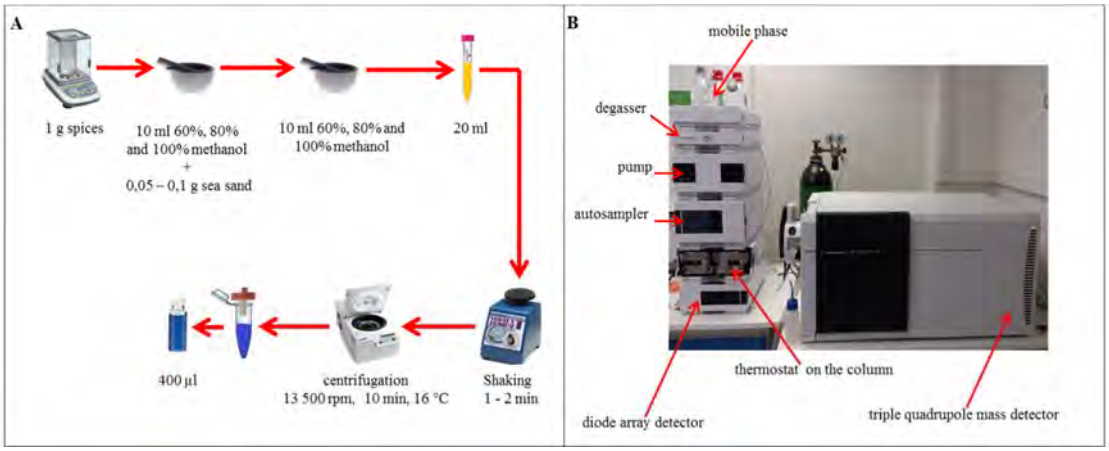
Antioxidants are defined as natural defensive agents that are used by an organism in the struggle against the free radicals and their oxidative effects. For example, they have the ability to slow down aging, protect against heart attack and cerebrovascular attack, cancer prevention, inhibit the growth of some tumors, and help to detoxify of anticancer drugs (Mundis and Macek 2010, Petrosova 2010).

For commonly used kinds of spices they were demonstrated to have antioxidant and antimicrobial effects, for sweet pepper, pepper and caraway seeds there were also demonstrated antibacterial effects, for marjoram, cinnamon and caraway seeds there were recorded anti-inflammatory effects, for cinnamon and caraway seeds there were found anticarcinogenic effects, the thyme, pepper and oregano are used as antifungal appropriations (Ramadan et al. 2013, Ben-Jabeur et al. 2015, El Ksibi et al. 2015, Hertwig et al. 2015, Martucci et al. 2015, Mnif and Aifa 2015, Shahwar et al. 2015, Yang et al. 2015).

MATERIAL AND METHODS

In this experiment eight kinds of ground spices (marjoram, sweet pepper, black pepper, caraway seeds, anise, thyme, cinnamon and oregano) were used. For determination of phenolic compounds from spices extraction with 60%, 80% and/or 100% methanol was used (Figure 1A).

Figure 1 (A) Scheme of preparing spices for HPLC analysis. (B) Scheme of high performance liquid chromatography coupled with mass detector.



To determine the selected phenolic compounds high performance liquid chromatography HPLC Agilent 1200 Series with diode array detector and a triple quadrupole mass detector 6460 Triple Quad LC / MS was used (Figure 1B). For the separation of phenolic compounds the column Zorbax EC 18 (Agilent Technologies, USA) of dimensions 50 x 3.0 mm and a particle size of 2.7 µm was used. The column was thermostated at 45 °C. Mobile phase A consisted of 100% methanol and mobile phase B was 0.2% acetic acid. Flow rate of mobile phase was 0.6 mL/min. The compounds were eluted with a linear upward gradient: 0.00 min (85% B) 0.17 min (85% B) 0.50 min (75% B) 1.70 min (70% B), 4.00 min (70% B), 6.00 min (85% B). Triple quadrupole mass detector operated in negative mode. The gas (nitrogen) temperature was 300 °C, gas flow rate was set to 12 L/min, pressure nebulizer had a value of 45 psi, the temperature of the focusing gas was 250 °C, flow rate of the focusing gas was set at 11 L/min and the voltage on the capillary tube amounted to 3500 V. All used chemicals were purchased from Sigma-Aldrich (St. Louis, USA).

Statistical analyses

Statistical analyses of content of selected phenolic compounds in eight kinds of spices were made using standard deviation behind three determinations.

RESULTS AND DISCUSSION

Determination of the presence and content of phenolic compounds was done by high performance liquid chromatography with mass detection. The results have been recalculated per 1 g of spice.

For oregano (Figure 2A) we found the highest content of caffeic acid (82.6 ± 4.0 µg/g), using extraction with 100% methanol. The lowest content was in cinnamic acid (0.8 ± 0.1 µg/g), using extraction with 60% methanol. Similar results were reported in study of Vallverdu-Queralt et al. (2014). The content of phenolic compounds in marjoram is shown in Figure 2B. The mostly represented phenolic compounds was caffeic acid (93.7 ± 3.8 µg/g), using extraction with 100% methanol. The least represented compounds was cinnamic acid (1.5 ± 0.1 µg/g), during the extraction with 80% methanol. In a study Kogiannou et al. (2013) the most represented in marjoram was caffeic acid (113.5 ± 9.5 µg/200 mL) determined by using LC-DAD-MS. Figure 2C shows the content of phenolic compounds in thyme. The most represented compounds was caffeic acid (115.8 ± 3.6 µg/g), during the extraction with 60% methanol. The least represented compounds in thyme was cinnamic acid (1.0 ± 0.1 µg/g), during the extraction with 80% methanol. Study Vallverdu-Queralt et al. (2014) states caffeic acid as the most represented phenolic compounds in thyme. From oregano, marjoram and thyme are used as a spice leaves (Decree no. 419/2000 Sb.). It can be assumed that the spices used as the leaves have the highest representation of caffeic acid as phenolic compounds.

Figure 2 Determination of selected phenolic compounds (A) for the extract of oregano, (B) for the extract of marjoram, (C), for the extracts of thyme

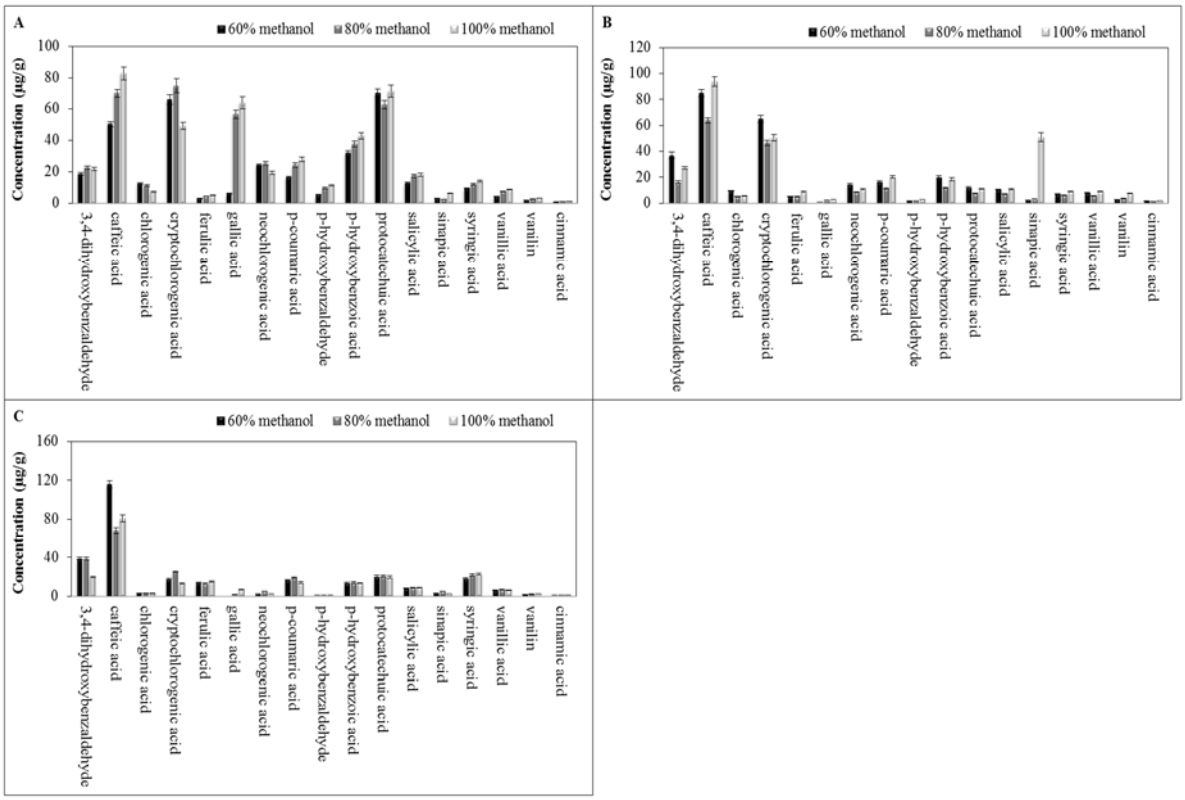
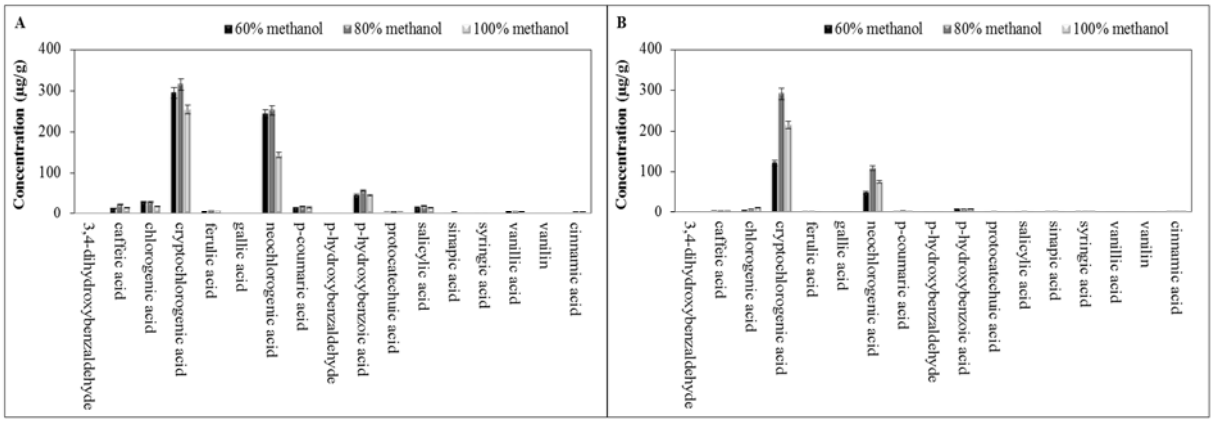
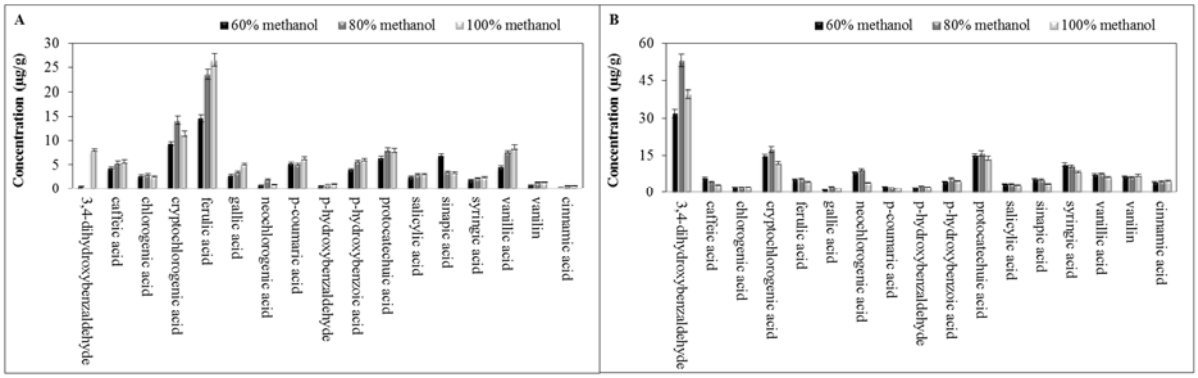


Figure 3 Determination of selected phenolic compounds (A) for the extract of anise, (B) for the extract of caraway seeds



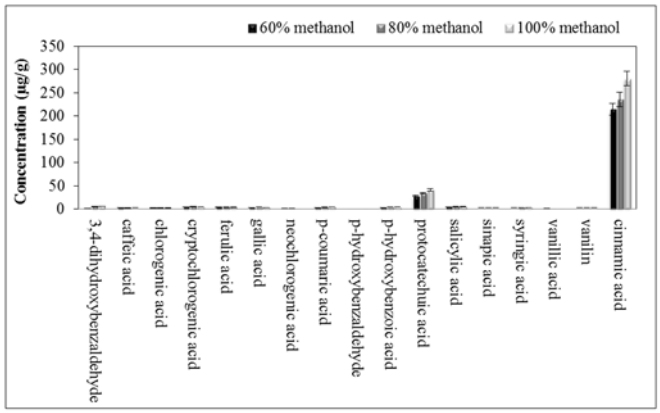
Representation of phenolic compounds in anise and caraway seeds was different than oregano, marjoram and thyme (Figure 3A and 3B). In anise the most represented was cryptochlorogenic acid (314.7 ± 14.0 µg/g), using extraction with 80% methanol. The lowest content was analyzed for 3,4-dihydroxybenzaldehyde (0.33 ± 0.1 µg/g), using extraction with 100% methanol. In the caraway seeds the highest content cryptochlorogenic acid (290.5 ± 14.0 µg/g) was found under the same conditions as anise (extraction with 80% methanol). As with anise, in caraway seeds the lowest proportion of 3,4-dihydroxybenzaldehyde (0.14 ± 0.0 µg/g) was determined. Extensive studies on the profile of phenolic compounds in anise and caraway seeds have not yet been published. Anise and caraway seed belongs to the family *Apiaceae*, which may explain a similar representation as cryptochlorogenic acid and also 3,4-dihydroxybenzaldehyde.

Figure 4 Determination of selected phenolic compounds (A) for the extract of sweet pepper, (B) for the extract of black pepper



In Figures 4 (A, B) there is shown the representation of phenolic compounds in sweet pepper and black pepper. The most represented compounds in sweet pepper was ferulic acid (26.5 ± 1.3 µg/g), using extraction with 100% methanol. The least represented phenolic compound was 3,4-dihydroxy benzaldehyde (0.01 ± 0.1 µg/g), using the extraction with 80% methanol. The largest representation of ferulic acid in sweet pepper confirmed by the study Vallverdu-Queralt et al. (2012). In the case of black pepper there was found the highest content of 3,4-dihydroxybenzaldehyde (53.0 ± 2.6 µg/g), using the extraction with 80% methanol. Gallic acid (0.9 ± 0.1 µg/g) was analyzed as represented at least a phenolic compound in black pepper in extracting with 60% methanol. The presence of a high content of ferulic acid was also demonstrated in a study Chandra et al. (2015). The content of phenolic compounds in black pepper may also affect his treatment (fermentation) (Kadlec et al. 2009).

Figure 5 Determination of selected phenolic compounds for the extract of cinnamon



Dominant phenolic compounds in the extracts of cinnamon was cinnamic acid (Figure 5), as the content was 278.0 ± 15.3 µg/g, using extraction with 100% methanol. The least represented phenolic compounds was *p*-hydroxybenzaldehyde (0.2 ± 0.0 µg/g), using the extraction with 60% methanol. The study Velisek and Hajslova (2009) and cited as the most represented phenolic compounds cinnamic acid.

CONCLUSION

Based on the results of measurement of phenolic compounds one may say that the most represented by phenolic compounds was caffeic acid in oregano, thyme and marjoram. The highest concentration of cryptochlorogenic acid was determined in anise and caraway seeds. The predominant phenolic compound of sweet pepper was ferulic acid. Dominant phenolic compound in cinnamon was cinnamic acid. For black pepper, the highest content was recorded for 3,4-dihydroxybenzaldehyde.

Our future aim will be to study, where we could evaluate the effect of the spices used in this study and their majority-occurring phenolic compounds on tumor cell lines. In these lines, we will evaluate

the ability of their growth and the effect on cell morphology, depending on the addition of various spices and their dominant phenolic compounds.

ACKNOWLEDGEMENT
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GC-FID ANALYSIS OF FOOD SAMPLES MADE OF HEMP

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Abstract: The aim of the work was to analyse different cannabinoids occurring in the food samples. The chromatographic data for the cannabinoids of interest namely Δ□-Tetrahydrocannabinol (THC), cannabidiol (CBD) and cannabinol (CBN) were determined using gas chromatography. Hemp seed powder, hemp oil and hemp protein in various degrees of concentration were used for the preparation of the food sample to be analysed. Hemp has a botanical relationship with drug/medicinal cannabis. Cannabis has psychotropic substances, which are wrongly attributed to hemp. The psychoactive compound THC was found in very low concentrations in the food samples.

Key Words: cannabis, hemp, cannabinoids, gas chromatography

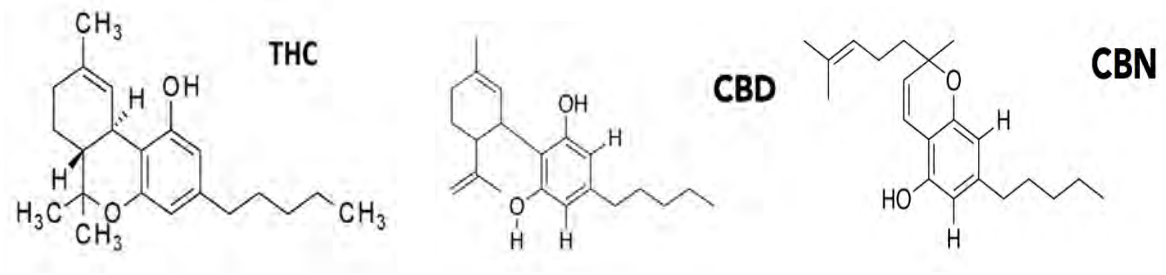
INTRODUCTION

The hemp plant *Cannabis sativa* L. is an annual plant in the Cannabaceae family. It has been an important source of food, fiber, medicine and psychoactive/religious drug since prehistoric times. Cannabis is mentioned as a medication in ancient Egyptian medical texts: Ramesseum III Papyrus (1700 B.C.), Eber's Papyrus (1600 B. C.), the Berlin Papyrus (1300 B.C.), and the Chester Beatty VI Papyrus (1300 B.C.) (Russo 2007, Maniche 1989).

Two main types of *Cannabis sativa* L. must be distinguished, the drug and non-drug types. The first is also known as marijuana, hashish or cannabis tincture and contains Δ9-Tetrahydrocannabinol (THC) (Figure 1) in concentrations between 1–20%, high enough to exhibit psychoactivity. The second type of *Cannabis sativa* L. is industrial hemp with THC concentrations < 0.3% so it has no psychoactive properties (Ross et al. 2000, Holler et al. 2008).

Structure of main cannabinoids

Figure 1 Shows the structure of main cannabinoids.



Legend: CBD - Cannabidiol, CBN – Cannabinol, THC- Δ9-Tetrahydrocannabinol

Hempseed possesses excellent nutritional value. It is very rich in essential fatty acids and other polyunsaturated fatty acids. It has almost as much protein as soybean and is rich in vitamin E and minerals such as phosphorus, potassium, sodium, magnesium, sulphur, calcium, iron, and zinc

(Callaway 2004). Cannabinoids are terphenolic secondary metabolites produced in sessile and stalked trichromes by cannabis plant. More than 100 cannabinoids have been discovered and studied until now (Turner et al. 1980). The most interesting and most studied compounds of this class are THC and CBD. The largest proportion of THC can be found on the surface of the seed coat. As a consequence, only very low THC concentrations are found inside of the seeds (less than 2 mg/kg with drug hemp and less than 0.5 mg/kg with fibre hemp) (Ross et al. 2000, Small and Marcus 2003). Cannabis is analysed for several different purposes. Identification and quantification of the main cannabinoids is the main objective for the analysis of hemp food products.

There are various methods available for analysing cannabinoids such as High performance liquid chromatography (HPLC) and gas chromatography. HPLC can be used to determine the decarboxylated form of THC, CBD and CBN. A rapid and simple method was optimized for the determination of THC, CBD and CBN contents in the food sample by gas chromatography with a flame ionisation detector using squalene as an internal standard.

MATERIALS AND METHODS

PCH Fisons instrument equipped with a flame ionisation detector (FID) was used for analysis of cannabinoids. The instrument was equipped with DB5 non-polar capillary column with 5% phenyl, 95% dimethylarylensiloxan (30 m length, 0.25 mm internal diameter, film thickness 0.25 μm). The temperature gradient started at 150 °C and increased at a rate of 3 °C/min until 270 °C. The thermostat injection temperature was 250 °C and the FID detector temperature was set to 270 °C.

The instrument was calibrated with three points from 0.05 to 0.5% CBD (cannabidiol, 1mg / ml in methanol, Sigma), CBN (cannabinol, 1 mg/ml in methanol, Sigma), and THC (10 mg/ml ethanol - Ipomed). They were established retention times cannabinol (CBN), cannabidiol (CBD) tetrahydrocannabidol (THC) and squalene (Table 1).

Table 1 Shows the retention time (tR) and the relative retention time (Rtr) of analytes.

Analyte	tR (min)	Rtr
Cannabidiol	26.4	0.825
Tetrahydrocannbidol	28.1	0.878
cannabinol	29.5	0.921
Squalene	32.5	1.000

Extractive solution used was 35 ml of squalene in 100 ml of hexane. 0.1 gm of each hemp food sample was homogenised with liquid nitrogen. After homogenisation, 5 ml of the extractive solution was added to the samples placed in a volumetric flask. The volumetric flask was then covered with aluminium foil and placed in an ultra sound for 20 mins. The extract was collected and centrifuged at 3000 rpm for 5 mins at 4 °C. The liquid phase was separated and stored in dark storage bottles at -20 °C in a freezer.

The samples to be analysed were provided by the Department of Food Technology of Mendel University in Brno. Different hemp components like protein powder, hemp grits and hemp flour were provided. The oil sample was not analysed. The hemp component was used in different proportions in the food to be analysed (Table 2). Each sample was analysed three times and an average values of mg/g of the cannabinoids were calculated.

Table 2 Shows the different concentrations of hemp powder used for each sample.

Bread Sample	Amount of wheat flour (g)	Amount of hemp flour (g)	Amount of hemp oil (ml)	Amount of hemp grits (g)	Amount of hemp protein (g)
B 1	500	0	0	0	0
B 2	500	0	5	0	0
B 3	475	0	0	25	0
B 4	450	0	0	50	0
B 5	400	0	0	100	0
B 6	400	0	0	100	0
B 7	475	25	0	0	0
B 8	450	50	0	0	0
B 9	475	0	0	0	25
B 10	450	0	0	0	50

RESULTS AND DISCUSSIONS

All the hemp samples were derived from the same source. The corresponding percentage values of cannabinoids for each sample were calculated (Table 3). The THC content of all the food samples analysed varied from 0 to 0.23% and were found to be below 0.3% whereas no CBN was found at all. CBN is the main chemical degradation product of Δ□-Tetrahydrocannabinol (Harvey 1990) which suggests that there was very little or no degradation at all. The food samples we analysed met the guidelines. Samples in the EU showed values ranged from 0.05% to 0.2% (El-Ghany 2002, Mechtler et al. 2004). The prescribed use of certified hemp planting seed by the EU and the increase of controls on manufacturers have obviously preserved low level of THC in EU food and feed products (Lachenmeier and Walch 2005).

Expectedly no cannabinoids were detected in B 1 as no hemp component was used. Surprisingly, B 4 did not show presence of any cannabinoids. As seen from the figure 2. Sample B 6 in which grits were utilized showed the highest concentration of cannabinoids 3.406 mg/g and 2.166 mg/g for CBD and THC respectively whereas sample B 5 in which similar quantity of grits were present showed much lower value for cannabinoids. Interestingly the grits when measured did not show any measureable amounts of cannabinoids. Hemp seed has very low concentrations of THC and the largest proportions of THC is found on the seed coat (Leson et al. 2001).

The sample B 9 and B 10 in which hemp protein component was used showed similar amounts of THC and CBD (Figure 2). The hemp protein when analysed showed lower values of cannabinoids than its final products. Similarly, with the analysed hemp flour the percentages of cannabinoids detected were lesser than the final product B 8. This could be either explained that the hemp component goes further decarboxylation during the baking process of the food samples (Heroudkova 2016). Hemp food products contain analysable amounts of THC. The presence of THC in the hemp containing food can raise concerns about psychoactive effects. According to the guidelines of European Union the hemp parts used for food production should not exceed 0.2% THC (Lachenmeier and Walch 2005, EU Commission 2008).

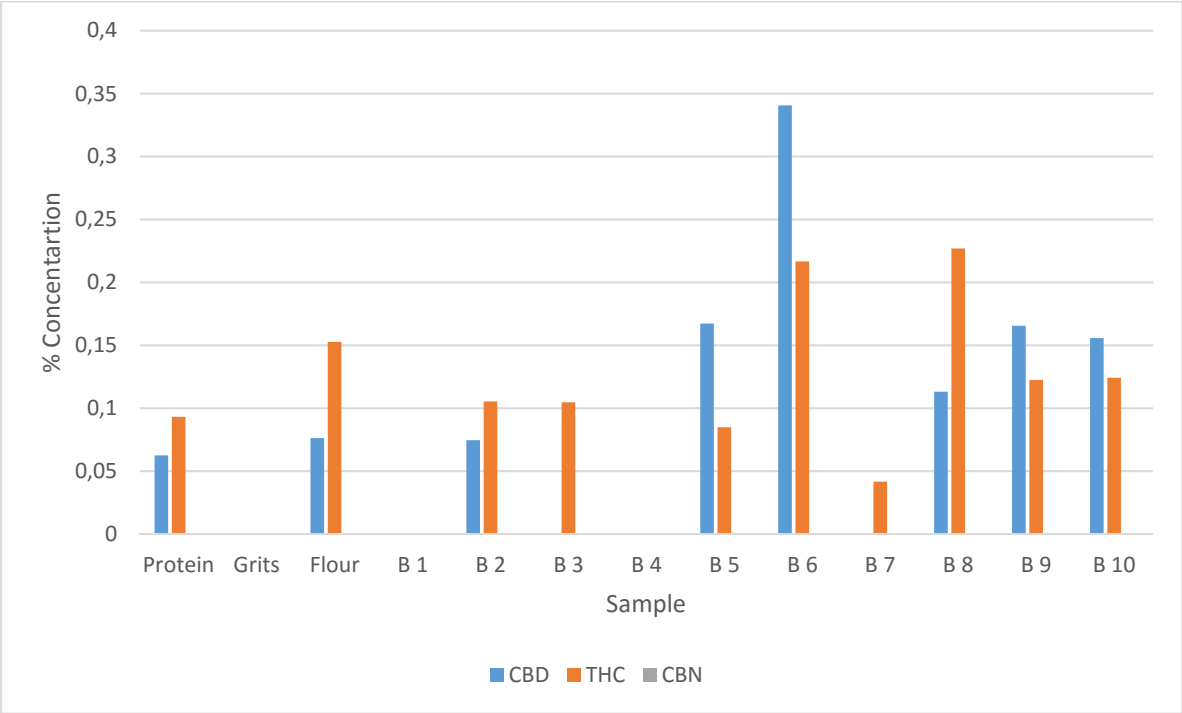
According to a similar research conducted where 10% of hemp component was used the highest amount of THC and CBD found were 3.548 mg/g and 25.323 mg/g respectively (Heroudkova 2016). From a forensic point of view, the presence of THC in hemp containing food stuff raised not only the problem of psychoactive effects, but it also leads to concerns about the validity of positive results of drug tests (Elsohly 2003).

Table 3 Cannabinoids content per sample.

Sample	CBD		THC	
	%	mg/g	%	mg/g
B 1	0.0000	0.0000	0.0000	0.0000
B 2	0.0764	0.764	0.1053	1.0530
B 3	0.0000	0.0000	0.1047	1.0470
B 4	0.0000	0.0000	0.0000	0.0000
B 5	0.1672	1.6720	0.0849	0.8490
B 6	0.3406	3.4060	0.2166	2.1660
B 7	0.0000	0,0000	0.0416	0.4160
B 8	0.1131	1.1300	0.2269	2.2690
B 9	0.1655	1.6550	0.1224	1.2240
B 10	0.1556	1.5560	0.1242	1.2420
Protein	0.0625	0.6250	0.0931	0.9312
Grits	0.0000	0.0000	0.0000	0.0000
Flour	0.0762	0.7620	0.1527	1.5270

Legend: CBD - Cannabidiol, CBN – Cannabinol, THC- Δ9-Tetrahydrocannabinol, B1-B10 see Table 2.

Figure 2 Shows the percentage concentrations for each sample.



Legend: CBD - Cannabidiol, CBN – Cannabinol, THC- Δ9-Tetrahydrocannabinol, B1-B10 see Table 2.

CONCLUSION

Hemp can be an excellent source of food and nutrition. Hemp containing food products are seeing a revival. The gas chromatography method used in the experiment was good for the detection of the main cannabinoids. The value of psychoactive compound was below or at par with the EU guidelines. In the future, we would analyse the food products made from different parts of hemp.

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TRACEABILITY OF CANNABIS DNA IN PASTRY

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Abstract: The cannabis plant is one of the economic plants providing fiber, seeds and aromatic resin. It produces different terpene phenolic compounds, including psychoactive Δ^9-tetrahydrocannabinol (THC). Cannabis plants used for food production should not exceed 0.2% THC. The presence of THC in food can be detected by chemical or biological analytical methods. This study is dealing with biological method of DNA profiling to detect and identify cannabis DNA in pastry by three SSR (Simple Sequence Repeat) markers, also called microsatellites. Results confirmed the thermal degradation of DNA in food during baking process and the reliability and usefulness of SSR markers. CAN0110 and CAN1690B markers appeared to be well suited for assessing the presence or absence of cannabis in pastry, while ANUCS204 marker showed a potential for genotyping, to determine the variety and origin of included cannabis material in the future.

Key Words: cannabis, pastry, DNA, SSR markers

INTRODUCTION

The cannabis plant (*Cannabis sativa* L.) is among the very oldest of economic plants providing humans with fiber for spinning, weaving cloth, making paper; seed for human food and animal feeding and aromatic resin containing compounds of medicinal value - cannabinoids. The biosynthesis of cannabinoid compounds is unique to cannabis (ElSohly 2007). It produces over 60 different terpene phenolic compounds, of which Δ^9-tetrahydrocannabinol (THC) is the main psychoactive constituent (Sirikantaramas et al. 2005). However, cannabis plant parts used for food products should originate from varieties allowed for industrial cultivation in Europe - they should not exceed 0.2% THC in dry matter of the upper 1/3 of the crop (Lachenmeier and Walch 2005, EU Commission 2008).

There are several approaches to detect the presence of THC in food by analytical methods. They can generally be divided to chemical methods including colour tests, immunoassays, ion mobility spectrometry and chromatography – (TLC, GC, HPLC ...) and biological methods - DNA profiling being the most common (UNODC 2009). Several DNA sequences present only in cannabis species were discovered. Based on their presence or absence it is possible to identify cannabis in a sample, such as 197bp mitochondrial *trn*L-*trn*F fragment (Linacre and Thorpe 1998) and SMT3 gene encoding an ubiquitin-like protein (Staginnus et al. 2014), or to distinguish fiber-type cannabis from drug-type by *THCA-synthase* gene polymorphism (Kojoma et al. 2006). Some of the most useful and widely used DNA markers are SSR, otherwise known as microsatellites, or short tandem repeats (STR). Microsatellites have become well suited for wide range of applications, including DNA fingerprinting and genotype identification (Alghanim and Almirall 2003, Ashkenazi et al. 2001).

Studies focused on detection of cannabis traces in food suggest that DNA methods are usually not very reliable when it comes to analysis of food samples subjected to a certain amount of heat treatment, such as cooking, baking etc. (Kitpipit et al. 2008). This paper investigates the detection of cannabis in pastry (dough and bread) and in hemp ingredients used for baking with several DNA markers.

MATERIAL AND METHODS

DNA isolation and samples

Altogether, 24 food samples were analysed – ten samples of dough with various proportions of hemp ingredients plus ten samples of bread made from this dough, and finally four samples of ingredients themselves – hemp oil, hemp flour, hemp grits (grinded hulled seeds) and hemp protein. Overview of dough samples and amount of hemp ingredients used in each one is shown in Table 1. All the samples were provided by the Department of Food Technology of Mendel University in Brno. Dough kneading and baking methods used were the same as described by Janečková et al. (2015).

DNA from all samples was isolated using DNeasy Plant Mini Kit (Quiagen) from 20 mg of material, homogenized by mortar and pestle and liquid nitrogen for frozen dough, bread and grits, without homogenization for oil, protein and flour. Concentration and purity of isolated DNA was measured via Picopet 1.0 spectrophotometer (Picodrop).

Table 1 Proportion of hemp ingredients in every sample

Dough/bread sample	Amount of wheat flour (g)	Amount of hemp flour (g)	Amount of hemp oil (ml)	Amount of hemp grits (g)	Amount of hemp protein (g)
D1/B1	500	0	0	0	0
D2/B2	500	0	5	0	0
D3/B3	475	0	0	25	0
D4/B4	450	0	0	50	0
D5/B5	400	0	0	100	0
D6/B6	400	0	0	100	0
D7B7	475	25	0	0	0
D8/B8	450	50	0	0	0
D9/B9	475	0	0	0	25
D10/B10	450	0	0	0	50

DNA markers

To confirm the general presence of cannabis DNA in samples, SMT3 sequence was used. It is a part of a housekeeping gene encoding ubiquitin-like protein in *Cannabaceae* species (*C. sativa, Humulus lupulus*) proposed by Staginnus et al., 2014. To see a possible polymorphism and variability in hemp DNA sequences, two types of microsatellite/SSR markers (Table 2) were evaluated. First type – ANUCS204 was taken from the work of Gilmore and Peakall (2003) as one of the microsatellites with high level of polymorphism in *C. sativa*. Other type, EST-SSR markers called CAN0110 and CAN1690B were used, as described in large scale development study of Gao et al. (2014).

Table 2 Types of SSR markers used

Name	Repeat motif	Forward primer (5'-3')	Reverse primer (5'-3')
ANUC204	$(CT)_{26}$	TGGAAGATATGCAACTGGAG	AACGAAGATAAGCACGAACA
CAN0110	$(AT)_{10}$	GGGTAAAGCTTACGCAAAGT	AACAAACAGTTGGACACCCT
CAN1690B	$(AAC)_6(ATC)_7$	TGTTTCTAAGGCTCAGTCCC	GGCAAAGGTAAAGCAAGTGT

PCR was performed in a total volume of 25 µl consisting of 0.5 U *Taq* polymerase (Promega), 1× aliquot buffer, 0.1 mM of each dNTP(Promega), 0.3 M of each primer and 20 ng of template DNA in T3 thermocycler (Biometra) for SSR markers and gradient thermal cycler QB-96 (Quanta Biotech)

for SMT3 housekeeping gene amplification. For all markers we used the same PCR protocols, as they are written in original articles of aforementioned authors.

Electrophoresis for SMT3 was done on 1% agarose gel with Tris-acetate-EDTA buffer stained with ethidium bromide. The amplification of SSR products was visualized on 8% non-denaturating polyacrylamide (PAA) gels in TBE (Tris-borate-EDTA) buffer followed by staining with silver (0.2% $AgNO_3$) (Musilová et al. 2013). All ten samples of dough were tested for the presence of SMT3 sequence and consequently, ten samples of bread as a final product were tested to see how the DNA behaves after heating. For accuracy, every DNA sample from pastry had three repetitions on the agarose gel. For the SSR markers on polyacrylamide gels, in addition to ten samples of dough and bread we also included the DNA of hemp ingredients – oil, grits, flour and protein.

RESULTS AND DISCUSSION

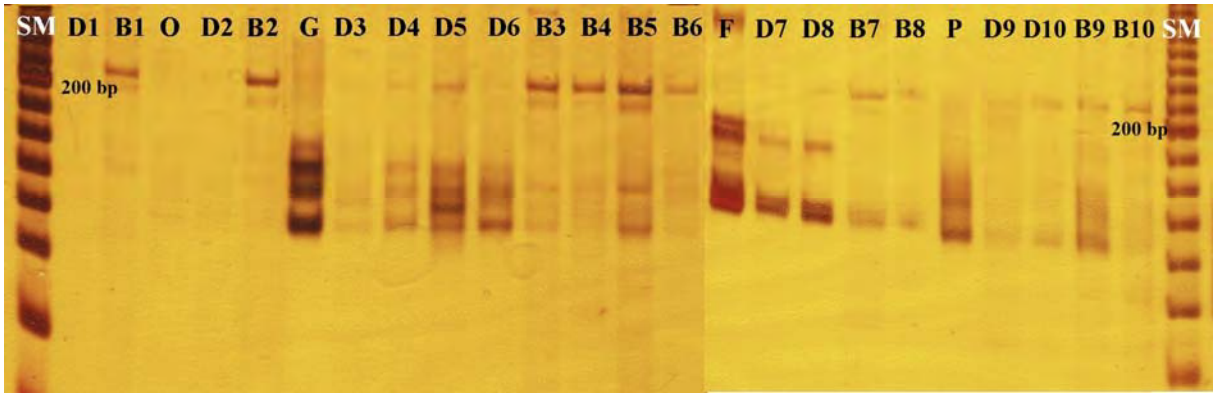
Variability of hemp DNA in food products

When compared to agarose gels with SMT3 gene, the intensity of DNA bands on polyacrylamide was higher and a bit more consistent with the real amount of hemp ingredient in sample, also in case of bread samples (Figure 1). This can be explained by more successful amplification due to the shorter sequences and relatively high abundance of microsatellite loci for the estimated length of cannabis genome (Alghanim and Almirall 2003, Korpelainen et al. 2008, van Bakel et al. 2011). Higher intensity and consistency was observed especially in case of CAN0110 and CAN1690B markers.

Interestingly, ANUCS204 marker, although having less intensive bands, was hypothetically showing some level of polymorphism between DNA from hemp ingredients (Figure 1). Allele size for this marker ranges from 128 to 184 bp (Gilmore and Peakall 2003). Different DNA band profile is visible, when comparing samples of grits, flour and protein. We can assume, that hemp ingredients did not originate from a single variety of hemp, as different profiles between oil and other hemp ingredient were additionally shown by CAN0110 marker.

Another interesting observation for the ANUCS204 marker was the presence of bands about 220 bp in all bread samples. These bands are outside of allele range, but were present only in bread – the only matrix that was heated up compared to other samples (Figure 1). This was observed even in samples B1 (pure wheat) and B2 (only oil included), but missing in oil sample. That most likely means that those bands got amplified due to the change of DNA structure – decaying to shorter fragments during heating process. Similar pattern was present also for CAN0110, but not for CAN1690B marker. The possible explanation is a different microsatellite sequence. In case of CAN0110 it is $(AT)_{10}$ sequence. It is known, that AT rich regions of DNA are less thermally stable compared to GC rich, due to weaker hydrogen bonds (only two bonds instead of three). However, the correlation between GC content and temperature was found in certain genomic regions while not in the others (Zheng et al. 2010).

Figure 1 Visualisation of PCR products for ANUCS204 marker



Legend: SM – 20 bp size marker, D – stands for dough samples, B – stands for bread samples, O – hemp oil, G – hemp grits (grinded hulled seeds), F – flour, P - protein; Numbers 1-10 – stand for the amount of hemp ingredient included in a sample according to Table 1

Evidence of hemp products in pastry

SMT3 sequence is a part of housekeeping gene, which means it is a gene required for the maintenance of the basal cellular function and is constitutively found in all cells of a certain organism – in this case *Cannabis sativa L*. (Eisenberg and Levanon 2003). Based on this, we can presume that the SMT3 products of size about 283 bp should be visible for the samples where some amount of hemp ingredient was included. Results for dough correlate with this theory, given that the bands were present in all samples except first two (D1, D2). D1 is a control, hemp-free sample containing only wheat flour. Even though sample D2 contained hemp oil, absence of the bands can be explained. Oil contains only trace amounts of DNA after filtration and it is not a good matrix for DNA isolation via DNeasy Plant Mini Kit (Testolin and Lain 2005). On the other hand, starch that is naturally present in all pastry samples has the tendency to clog the isolation kit columns as well (Pervaiz 2011). It did affect the DNA quality in samples, but the bands were still present. In addition, the intensity of DNA bands was the highest in samples with largest percentual proportion of hemp grits (D5 and D6), which seems to be a better isolation matrix. DNA band intensity in the samples containing hemp flour can be compared with that of samples containing hemp protein. As expected in case of bread samples, when the agarose gel was subsequently compared to the previous with dough, the intensity of DNA bands was only roughly corresponding and mostly they were not very clearly visible. This result of course, can be attributed to DNA degradation during baking process, when temperature rose to 230 °C with oven being steamed with 50ml of water (Janečková et al. 2015). Under dry conditions, complete DNA degradation occurs at above 190 °C (Karni et al. 2013).

CONCLUSION

This study evaluated several DNA markers for the ability to detect the cannabis DNA in pastry with certain amount of hemp ingredients used. Results confirmed that some degradation of DNA occurs during baking process. SSR markers showed to be a reliable tool not only to detect the presence of cannabis in food, but also in possible identification of hemp origin. In the future, panel of microsatellites should be developed to reliably identify the variety of cannabis used in food and possibly distinguish fiber-type from drug-type based on DNA profiling.

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USE OF HEMP RAW MATERIALS IN COMMON BAKERY PRODUCT RECIPES

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Abstract: This paper deals with the possibilities of using products derived from industrial hemp in creating recipes of common bakery products. The food industry may use only industrial hemp products containing less than 0.3% of THC. Nine recipes were created during testing, in which addition of hemp oil, hemp grits, hemp flour and hemp protein added to wheat flour, or combinations thereof, were evaluated. The influence of the recipe composition on the bakery product characteristics was evaluated by the baking test (Rapid-Mix-Test or RMT). Lowest baking loss (10.62%) and the highest yield of bread (147.81%) was in recipe with 10% share of hemp flour. The highest volume yield was recorded after the addition of hemp oil. Index number that characterizes arching of baking goods was highest for variants with 5% and 10% of hemp grits. Sensory analysis evaluated descriptors of shape, crust colour, aroma, elasticity of the crumb, crumb colour, ease of bite, mouthfeel after brief chewing, consistency, crumb moisture, taste and overall impression. Addition of the hemp oil in seven descriptors out of eleven achieved a better evaluation than in the control variant. The addition of hemp flour and protein has positively influenced the crumb moisture.

Key Words: hemp flour, hemp grits, hemp protein, hemp oil, baking test, sensory analysis

INTRODUCTION

Bakery products belong to the base of our diet. Lately, we have been encountering enrichment of traditional flours with alternative products containing substances beneficial to health.Enrichment of recipes with new raw materials gives a product a higher nutritional value (Pejcz et al. 2015). It is important to modify the recipe so that there is not much change in the technological process of baking and the sensory quality of the product is similar to or even better than the original product.

Hemp is an annual dioecious herb, having both male and female plants. The main two types of hemp we can encounter are Indian hemp (*Cannabis indica,* Lam) and "common" hemp (*Cannabis sativa* L.*)*.Indica is grown exclusively for narcotic substances, which are contained in the green parts of plants, predominantly in the resin of female inflorescences.Sativa is the most widespread variety, which is grown for strong fibres and forms a very little resin (Šnobl and Pulkrábek 2005).

The common hemp contains 533 compoundsincluding 103 monoterpene phenolic compounds that are present only in this plant.

Hemp is a crop used since ancient times. It was used for its therapeutic effects and technical applications. In the Czech lands, it appeared as early as the Middle Ages. It was normally used for the production of hemp fabrics, paper, or oil extraction. Recently, there have been efforts to use hemp in food and medicine. In the food industry, we can now encounter products that contain it, such as chocolates. Currently, the food industry may use products such as hemp protein, flour, seeds, or flowers.

Hemp seeds contain a number of bioactive substances, such as flavones, polyphenols, albumin and edistinproteins (Norajit et al. 2011), manganese, potassium, iron, zinc, or magnesium (Cozea et al.

2016), vitamins A, B, C, and E (Pejcz et al. 2015). It is known that hemp seeds lower blood pressure, reduce cholesterol, and boost the immune system (Pejcz et al. 2015). Hemp oil contains high amounts of linoleic acid as well as α-linoleic and oleic acids (Galasso et al. 2016). The ratio of linoleic acid and α-linoleic acid is 3: 1 (Leizer et al. 2000). Hemp seeds contain a number of essential oils such as myrcene, trans-caryophyllene, trans-β-ocimene, and α-humulene (Novak et al. 2000). By adding hemp flour we can increase the amount of protein, as it succeeded in the energy bars from extruded rice (Norajit et al. 2011). The above facts support the idea of using hemp products for fortification of bakery products. Due to the composition of hemp seeds, we can expect technologicaland nutritional, as well as sensory benefits. This paper also validates these assumptions as it is focused on enriching traditional recipes of bakery products with hemp products, namely flour, protein, oil, and grits.

MATERIALS AND METHODS

The test was carried out according to the outline presented in Table 1. The baking used ordinary fine-grain wheat flour, water, yeast, sugar, salt, oil, and hemp component in different proportions.

Table 1 Type and amount of hemp component in the recipe

Variant	Wheat flour [g]	Hemp flour [g]	Hemp oil [ml]	Hemp grits [g]	Hemp protein [g]
1	500	-	-	-	-
2	500	-	5	-	-
3	475	-	-	25	-
4	450	-	-	50	-
5	400	-	-	100	-
6	400	-	-	100	-
7	475	25	-	-	-
8	450	50	-	-	-
9	475	-	-	-	25
10	450	-		-	50

The dough was prepared from all raw materials mixed together at once. It was kneaded in a high-speed kneader for approximately one minute.Rising took place at a temperature of 32 °C ± 1 °C and humidity 80 ± 5% for 20 minutes. After removal from the proofer, the dough was let to rest for 10 minutes and then weighed. The dough was shaped into 80-g loaves and again allowed to rise at 32 °C ± 1 °C and humidity 80 ± 5% for 25 minutes. Before loading them into oven, the loaves were sprinkled with water and baked at 230 °C to 240 °C in a laboratory oven with a proofer. At the beginning of the baking, the oven was steamed with 50 ml of water. The baking time was 20 minutes.

A baking test was subsequently carried out. It evaluated dough yield (%), baking loss (%), yield of bread (%), volume yield (ml/100 g), and bread arching characterized by an index number. One hour after baking a sensory analysis by experienced sensory evaluators (n = 10) was carried out. The bakery products were evaluated for shape, crust colour, aroma, crumb elasticity, crumb colour, ease of bite, mouthfeel after brief chewing, consistency, crumb moisture, and taste. Graphic evaluations used 100-mm unstructured scales, where 1 mm on the scale corresponded to one point.

Statistical evaluation of the gained data was carried out using Microsoft Excel and Statistica 12. The calculation used the one way ANOVA method, which is used for evaluation of the analysis of variance. It calculated averages and standard deviations of gained data and determined conclusiveness of the differences in individual evaluated descriptors.

RESULTS AND DISCUSION

Baking test

The results shown in Table 2 indicate that the highest dough yield was achieved after adding hemp oil (166.3%) and a very good yield was achieved in recipes with the added hemp flour (164.46–165.38%), and 5% of hemp protein (163.91%).The achieved yield of these variants corresponds to the

results indicated by Dvořáková et al. (2005) in assessing the standard recipes for making bread and common pastries. In her case, the yield ranged from 155 ± 10%. This means that the addition of hemp material did not have a negative influence on the dough yield.

The addition of hemp flour at a level of 10% influenced mostly the loss of water through baking. In this variant, the water loss was very low and amounted to 10.62%. However baking loss may be very variable related to used raw materials. Dvořáková et al. (2005), who also addressed this issue, provided a relatively wide range of 15 ± 5%.

The lower loss through baking in this variant was also positively reflected in the bread yield, which significantly exceeded the control variant, which used only wheat flour (147.81%).

High volume yield was determined after addition of the hemp oil (424 ml/100 g), and up to 5 per cent of hemp protein (416 ml/100 g) was also a favourable addition. However, a higher proportion (10%) already had a negative effect and specific volume was very small (277 ml/100 g). The negative influence is clearly seen in Figure 1. The addition of hemp flour decreased the volume yield. Its values decreased with a higher content. However, we cannot evaluate our results negatively compared to those in the study by Pojićet al. (2015). In their case, a mere addition of 5% of hemp flour decreased the volume yield to 218 ml/100 g, and in the variant with the share of 10%, the volume yield was further reduced by approximately 63 ml/100 g.

If we generalize the results, then the addition of hemp material has one thing in common, except for variants with addition of 10% of hemp protein, namely the increase of value of the index number compared to the control. Variations with the addition of hemp grits showed the best results.

Table 2 Results for the baking test

Variant	Dough yield (%)	Baking loss (%)	Bread yield (%)	Volume yield (ml/100 g)	Index number
1	163.85	14.99	139.29	402.00	0.61
2	166.03	15.85	139.72	424.00	0.69
3	159.35	12.44	139.52	370.00	0.72
4	156.02	15.06	132.52	388.00	0.72
5	143.59	14.87	122.23	294.00	0.67
6	148.01	12.97	128.82	296.00	0.64
7	164.46	14.77	140.17	396.00	0.63
8	165.38	10.62	147.81	344.00	0.65
9	163.91	15.37	138.72	416.00	0.69
10	162.24	12.71	141.62	277.00	0.61

Legend: Variant 1: control variant, Variant 2: 5 ml of hemp oil, Variant 3: 5% of hemp grits, Variant 4: 10% of hemp grits, Variant 5: 20% of hemp grits, Variant 6: 20% of hemp grits without oil, Variant 7: 5% of hemp flour, Variant 8: 10% of hemp flour, Variant 9: 5% of hemp protein, Variant 10: 10% of hemp protein.

Sensory analysis

Figure 1 shows that the *shapes* of the products were all similar. The *colour of the crust* has received conclusively the best evaluation in variants with added hemp oil (Table 3). Although it has a dark green colour, it did not show up in the crust colour. Samples with added hemp protein (variants 9 and 10) and hemp flour (variants 7 and 8) have received the worst evaluations. Hemp flour and protein are at first sight quite similar materials. Their colour is dark green, which is reflected in the colour of bread and lower ratings in sensory analysis. These materials are not light components and their use is to be expected to cause an atypical colour in the final product. The colour with an increasing amount of hemp flour was evaluated in studies by Apostol et al. (2015) and Pejcz et al. (2015). Their evaluations were also worse than the control, and even the addition of 5% of hemp flour was rated worse than adding 10 and 15%.

All hemp raw materials have worsened the *aroma* of the product. This is because hemp ingredients have strong, typical aroma due to the presence of essential oils, such as α-humulene, caryophyllene, or caryophyllene oxide (Bertoli 2010).

Figure 1 Product appearance and shape in cut

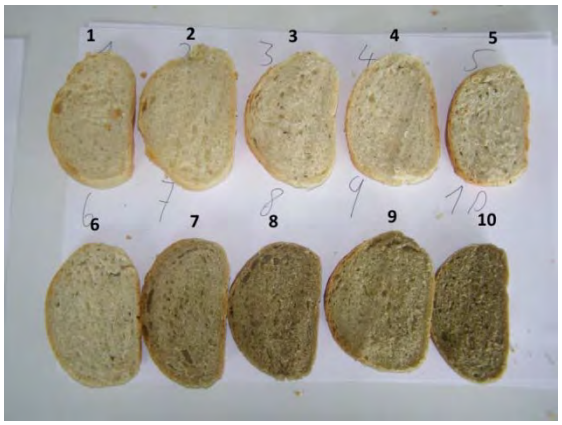

Figure 2 Overall impression

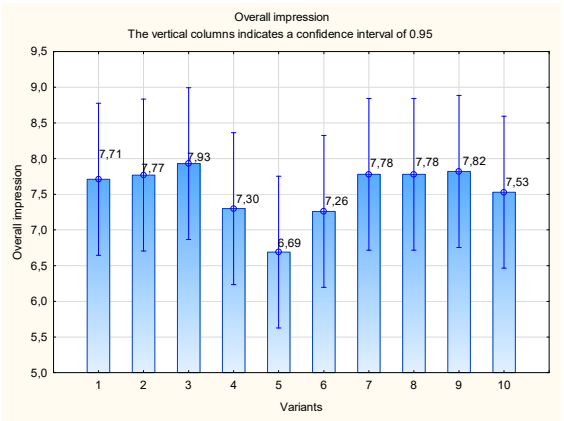

Table 3 Results of sensory analysis

Variant	Shape	Crust colour	Aroma	Crumb flexibility	Crumb colour	Ease of bite
1	8.5^{a}	7.3^{a}	8.2^{b}	8.3^{ab}	8.4^{a}	7.5^{a}
2	8.37^{a}	7.75^{b}	7.05^{ab}	8.31^{b}	8.26^{a}	7.64^{a}
3	8.02^{a}	7.24^{ab}	6.35^{ab}	7.56^{ab}	8.21^{a}	7.87^{a}
4	7.3^{a}	7.2^{ab}	4.93^{a}	7.87^{ab}	8.15^{a}	7.5^{a}
5	7.86^{a}	7.28^{ab}	5.9^{a}	7.32^{ab}	8.04^{a}	7.2^{a}
6	7.63^{a}	6.89^{ab}	5.85^{ab}	6.84^{ab}	8.1^{a}	7.53^{a}
7	6.95^{a}	6.02^{ab}	6.89^{ab}	6.99^{ab}	7.32^{a}	6.7^{a}
8	6.97^{a}	5.29^{ab}	6.35^{ab}	6.12^{ab}	6.59^{a}	6.96^{a}
9	7.8^{a}	5.58^{ab}	6.24 ^{ab}	7.31^{ab}	7.16^{a}	6.99^{a}
10	7.42^{a}	4.61^{a}	6.99^{ab}	6.02^{a}	6.68^{a}	6.94^{a}

Table 3 (Continuation): Results of sensory analysis

Variant	Mouthfeel after a short chewing	Consistency	Crumb moisture	Taste	Overall impression
1	7.3^{a}	7.1^{a}	7.7^{a}	7.3^{a}	7.71^{a}
2	7.8^{a}	7.47^{a}	7.75^{a}	6.56^{a}	7.77^{a}
3	8.04^{a}	6.67^{a}	8.1 ^{a}	7.96^{a}	7.93^{a}
4	7.77^{a}	6.27^{a}	8.01^{a}	7.49^{a}	7.30^{a}
5	6.87^{a}	7.21^{a}	7.36^{a}	5.16^{a}	6.69^{a}
6	7.52^{a}	6.63^{a}	7.65^{a}	6.8^{a}	7.26^{a}
7	7.3^{a}	6.61^{a}	7.86^{a}	5.43^{a}	7.78^{a}
8	7.2^{a}	6.83^{a}	7.74^{a}	6.05^{a}	7.78^{a}
9	7.83^{a}	6.25^{a}	7.93^{a}	6.9^{a}	7.82^{a}
10	7.3^{a}	6.11^{a}	7.83^{a}	6.52^{a}	7.53^{a}

Note: Averages of the individual variants are not significantly different ($p > 0.95$) if they are followed by identical superscript.

The best *crumb flexibility* was found in the control and variants with added hemp oil. Addition of 10% of hemp flour and protein was manifested in less crumb flexibility.In the study by Apostol et al. (2015), the flexibility of the sample with 5 and 10% of hemp flour compared with the control was unchanged. The worse flexibility was in variants with a higher content of hemp flour (15, 20, and 100%). Flexibility in the study by Pejcz et al. (2015) was similar in variants with 5 and 15% of hemp flour, but with 10%, it was worse.

Just as in the case of the crust colour, the *crumb colour* was evaluated worst for variants with higher content of hemp flour and protein (Figure 1). So it was in the studies by Apostol et al. (2015) and by Pejcz et al. (2015).

The *ease of bite* was not significantly affected by the recipe composition. Variants with added hemp flour and protein received only slightly worse ratings. These constituents most likely cause greater firmness of the product.

Mouthfeel after brief chewing was evaluated very well especially in the variant with the addition of hemp oil and 5% of hemp grits.

Hemp oil and the addition of 20% of hemp grits improved *product consistency,* while the addition of hemp ingredients in other variants was worse than in the control.

Very good *crumb moisture* occurred after the addition of hemp grits. Likewise, it was so with the *taste* descriptor. Hemp grits are used as additives in chocolates or are eaten separately, so consumers may be already accustomed to their taste.

Best *overall impression* was achieved by variant 3 (Figure 2) with 5% addition of hemp grits. In contrast, Apostol et al. (2015) sees as ideal from a sensory perspective the recipe with % addition of hemp flour. However, they see the increase of its portion negatively, which was confirmed in our tests. Likewise Pejcz et al. (2015) indicate worsening of the overall sensory quality of the product with increasing amounts of hemp flour.

CONCLUSION

Baking test confirmed higher dough yield and volume yield compared to the control after the addition of hemp oil. The addition of hemp flour up to 10% reduced baking loss and increased bread yield. Adding hemp grits most affected bread arching and index number.

The most suitable hemp ingredient in sensory analysis proved hemp oil, which improved crust colour, crumb elasticity, ease of bite, crumb moisture, consistency and overall impression when compared to the control variant. The addition of hemp grits also had a very good effect. Parameters related to aroma and look received the worst evaluations. Green colour and the typical, strong odour of hemp pose certain disadvantages when used for baking.

If we look at the results of the RMT test and sensory analysis comprehensively, we can conclude that hemp oil appears to be an ideal addition.In terms of technology but also nutrition, hemp flour as supplement is also recommended, however the amount should not exceed 5%.

ACKNOWLEDGEMENT

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THE EFFECT OF FISH AND PALM OIL ADDITION ON FATTY ACIDS CONTENT OF PIG TISSUES

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Abstract: Thirty-two piglets at the age of eight weeks were divided into two groups for 60 days of fattening. The experimental group (FO) was fed the basic feed mixture with addition of fish oil (2.5%) and the control group (PO) was fed basic feed mixture with addition of palm oil (2.5%). Fish oil is characterized by high proportion of n–3 polyunsaturated fatty acids. Palm oil has high level of palmitic, oleic and linoleic acids. At the end of fattening period, fatty acids content in the liver, muscle and adipose tissues were determined. FO-diet caused increase of PUFA n–3 in all observed tissues ($P < 0.01$). PO-diet had effect only in adipose tissue, where significant increase of palmitic, stearic and linoleic acids was found ($P < 0.05$).

Key Words: fatty acids, EPA, DHA, pig, fish oil, palm oil, liver, adipose tissue, muscle

INTRODUCTION

Component of total lipids in the human nutrition is important, because it depends on representation of fatty acids. Its chemical composition determines their physiological effects. Fatty acids are divided to groups according to number of double bounds in a molecule (saturated fatty acids – SFA, no double bound; monounsaturated fatty acids – MUFA, 1 double bound; polyunsaturated fatty acids – PUFA, 2 and more double bounds).

From the point of view of human nutrition are important PUFA n–3 and n–6 groups, which are characterized by different physiological effects. Representative of PUFA n–3 is essential α-linolenic acid (LNA; 18:3n–3) and their important metabolites eicosapentaenoic acid (EPA; 20:5 n–3) and docosahexanoic acid (DHA; 22:6n–3). The family of PUFA n–6 contains essential fatty acid linoleic acid (LA; 18:2n–6) and it`s important metabolite arachidonic acid (AA; 20:4n–6). The final metabolites of AA and EPA and DHA, respectively, are eicosanoids, which play important role in vasodilatation, resistance, evolution, inflammatory response and cholesterol homeostasis (Mourek 2007).

Consumption of long-chain polyunsaturated fatty acids of the n–3 group (EPA, DHA) is a possible dietary strategy to decrease risk of diseases with a chronic inflammation (Givens and Gibbs 2008). In human nutrition, ratio of PUFA n–6/n–3 under 5 : 1 or lower is recommended (Kouba and Mourot 2011). One of the possibilities to increase PUFA n–6/n–3 ratio is consumption of fish oil due to its amount of EPA and DHA.

Nowadays it is discussed using of palm oil, which has higher content of saturated fatty acids and very low content of PUFA n–3. Based on their different percentages of fatty acids we used these oils on our project for dietary intervention on animal model organism close to human – pig.

The aim of the research was to evaluate in a model organism an effect of fatty acids in animal diet on their deposition in physiologically important tissues, which could be apllied to human nutrtition.

MATERIAL AND METHODS

The experiment was carried out on 32 piglets (16 males, 16 females; Large White x Landrace; Bioprodukt Knapovec a.s., Ústí nad Orlicí, Czech Republic) at the age of eight weeks with the mean live weight of 25.5 ± 1.15 kg. The pigs were housed in an experimental stable in floored indoor pens of four animals each. The experiment was performed in compliance with the Czech National Council Act No. 246/1992 Coll. to protect animals against cruelty, the Amended Act No. 162/1993 Coll., and was approved by the "Commission to protect animals against cruelty" of the Mendel University in Brno and of the Ministry of Agriculture of the Czech Republic.

Average ambient temperature and relative humidity were 19 ± 3 °C and 55 ± 10%, respectively. The animals were allocated into two groups based on individual live body weight and sex. The experimental group (FO) was fed the basic feed mixture with addition of fish oil (2.5%) and the control group (PO) was fed basic feed mixture with addition of palm oil (2.5%). During the course of the experiment (60 days) the pigs were fed *ad semi-libitum.*

At the end of the experiment, the pigs were anesthetized by the intramuscular application of the TKX (12.5 mg/ml ketamine, 12.5 mg/ml xylazine, 12.5 mg/ml tiletamin, 12.5 mg/ml zolazepam) and sacrificed by bleeding. Aliquots of the liver, adipose tissue and muscle (*m. quadriceps femoris*; 25 g, 25 g, and 10 g, respectively) were taken and stored at -20 ºC for fatty acids analysis.

Tissue lipids and fatty acids determination

Total lipids were extracted by hexane/isopropanol solvent according to Komprda et al. (2015). Derivatization of the samples were performed according to Komprda et al. (2013). Methyl esters of fatty acids (FAME) were separated using an Fisons GC 8000 series chromatograph with capillary column DB-23 (60 m x 0.25 nm x 0.25 µm; Agilent Technologies, J&W Scientific, USA). The injector was heated to 250 ºC and detector (FID) to 260 ºC. The temperature program was 140 ºC/1 min, gradient 5ºC/ min to 200 ºC held for 1 min, gradient 3 ºC/min to 240 ºC and held for 15 min. The carrier gas was nitrogen, flow rate of 1.5.mL/min, the pressure was 200 kPa and split ratio was 20 : 1. FAME identification was performed by using GLC–455 reference standards (Nu–Chek–Prep, USA). The content of fatty acids (FA) in fish and palm oil, respectively, was expressed as percentage ot the sum of determined fatty acids (Table 1). FA content in the diet and in the analyzed tissues was expressed in mg/100 g of the diet or the analyzed tissues.

Table 1 Percentage of fatty acids in fish and palm oil (% of the sum of total fatty acids)

Fatty acids	14:0	16:0	16:1	18:0	18:1	18:2n6	18:3n3	20:2n6	20:3n6	20:4n6	20:5n3	22:4n3	22:5n3	22:6n3
F	5.6	15.3	11.7	2.6	25.1	3.5	1.6	0.7	0.2	0.7	11.8	0.3	2.1	18.5
P	1.5	40.3	0.2	1.6	43.8	11.3	0.3	0.1	0	0.1	0.3	0.1	0.1	0.1

Legend: F - fish oil; P - palm oil

The differences in absolute amount of fatty acids in the analyzed tissues and pig`s feed (FO, PO) were evaluated by one-way analysis of the variance ratio test including *post–hoc* Tukey`s test using STATISTICA 12 package (StatSoft, USA).

RESULTS AND DISCUSSION

The experiment was focused on the effect of dietary fis oil and palm oil, respectively on deposition of fatty acids in the tested tissues. The composition of fatty acids in the diets with added palm oil and fish oil is shown in table 2.

Table 2 The composition of fatty acids in pig diets (mg/100 g of fresh matter ± mean error)

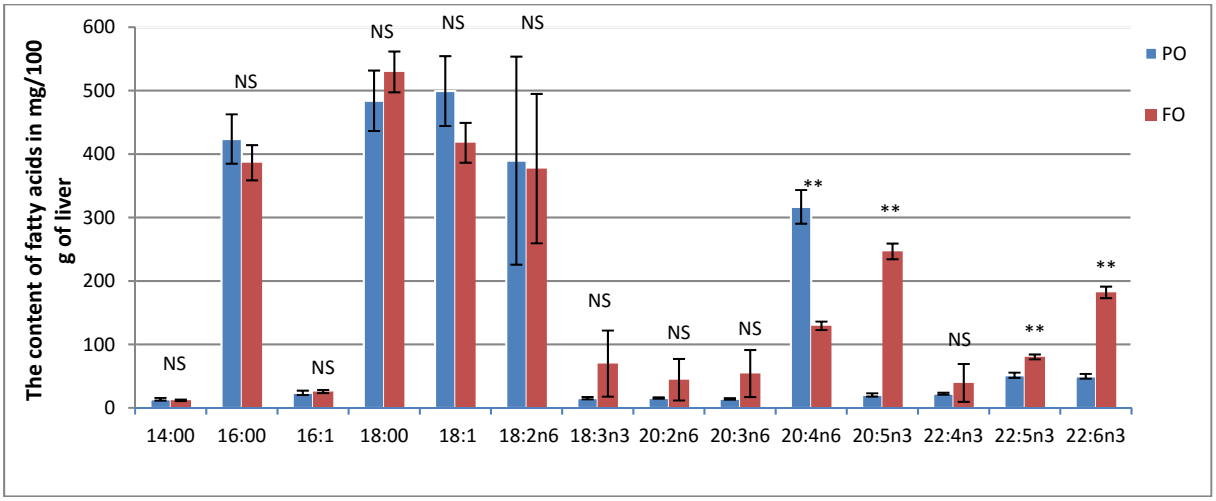
Fatty acids	FO	PO
C 14:00	92^{A}±3.8	34^{B}±2.2
C 16:00	679^{A}±1.9	1472^{B}±26.9
C 16:1	186^{A}±5.7	43^{B}±6.7
C 18:00	130^{A}±49.8	65^{A}±6.7
C18:1	957^{A}±65.1	1389^{B}±6.7
C 18:2n6	1156^{A}±0.5	1356^{B}±4.5
C 18:3n3	88^{A}±9.3	76^{A}±6.1
C 20:2n6	11^{A}±0.1	4^{B}±0.2
C 20:3n6	4^{A}±0.5	2^{A}±1.2
C 20:4n6	17^{A}±1.9	11^{B}±2.2
C 20:5n3	176^{A}±3.8	4^{B}±0.0
C 22:4n3	8^{A}±0.2	4^{B}±0.0
C 22:5n3	36^{A}±1.9	9^{B}±0.0
C 22:6n3	270^{A}±1.9	4^{B}±0.0

Legend: FO – basic feed mixture with addition 2. 5 % fish oil; PO – basic feed mixture with addition 2.5 % palm oil; ; A, B – means with different superscripts differ at P < 0.01; one way ANOVA with post–hocTukey`s test; n = 4.

The PO group had twice higher intake of palmitic acid (P < 0.01) oleic acid and linoleic acid than the FO group. Addition of fish oil to basic feed mixture caused significant increase (P < 0.01) of content of polyunsaturated fatty acids n–3 (eicosapentaenoic acid–EPA, docosatetraenoic acid–DTA, docosapentaenoic acid–DPA, docosahexaenoic acid–DHA) compared with addition of palm oil.

The composition of fatty acids in liver, muscle and adipose tissues of animals with different addition of oils are shown in Figures 1–5.

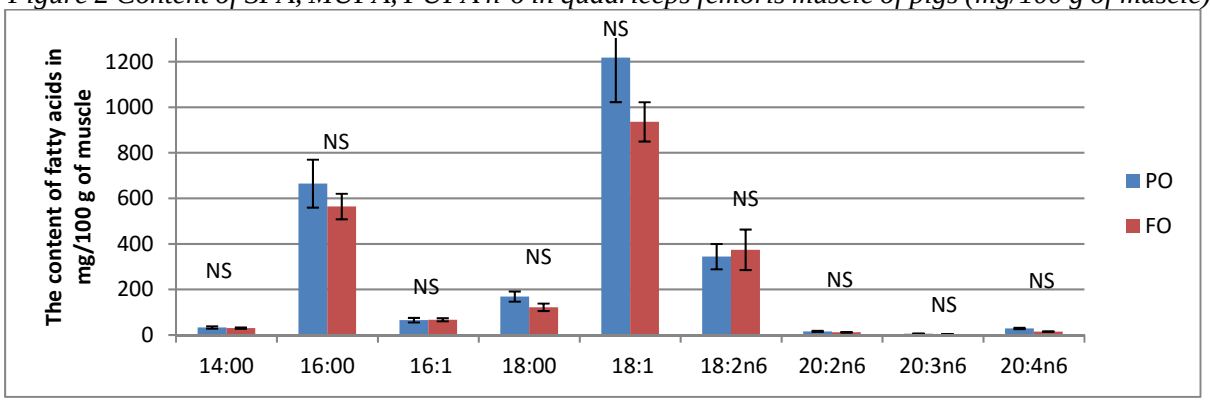
Figure 1 Fatty acid content in the liver of pigs (mg/100 g of liver)



*Legend: FO – basic feed mixture with addition of 2.5 % fish oil; PO – basic feed mixture with addition of 2.5% palm oil; ** – differences significant at P<0.01; one way ANOVA with post-hocTukey`s test; n = 32; NS – not significant (P > 0.05).*

The differences between the dietary groups in saturated FA and monounsaturated fatty acids, respectively were not significant (P > 0.05). Ayleso et al. (2012) also did not report increase of saturated fatty acids in the liver tissue due to addition of palm oil to the feeding mixture. Significant differences were found in the present experiment between amounts of n-3 polyunsaturated fatty acids. The increase of EPA, DPA and DHA was significant (P < 0.01) in the liver tissue of FO group in comparison with the PO group.

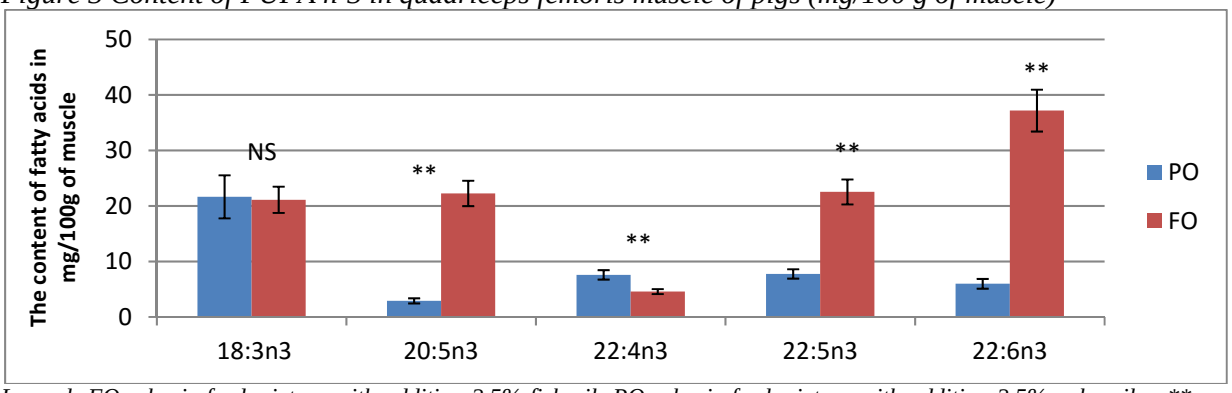
Figure 2 Content of SFA, MUFA, PUFA n-6 in quadriceps femoris muscle of pigs (mg/100 g of muscle)



*Legend: FO – basic feed mixture with addition 2.5% fish oil; PO – basic feed mixture with addition 2.5% palm oil; ; ** – differences significant at P < 0.01; one-way ANOVA with post-hocTukey`s test; n = 32; NS – not significant (P > 0.05).*

The type of the added dietary oil did not influence content of saturated and monounasaturated fatty acids either in the liver (Figure 1) or muscle (Figure 2).

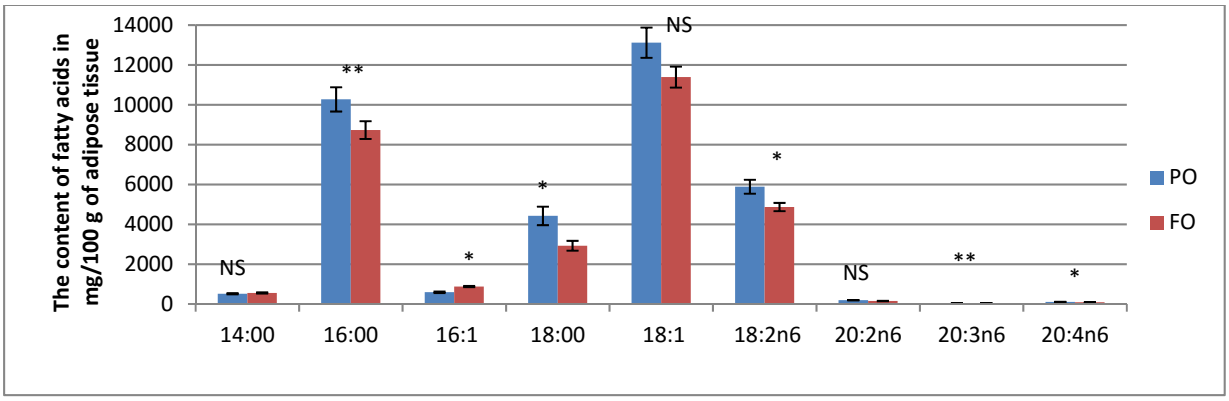
Figure 3 Content of PUFA n-3 in quadriceps femoris muscle of pigs (mg/100 g of muscle)



*Legend: FO – basic feed mixture with addition 2.5% fish oil; PO – basic feed mixture with addition 2.5% palm oil; ; ** – differences significant at P < 0.01; one-way ANOVA with post-hocTukey`s test; n = 32; NS – not significant (P > 0.05).*

Addition of fish oil significantly (P < 0.01) increased amount of arachidonic, eicosapentaenoic, docosatetraenoic, docosapentaenoic and docosahexaenoic acids in comparison with PO group.

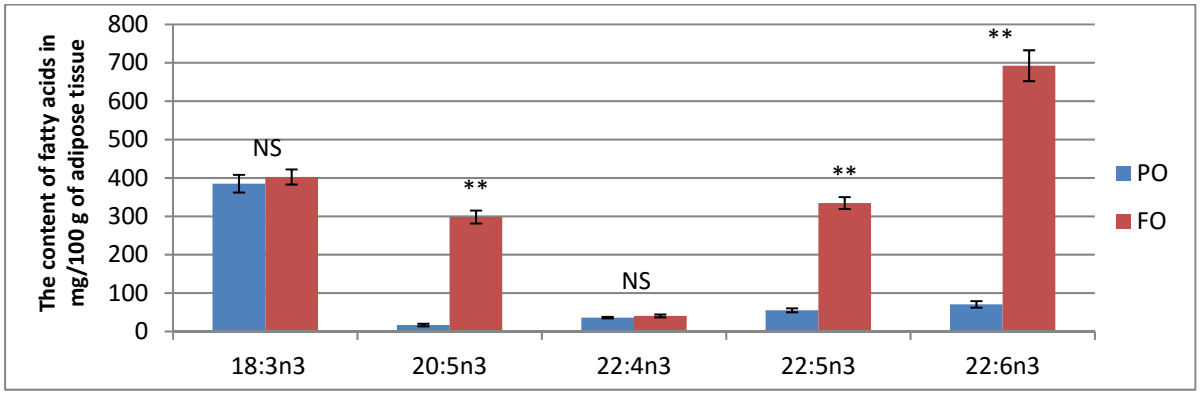
Figure 4 Content of SFA, MUFA, PUFA n-6 in visceral adipose tissue of pigs (mg/100 g)



*Legend: FO – basic feed mixture with addition of 2.5% fish oil; PO – basic feed mixture with addition of 2.5% palm oil; ; * - differences significant at P < 0.05; ** - differences significant at P<0.01; one-way ANOVA with post-hoc Tukey`s test; n = 32; NS – not significant (P > 0.05).*

Addition of palm oil to the animal diet increased significantly content of palmitic (P < 0.01), stearic (P < 0.05), linoleic (P < 0.05) and eicosadienoic (P < 0.01) acids in adipose tissue of the PO group.

Figure 5 Content of PUFA n-3 in visceral adipose tissue of pigs (mg/100 g)

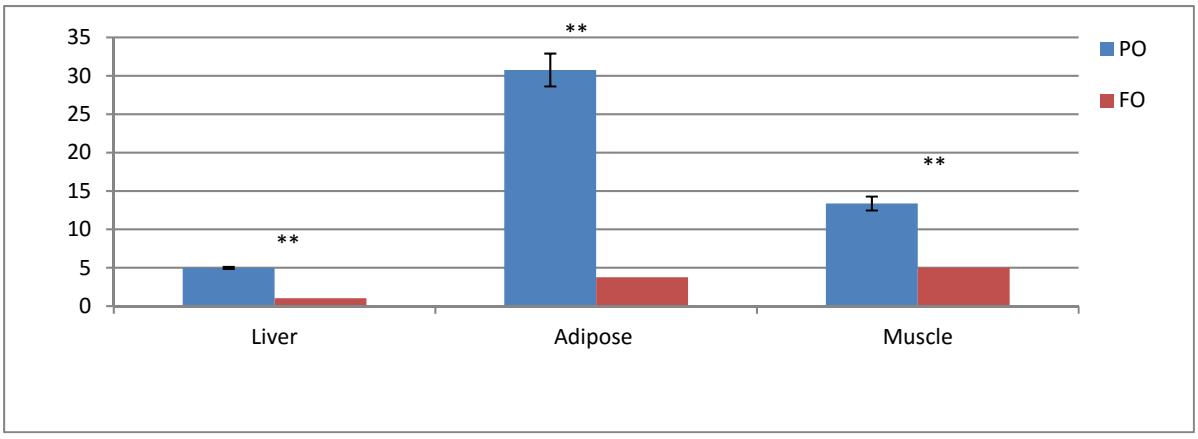


*Legend: FO – basic feed mixture with addition of 2.5% fish oil; PO – basic feed mixture with addition of 2.5% palm oil; ; * - differences significant at P < 0.05; ** - differences significant at P<0.01; one-way ANOVA with post-hoc Tukey`s test; n = 32; NS – not significant (P > 0.05).*

The FO group had higher amounts of EPA, DTA and DHA (P < 0.01) than the PO group. In pigs, the composition of fatty acids stored in adipose tissues largely reflects that of ingested lipids (Kouba and Mourot 2011).

The addition of palm oil increased level of linoleic acid (PUFA n–6; Table 1) in the feed mixture. On the other hand, fish oil had higher amount of α-linolenic acid (LNA), EPA, DTA and DHA (PUFA n–3; Table 1). The ratio of PUFA n–6 and n–3 is important for nutritional point of view on the grounds of production of eicosanoids and other biochemically active molecules, which has affect on cholesterol homeostasis, blood vessels, arthritis and inflammatory responses. The ratio of PUFA n–6 and PUFA n–3 in the tested tissues of is shown in Figure 6.

Figure 6 The ratio of PUFA n-6/PUFA n-3 in the tested pig tissues



*Legend: FO – basic feed mixture with addition of 2.5% fish oil; PO – basic feed mixture with addition of 2.5% palm oil; one-way ANOVA with post-hoc Tukey`s test; n = 16; NS – not significant, ** P < 0,01.*

All tissues had significantly lower ratio (P < 0.01) of PUFA n-6/PUFA n–3 in the FO group compared with the PO group. Liver tissue had recommended ratio (5 : 1) in the PO group, but even lower ratio in the FO group. Harnack et al. (2009) suggested the LA/LNA ratio 1 : 1 positive for formation their metabolites. Determining content of PUFA in the liver of model animals, including the ratio of n–6/n–3 is significant for evaluation of cholesterol metabolism with the consequence of estimated risk of cardiovascular diseases (Komprda et al. 2015). The effect of fish oil to decrease ratio of n–6/n–3 in animal tissues was also confirmed by Feillet-Coudray et al. (2013).

CONCLUSION

The addition of fish oil affected the deposition of fatty acids in tested tissues. Deposition of EPA, DPA, DHA significantly increased (P < 0.01) in the liver, muscle and adipose tissues under the fish oil diet rich in PUFA n–3 compared with the control group (PO). Increased amount of PUFA n–3 in the

diet (FO) decreased the ratio of PUFA n–6/n–3 to a recommended value, which is associated with reduced risk of cardiovascular diseases. On the other hand, palm oil added to the feeding mixture increased ($P < 0.01$) amount of arachidonic acid in the liver and muscle tissues with no effect on saturated fatty acids, especially palmitic acid. Fatty acids content in adipose tissue mirrors dietary intake of these fatty acids.

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INFLUENCE OF RIPENING ON THE PHYSICOCHEMICAL AND SENSORY PROFILE OF SEMI-HARD CHEESE

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Abstract: The aim of this paper deals with the physicochemical changes and sensory profile of semi-hard cheese during ripening. Accurate cylindrical samples of cheese with different surface treatment (wax vs. oil) were prepared. The first group of cheese was treated by wax and second group was oiled. From these groups, there were picked parts of cheese after 10, 20, 30, 40, 60 and 85 days of ripening to physicochemical analysis. Dry matter was determined by drying the sample to a constant weight at 102 °C, content of fat, protein content by Kjeldahl method, content of salt and titratable acidity, water activity (25 °C) and pH. It was evaluated colour, texture and overall appearance by sensory evaluation at Department of Food Technology Mendel University in Brno. There were statistically significant differences in all physicochemical parameters during ripening between waxy and oiled cheese. Similarly, there were statistically significant differences between each group (waxy vs. oiled) in different stages of ripening. Statistical differences were caused by different water content during ripening which has influenced content of fat, protein and other components. The wax keeps whey in the cheese which caused better ripening in the entire volume of cheese. The wax did not have any negative influence on ripening of cheese. The oiled cheese were gradually drying and thus was influenced ripening which was negatively assessed evaluators.

Key Words: cheese, sensory evaluation, physicochemical analysis, surface treatment of cheese

INTRODUCTION

The group of semi-hard cheeses is very large group, which is divided into a few smaller groups of cheeses. Into these groups belong semi-soft cheeses, white cheeses, pasta filata cheeses, blue-veined cheeses and etc. (Keresteš et al. 2016). A wide variety of semi-hard pasta filata cheeses, including Italian "Caciocavallo Silano" – obtained PDO, "Caciocavallo Podolico" etc., Russian "Kashkavalo" and Balkanian types "Kashklaval Balkan" fall under the common name "Caciocavallo". Other pasta filata cheeses, which are made by using a similar method, are marketed under different names including the PDO "Provolone Valpadana" (Piraino et al. 2005). Between pasta filata cheeses that obtained PGI in Slovak Republic belong "Klenovecký syrec", "Oštiepok" etc. (Keresteš et al. 2016).

Ripening of cheeses includes all chemical changes processing in the cheeses. Some of these changes start before the curd making is stopped. During cheese ripening are changed organoleptic properties – as a structure, colour and composition of cheese (Jarošová and Cwiková 2014, Cwiková and Nedomová 2007), biochemical and microbial aspects, chemical and physical properties (Walstra et al. 2006, Nedomová 2009, Nedomová 2010a, Nedomová 2010b). Jarošová and Cwiková (2014) say that the best sensory quality of smear-ripened cheeses (smell, colour and appearance, degree of ripeness, consistency and taste) is in storage at 4–8 °C until the end of their shelf-life.

Cheese wax was used for surface treatment and preservation of cheese blocks. Cheese wax is suitable for waxing semi-hard and hard cheeses. Wax had a good adhesion, does not crack and sets quickly. Cheese wax protected the cheese from mould and moisture loss, and therefore weight. It can be applied at all stages of ripening. Under the wax, cheese ripened further. The aim of waxing semi-hard and hard cheeses is to give clean and nice appearance to cheeses, prevent water loss to reduce waste and prevent the development of microorganisms on the cheese surface (Çetinkaya and

Atasever 2015). The next technique for ripening is ripening in plastic films produces cheeses without a rind, but this technique does not allow surface water evaporation (Bertola et al. 2000).

The aim of this paper was a comparison of the physicochemical changes and sensory profile of semi-hard cheeses during ripening, where was used a different surface treatment of cheese (wax vs. oil).

MATERIAL AND METHODS

Cheese making

Cheese was made in manufacture place in Mendel University in Brno at Department of Food Technology. Cheese was made from the milk of Holstein dairy cows from South Moravian region. This milk had a composition: dry matter 12.68% (gravimetry) (ISO 6731:2010), fat content by Gerber 3.6% (ISO 2446:2008), protein content by Kjeldahl 3.19% (EN ISO 8968-1:2002), lactose by polarimetry 4.92% and titratable acidity 6.5 SH (ČSN 57 0530). This milk fulfilled all the requirements for total bacterial count and somatic cells count.

Cheese was manufactured using pasteurized milk (72 °C/30 sec), milk cooling to 33°C and adding cheeses culture TM 1 (Bulgaricus, Czech Republic) and cheeses culture MC 1 (Bulgaricus, Czech Republic). Milk was held at 32 °C during 40 min, adding 10 ml 36% of $CaCl_2$. For the renneting was used commercial chymosin rennet Naturen 145 IMCU (CHR. HANSEN, Denmark). For vat (70 l) was used 40 ml chymosin rennet. Forty minutes later the curd was cut into small cubes 15 x 15 mm. After 15 min was separated 18 l of whey and 15 l of warm water (40 °C) was added. This mixture was heated to 40 °C and stirred for 30 min. After 30 min was curd filled into form and whey was separated. The curd was formed and pressed 45 min, then was curd turned and pressed for another 20 min. The curd was fermented 20 hours. Next day was the cheese placed into vat with warm water (85 °C) for 2 min. These cheeses got a pasta filata surface. After this step the cheeses were placed into salt brine (18% NaCl) for 20 hours at 10 °C. Then the cheeses were dried and kept in a chamber (12 °C) for 2 days. Cheeses were divided into two halves. First half was waxed by cheese wax (Driml, Czech Republic, BB: 1/2018). The second half of cheeses was oiled (rape seed oil, Czech Republic). These treated cheeses were placed in ripening chamber with condition 12 ± 1 °C and relative humidity 85% for 85 days. The oiled cheeses were oiled when was necessary as well as removing surface moulds.

A total amount of 40 samples of cheese were used to study chemical parameters and sensory evaluation during ripening. From these groups part of cheeses was picked after 10, 20, 30, 40, 60 and 85 days of ripening to physicochemical analysis and sensory evaluation. The cheeses had a cylindrical shape, which had average width a 71.8 mm and average height 33.7 mm. The weights of these cheeses were in range from 112 g to 140 g.

Physicochemical analysis

For physicochemical analysis was used full load of waxing and oiled cheese. Dry matter was determined by drying the sample to a constant weight at 102 °C. Content of fat was determined by the Gerber-van Gulik method according to the International organization for Standardization (ISO 3433:2008). Protein content was determined by Kjeldahl method (total nitrogen multiplying by factor 6.38) (EN ISO 8968-1:2002), content of salt and titratable acidity was determined by ČSN 57 0170. Water activity (25 °C) by a_w LabSwift – slow program (Novasina LabSwift-AW, United Kingdom) and pH was measured by pH multi 907 PORTAVO with glass probe SE 104N (Knick, Germany). Each analysis was performed twice, water activity and protein content was performed for three times.

Sensory analysis

Sensory evaluation was carried out at Department of Food Technology Mendel University in Brno in the sensory laboratory, which is equipped in according of ČSN ISO 8589 (2008). The 10 assessors with Assessor´s Certificate evaluated these descriptors of colour, texture and overall appearance. Cheeses for sensory evaluation were tempered at 18 °C and served on the plastic white plate.

The sensory attributes were analysed using a method of sensory profile with line unstructured scales (100 mm, 1 mm = 1 point) with verbal descriptions of the end points (0 points the worst value, or the lowest, 100 points the best value, or the highest).

Statistical analysis

The results were statistically processed by program STATISTICA 12 (Statsoft, Praha), where was performed ANOVA (Duncan´s test) to determine influence of ripening to physicochemical properties. The results of sensory evaluation were processed by Microsoft Excel 2010.

RESULTS AND DISCUSSION

At first, there were laid down analytical characteristics of cheeses using methods which are described above. Chemical composition and physicochemical parameters are shown in the Table 1 and Table 2.

Table 1 Chemical composition of cheeses during ripening

Days of ripening	Content of dry matter (%)		Content of fat (%)		Content of protein (%)		Content of NaCl (%)	
	Wax	Oil	Wax	Oil	Wax	Oil	Wax	Oil
10	57.85^{c}	67.74^{b}	29.00^{ab}	35.50^{c}	20.38^{c}	24.33^{c}	4.26^{c}	4.67^{c}
20	59.23^{a}	69.97^{c}	29.00^{ab}	39.00^{b}	21.73^{b}	27.51^{ab}	4.03^{b}	5.48^{b}
30	59.07^{a}	71.75^{d}	29.00^{ab}	38.00^{a}	23.33^{a}	27.59^{a}	4.01^{b}	5.30^{a}
40	57.44^{b}	73.40^{e}	26.50^{d}	38.00^{ab}	23.18^{b}	27.56^{a}	4.52^{c}	5.36^{a}
60	60.27^{d}	74.49^{a}	31.00^{c}	39.00^{ab}	23.90^{a}	27.93^{d}	3.86^{a}	5.19^{a}
85	59.06^{a}	75.39^{a}	31.50^{c}	38.50^{ab}	23.71^{a}	28.09^{b}	3.90^{a}	5.53^{b}

$^{a, b, c, d, e}$ *– different superscripts in a column indicate a statistically significant difference at P < 0.05*

There were statistically significant differences in all physicochemical parameters during ripening between waxy and oiled cheese. These cheeses had a dry matter in a range 57.44% (wax, 40 days) to 75.39% (oil, 85 days). Content of fat was in range 26.50% to 31.50% for wax cheese. Content of fat of oiled cheese was higher, due to using oil for surface treatment. Average fat content of oiled cheese was 38.00%. The lowest content of protein was 20.38% (wax, 10 days) and the highest 28.09% (oil, 85 days). Content of salt was in range 3.86% to 5.53% during ripening. The values of pH were the lowest 5.20 (oil, 60 days) and the highest 5.43 (wax, 40 days). The highest titratable acidity was 66.60 SH for oil cheese (10 days) and the lowest 26.60 SH (oil, 40 days). The water activity was decreased during ripening. The wax cheese had after 10 days of ripening a_w = 0.930 and the lowest a_w = 0.822 had an oil cheese after 85 days of ripening. Similarly, there were statistically significant differences between each group (waxy vs. oiled) in different stages of ripening. Statistical differences were caused by different water content during ripening which has influenced content of fat, protein and other components.

Table 2 Physicochemical parameters of cheeses during ripening

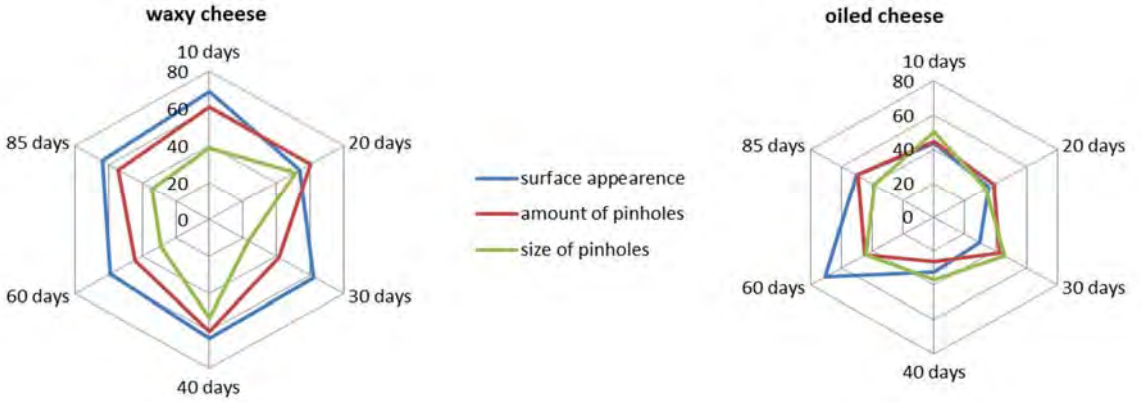
Days of ripening	pH (-)		Titratable acidity (SH)		Water activity (25 °C)	
	Wax	Oil	Wax	Oil	Wax	Oil
10	5.36^{b}	5.25^{a}	55.20^{f}	66.60^{f}	0.930^{c}	0.877^{e}
20	5.35^{b}	5.28^{a}	35.90^{b}	40.10^{c}	0.922^{a}	0.860^{d}
30	5.42^{a}	5.28^{a}	36.70^{c}	35.90^{b}	0.917^{d}	0.826^{a}
40	5.43^{a}	5.34^{c}	39.60^{d}	26.60^{a}	0.929^{bc}	0.838^{b}
60	5.42^{a}	5.20^{b}	32.30^{a}	40.60^{d}	0.927^{b}	0.826^{a}
85	5.41^{a}	5.22^{a}	43.20^{e}	45.80^{e}	0.920^{a}	0.822^{c}

$^{a, b, c, d, e, f}$ *– different superscripts in a column indicate a statistically significant difference at P < 0.05*

The best overall appearance had a waxy cheese, which had a higher value a surface appearance,

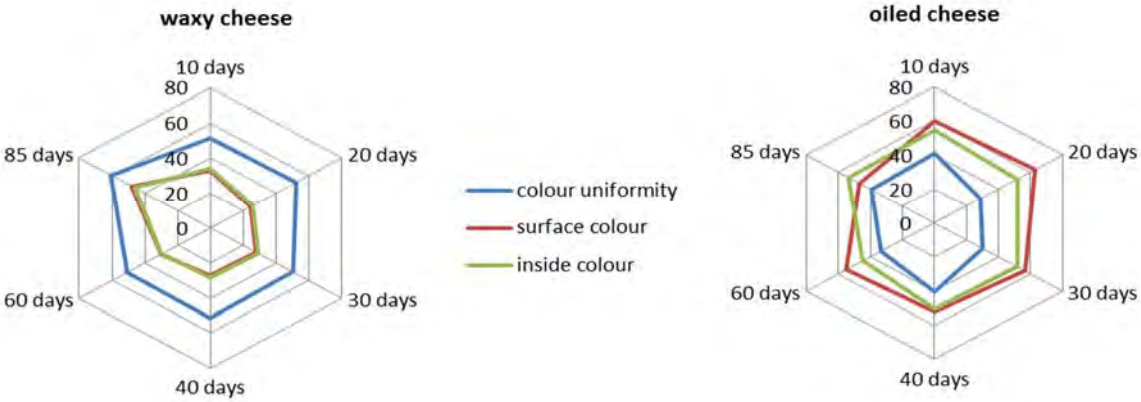
but this cheese had a higher amount of pinholes and their size was bigger. The oiled cheese had a worse evaluation of surface appearance, but amount of pinholes was lower and their size was smaller (Figure 1).

Figure 1 Comparison overall appearance of waxy cheese and oiled cheese during ripening



In the Figure 2 is seen, that surface colour and inside colour of waxy cheese is balanced and colour uniformity is comparable during ripening. Oiled cheese had a worse colour uniformity. Surface colour and inside colour oiled cheese was equalized after 40 days of ripening. After 85 days of ripening was better evaluated oiled cheese inside colour, until that time was better evaluated surface colour.

Figure 2 Comparison colour parameters of waxy and oiled cheese during ripening



The waxy cheese was better appreciated in the texture attributes than oiled cheese (Figure 3, Figure 4). Values of firmness and elasticity of waxed cheeses were higher; however in this cause higher values mean that the cheeses are better acceptable for assessors.

Figure 3 Comparison texture attributes of waxy and oiled cheese by hand

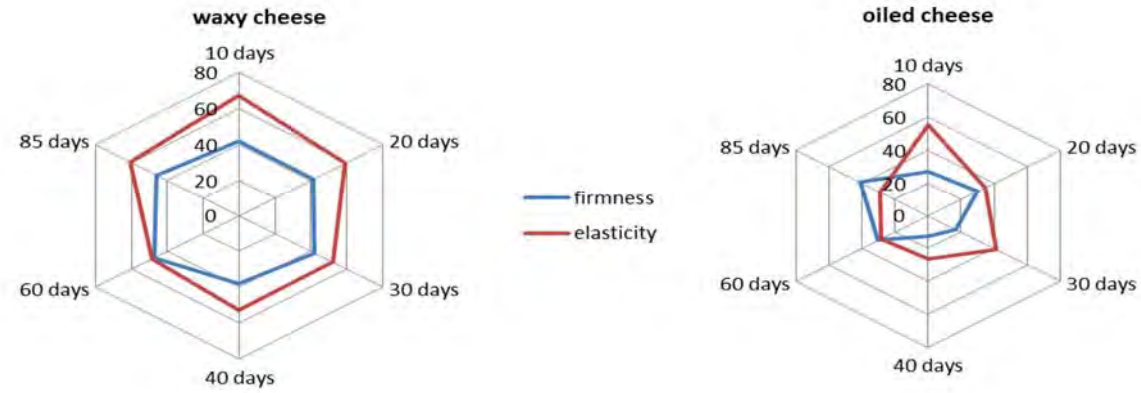
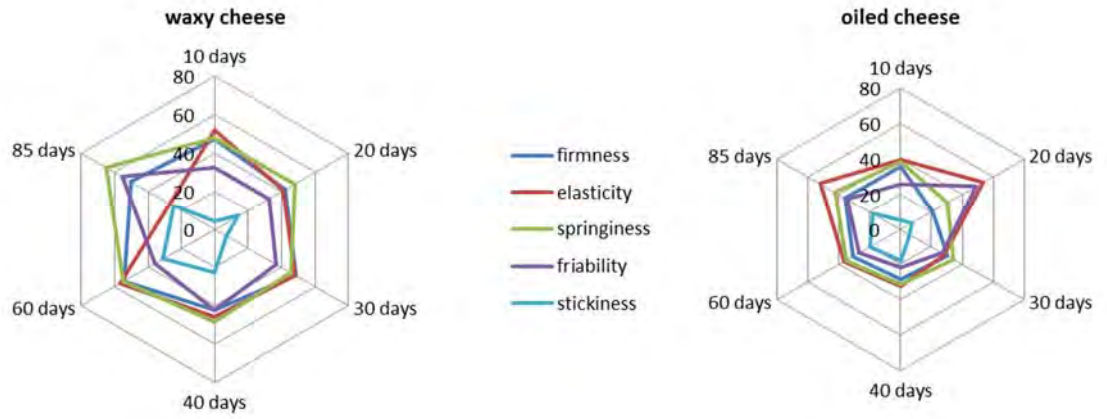
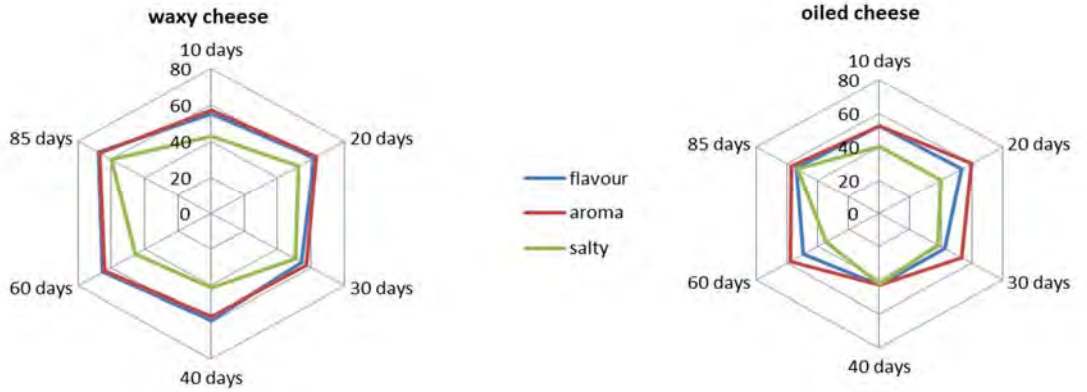


Figure 4 Texture comparison of waxy and oiled cheeses by mouth



The flavour and aroma of waxy cheeses was equalized all the time ripening. Salty flavour was higher at 20, 30 and 85 days after ripening (Figure 5). The aroma oiled cheeses was equalized all time ripening too. The flavour was worse at 20, 30 and 60 days after ripening. The highest value of salty flavour was perceived after 85 days of ripening. The flavour and aroma were best at the end of shelf-life of these cheeses as well as smear-ripened cheeses (Jarošová and Cwiková 2014).

Figure 5 Compare flavour of waxy and oiled cheese



Cheeses which were treated by wax were better evaluated than the oiled cheeses. The wax keeps whey in the cheeses which causes better ripening in the entire volume of cheese. The wax did not have any negative influence to ripening of cheeses. According by Çetinkaya and Atasever (2015) cheese wax and vacuum packaging did not have any adverse effect to cheese quality and did not delay cheese ripening. Hence, cheese wax is recommended as alternative material for packaging of cheeses. The oiled cheeses were gradually dried and thus the ripening was influenced and this was negatively influenced assessors.

CONCLUSION

This paper compared cow´s semi-hard cheese which was made in Mendel University in Brno. These cheeses were differed by surface treatment. The first group was treated by wax and second group was treated by oil. Cheeses were used to study chemical parameters and sensory evaluation during 85 days of ripening. Among the measured results, there was significant difference in the physicochemical analysis (dry matter, content of fat, protein and NaCl, pH, titratable acidity and water activity) during ripening. Colour, texture and overall appearance were evaluated by sensory analysis. Cheeses which were treated by wax were better evaluated than the oiled cheeses. The wax keeps whey in the cheeses which caused better ripening in the entire volume of cheese. The wax did not have any

negative influence to ripening of cheeses. The oiled cheeses were gradually dried and thus the ripening was influenced which was negatively evaluated by assessors.

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SELECTED ELECTRIC PROPERTIES OF PERGA

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Abstract: Measurement of electrical properties is one of the most important operations in the collection, storage and processing of the agricultural production. The physical properties of biological materials affect measurement accuracy. Bee bread – also called "Perga" – are fermented bees pollen in the comb, which store bees as protein supply. Bee bread is considered the most perfect of all natural foods – used by the Greeks as ambrosia. It includes many vitamins and trace elements. We measured several electrical properties, such as capacitance, resistance, impedance and loss factor. From measured and calculated values are constructed graphical dependencies of electrical quantities on frequency and moisture content. We found out that all measured electrical properties decreased with frequency, increase of the moisture content caused the growths of electrical properties.

Key Words: perga, electrical properties, frequency, moisture content

INTRODUCTION

Bee bread – also called "Perga" – are fermented bees pollen in the comb, which store bees as protein supply. The bee bread is harvested by hand from the honeycomb and thus is not only the most valuable, but also the most expensive bee products (McIntyre and Snyder 1978). Bee bread is considered the most perfect of all natural foods – used by the Greeks as ambrosia. It includes many vitamins (A, B1, B2, B6, B12, C, D, E, P, PP) and trace elements (potassium, magnesium, calcium, copper, iron, silicon, sulphur, chlorine, manganese) (McIntyre and Snyder 1978). In the perga there is everything that is required for the normal growth and existence of a live organism. Good results are noted by specialists at treatment of patients with many diseases (Georgiev et al. 2004).

The dielectric properties of foods and biological products have become valuable parameters in food engineering and technology. The investigation of material and moisture measurement using electromagnetic waves in wide spectrum serve for quality control and improvement in many branches like industry, forest and wood–working industry, civil engineering, agriculture, commerce and also foods e. g. for quality evaluation of meat, fruits, coffee etc. (Hlaváčová 2003, Venkatesh 2005).

The study of electrical properties is important for predicting the behaviour of a material in electric field or for knowing how the presence of material can influence the field or an associated electrical circuit. Electrical measurements on these materials are of fundamental importance in relation to the analysis of quantity of absorbed water and dielectric heating characteristics. Dielectric properties of materials represent an important part of electrical properties. The research of electrical properties is finding utilisation in a lot of technical applications. Results of measurements are used for determining the moisture content, the surface level of liquid and grainy materials, for controlling the presence of pests in grain storage, for the quantitative determination of mechanical damage, and in many other cases (Hlaváčová 2003, Kuang and Nelson 1998). It was discovered that electric properties of these materials are very affected by moisture content of the material. Small quantities of adsorbed water can cause large changes in electrical properties of hydroscopic materials. An extensive review of the literature on

dielectric properties of agricultural materials was published by Nelson and Trabelsi (2008) that includes a number of potential applications in which the dielectric properties of such products are of interest.

Every material influences the electric field to which it is subjected. The relation between parameters of electromagnetic field and properties of material medium is described with permittivity ε and permeability μ of this medium. Both parameters describe electromagnetic properties of matter. A work of Barbosa–Canovas et al. (2006) deals with the measurement of these parameters. Permittivity is complex quantity

$$\varepsilon^* = \varepsilon' - j\varepsilon'' \tag{1}$$

The real part of complex permittivity ε^* is the permittivity of dielectric ε'. The coefficient of imaginary part ε'' characterises losses in dielectric. Dielectric properties of materials are generally formulated by relative complex permittivity

$$\varepsilon_r^* = \frac{\varepsilon^*}{\varepsilon_o} = \frac{\varepsilon'}{\varepsilon_0} - j\frac{\varepsilon''}{\varepsilon_0} = \varepsilon_r' - j\varepsilon_r'' = \varepsilon_r'(1 - tg\delta) \tag{2}$$

where

$$tg\delta = \frac{\varepsilon_r''}{\varepsilon_r'} = \frac{\varepsilon''}{\varepsilon'} = \frac{\sigma}{\omega\varepsilon'} \tag{3}$$

is loss tangent of loss angle δ, it is the angle completing to $\frac{\pi}{2}$ phase difference between voltage and current flowing through the dielectric. ε_o is vacuum permittivity ($8.85 \cdot 10^{-12}$ F/m), ε_r' or ε_r is relative permitivity or dielectric constant, j is imaginary unit, σ is conductivity of material, ω is angular frequency. To measure the dielectric properties of samples, a resonant method has been used. This method for measuring the dielectric properties of seeds and liquids in a frequency range from 100 kHz to 300 kHz was developed by Nelson et al. (2007).

MATERIAL AND METHODS

Perga

Bee bread – also called "Perga"– are fermented bees pollen in the comb, which store bees as protein supply. Perga samples of different plant species were collected by beekeepers from selected regions of Ukraine (Poltava and Dnepropetrovsk) according to a new patented technology developed by the research team of the Department of Apiculture at the National University of Life and Environmental Sciences of Ukraine, with offices in Kiev. All samples were polyfloral Perga.

Electrical properties measurement

All measurements were carried out under an air temperature of 20 °C and of 60% relative humidity. Bulk density was determined by the mass of constant sample volume. We used two methods of measurement by LCR meter and Q meter.

Low–frequency electrical properties of perga were measured by an instrument GoodWill Instek LCR meter 821 at different frequencies using four-electrode (tetra polar) system. The sample was placed in the sensor with parameters: diameter of electrode 37.8 mm, electrodes spacing 49.2 mm, mass of empty sensor 208.89 g. We measured capacitance, resistance, impedance and loss factor. Each property was measured in the frequency range from 0.1 kHz to 200 kHz, at all frequencies three times. Average value has been computed from these ones and standard deviation was calculated. The measured values were loaded by PC.

The capacitance of the testing capacitor was measured by the Q meter TESLA MB 560. By measuring the permittivity ε' of testing capacitor, the real capacitor can be considered as a lossless capacitor connected with active resistance in a parallel or serial configuration. The substance of measurement is to determine the magnitude of capacitance and resistance of parallel or serial configuration of dielectric at a specific frequency. The Q meter was connected with the testing coaxial

capacitor, which was used as a sample holder. The measurement was performed in a frequency range from 1 MHz to 16 MHz. Dielectric constant ε_r was calculated according following relations

$$\varepsilon_r = \frac{C - C_x}{C_o} \tag{4}$$

$$C = C_1 - C_2 \tag{5}$$

where

C – capacitance of testing capacitor with a sample, F

C_o – capacitance of empty testing capacitor free of interconnector capacitance, F

C_x – capacitance of interconnector, F

C_1 – capacitance of tuning capacitor by resonance and by non-connection of testingcapacitor, F

C_2 – capacitance of tuning capacitor by resonance and by connection of testing capacitor, F

The resistance R of testing capacitor was measured using multimeter MASTER M830BUZ. Conductivity of the sample σ was determined according following relation

$$\sigma = \frac{\ln \frac{r_1}{r_2}}{2\pi\, l\, R} \tag{6}$$

where

r_1 – radius of capacitor outer electrode, 0.033 m

r_2 – radius of inner electrode, 0.008 m

l – length of capacitor, 0.06 m

Moisture content measurement

Moisture content wet basis was determined by gravimetric method. The samples were weighed by Sartorius Basic electronic analytical and precision balance (Sartorius AG) on the beginning and after drying andreaching constant mass. Moisture content was calculated according the following relation

$$w = \frac{m_1 - m_2}{m_1} 100\ \% \tag{7}$$

where

m_1 – initial mass of sample

m_2 – mass of dry sample

Different moisture content w of samples was achieved by natural drying.

RESULTS AND DISCUSSION

Measurement by RLC meter

From measured values at initial moisture content are constructed graphical dependencies of electrical quantities on frequency. For illustration, the resistance and impedance versus frequency curves are shown on Figure 1. The resistance and impedance of perga sample decreases in this frequency range. The regression equation for resistance and impedance has the shape of decreasing power function

$$R = R_0 \left(\frac{f}{f_0} \right)^{-k} \tag{8}$$

where

R – resistance, kΩ

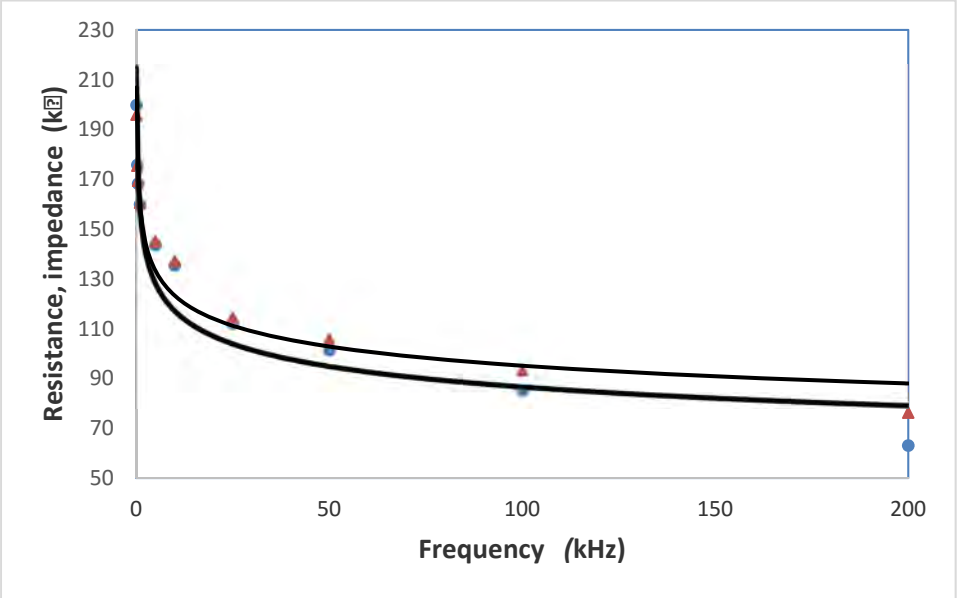
R_0 = 158.98 kΩ – reference resistance,

f – frequency,

f_0 = 1 kHz,

k = - 0.131 – constant.

Figure 1 The resistance (●) and impedance (▲) versus frequency curves

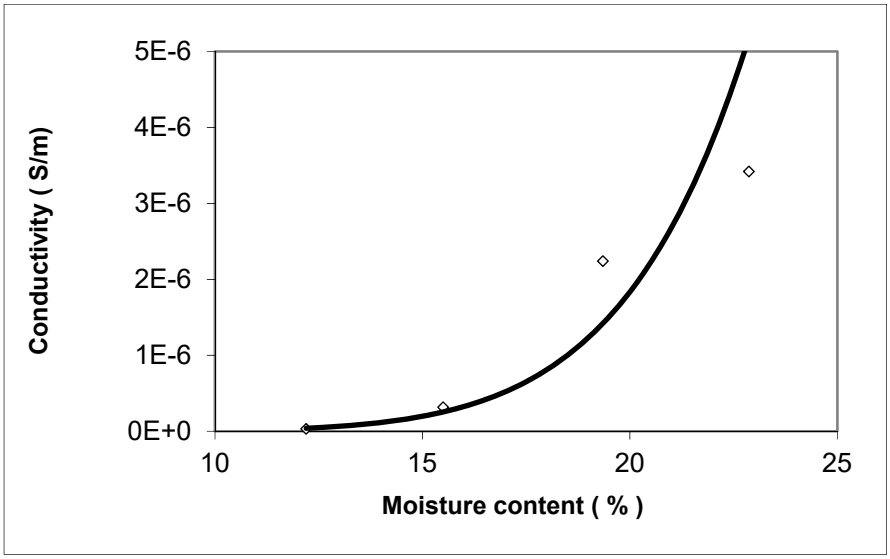


The similar equation can be write also for impedance. The coefficient of determination has high value R^2 = 0.9089 for resistance and 0.9427 for impedance. The change of resistance and impedance at low frequency is significant, compared to the higher frequencies. The impedance has a slightly higher values as resistance, which is caused by the capacitance of perga. Also the capacitance decreases with frequency according to power function. Only loos factor increased in this frequency range.

Measurement by Q meter

The effect of moisture content on conductivity and dielectric constant are shown in Figure 2 and 3.

Figure 2 Effect of moisture content w on the conductivity σ of perga samples



This increasing dependency can be also modelled by a power function

$$\sigma = \sigma_0 \, w^k \tag{9}$$

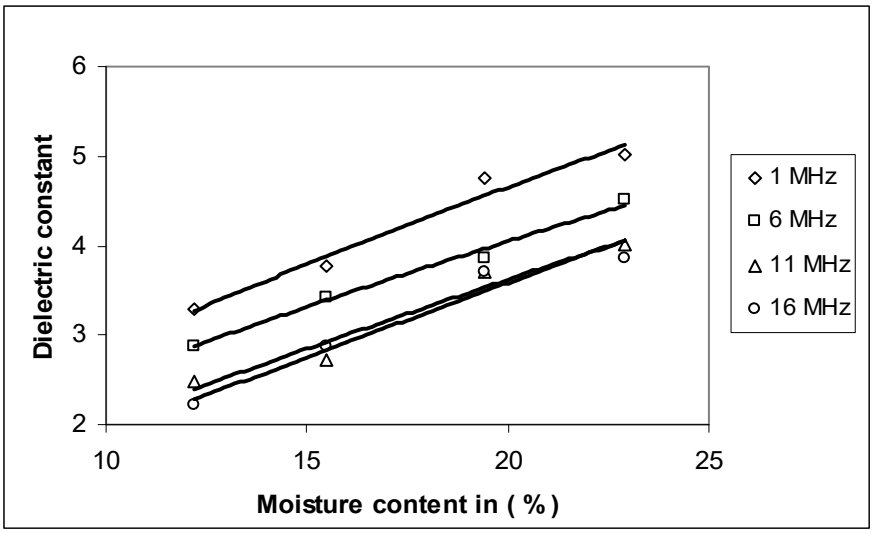
where

$\sigma_0 = 2 \cdot 10^{-16}$ S/m – reference conductivity

k = 7.7523 – constant

Regression coefficient has also high value $R^2 = 0.9645$.

Figure 3 Moisture content w dependences of dielectric constant ε for perga samples measured at different frequency f



We used also power function for modelling these dependencies

$$\varepsilon_r = \varepsilon_{r0}\, w^k \tag{10}$$

where

ε_{r0} – reference dielectric constant

k – constant

Coefficients of regression equation (10) and coefficient of determination are displayed in Table 1.

Table 1 Coefficients of regression equation (10) and coefficient of determination

Frequency (MHz)	ε_{r0}	k	R^2
1	0.5526	0.7113	0.9726
6	0.508	0.6928	0.9905
11	0.2964	0.8353	0.9512
16	0.2293	0.9176	0.9645

Also in case of equation (10), it can be see that the coefficient of determination has high value. Values of dielectric constant increase when the moisture content of samples increases. This effect is the consequence of a very high magnitude of dielectric constant of water in comparison with other components of perga (Nelson and Trabelsi 2008).

CONCLUSION

The measurements indicate that perga must be included in the most complex objects. It is organic heterogeneous multi–component dielectrics. The factors having the highest effect on these effects are also specified.

We found out that the resistance and impedance decrease with frequency. The change of resistance and impedance at low frequency is significant, compared to the higher frequencies. The impedance has a slightly higher values as resistance, which is caused by the capacitance of perga. Also the capacitance decreases with frequency according to power function. Only loos factor increased in this frequency range. It was determined that the dielectric constant of perga increases when the moisture content of perga increases. The minimal decrease is at higher frequencies. These relationships are caused by the dipole moment of water molecules and may be the orientation of charged groups of macromolecules. In this case, an orientation polarisation occurs in electric field. This type of polarisation is highly dependent on frequency. Dipole macromolecules are not able to follow changes in the polarity

of electric field. Conductivity values increase when the moisture content of samples increases. This effect is caused by improvement of conditions for an electrolytic transport of charges by dissociated ions in damp medium.

ACKNOWLEDGEMENTS

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THE STUDY OF ANTIMICROBIAL EFFECT OF GRAPE SEED EXTRACT

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Abstract: In this study, an experiment on the antimicrobial activity of grape seeds from *Vitis vinifera* L. was performed. *Vitis vinifera* L. is known for its health benefits because of its high content of phenolic compounds. Grape seed extract was made from interspecific wine cultivars. Three pathogens (*Candida tropicalis*, *Escherichia coli*, and *Enterococcus faecalis*) were selected for the experiment. The antimicrobial effect was determined using a disc diffusion method. The high efficiency of grape seed extract was observed on *Candida tropicalis* (12.9 mm) and *Enterococcus faecalis* (9.1 mm). Grape seed extract was proven to be effective in inhibiting these pathogens.

Key Words: grape seed extract, antimicrobial activity, disc diffusion method

INTRODUCTION

Vitis vinifera L. is one of the most grown fruits in the world, with more than 67 million tons produced annually. There are many proven health benefits from consuming raw grapes, or grape products such as wine or juices (Friedman 2014). Grape seeds contain 60–70% of the grape's total extractable phenolics (Godevac et al. 2010). Due to their high phenolic compound, grape seeds have the ability to decrease oxidative stress, which makes them beneficial to human health. They also have the ability to promote the growth of beneficial bacteria in the intestinal tract (Brenes et al. 2016). The effect of grape seed extract (GSE) on three common pathogens, and its utilisation in practice, will be evaluated in this study.

MATERIAL AND METHODS

Biological Material

Key material for the preparation of tested extracts was a mixture of seeds of interspecific *Vitis vinifera* L. cultivars ('Cerason' (red), 'Marlen' (red) and 'Erilon' (white)) provided by the Department of Viticulture and Enology at Mendel University in Brno (MENDELU). Seeds were separated from other fractions (impurities, parts of plant), sorted, and dried to 8% moisture. 100 g of fine crushed grape seeds, with 1 litre of 75% methanol, were put into glass jars and stirred regularly. The extraction of substances took place in the dark, at a temperature of 21 °C for 168 hours.

Decanted extract from the jar was centrifuged. Then the evaporation of methanol from the extract was performed at 70 °C in a vacuum evaporator (IKA, Germany) with a constant rotation (70 rpm). 100% pure GSE was tested.

Disc Diffusion Method

Three microorganisms were tested for GSE antimicrobial activity: *Candida tropicalis* (CCM8223), *Escherichia coli* (CCM7229), and *Enterococcus faecalis* (CCM4224). Petri plates with two types of media (Biokar Diagnostics, France) - CHL (for *C. tropicalis*) and PCA (other

microorganisms) - were inoculated (density of inoculum was 0.27 MF). Inoculum was spread evenly on the surface and left to rest for 15 minutes.

The disc diffusion method was used to evaluate the antimicrobial effects of GSE. Paper discs (Fisher Scientific, Czech Republic) 9 mm wide in diameter were saturated by 30 µl of extract and inserted into Petri plates with pathogens. Three Petri plates were used for testing and in each plate there were three discs. Additional Petri plates with each pathogen (but without the discs) were prepared for use as a standard. Also, one plate with discs saturated with distilled water was used for each microorganism as a control.

RESULTS AND DISCUSSION

Petri plates were incubated in suitable conditions of thermostats (*Candida tropicalis* at 25 °C, *Escherichia coli* and *Enterococcus faecalis* at 37 °C). Two measures of inhibition growth zones' diameters were taken after 24 and 48 hours of incubation.The experiment conducted on 23. 2. 2016 studied the effects of GSE on three model pathogens: *Candida tropicalis*, *Escherichia coli*, and *Enterococcus faecalis*. Results depicted in Figure 1 and Figure 2 are average of the measurements of zones taken in Petri plates. If this average was less than 9 mm (diameter of the paper disc), GSE did not exhibit the expected inhibition. Standard deviation was used in Figures 1 and 2 (marked as error segment in each column). Conclusiveness of inhibitory effects of GSE against microorganisms was evaluated by Wilcoxon signed-rank test. *P*-value was 0.01 in *Candida tropicalis* (both 24 hours and 48 hours measurements), in other microorganisms were measurements identical, so test could not be made.

Positive results were observed in Petri plates with *Candida tropicalis*. After 24 hours there were inhibitory zones with a diameter of 12 mm. There was no sharp distinction between the microbial colony and the discs, so we can assume that growth was weakened, but not entirely inhibited. Zones expanded after 48 hours to a diameter 12.9 mm. It is obvious that the inhibitory effect increases with a longer duration of application.

The inhibition of *Escherichia coli* was barely noticeable. After 24 hours, no negative effect on growth was observed. Yet *Escherichia* was growing more intensely around the discs. There was no increased growth around the discs visible after 48 hours. Again, there was no sharp distinction between the bacteria and the disc.

Better measurements were taken in the colony of *Enterococcus faecalis*. After 24 hours there was an average diameter of zones 9 mm, but the antimicrobial activity increased after a longer period of application to 9.1 mm.

Figure 1 Diameter of inhibitory zones in mm (on Y axis) according to microorganism (on X axis) after 24 hours

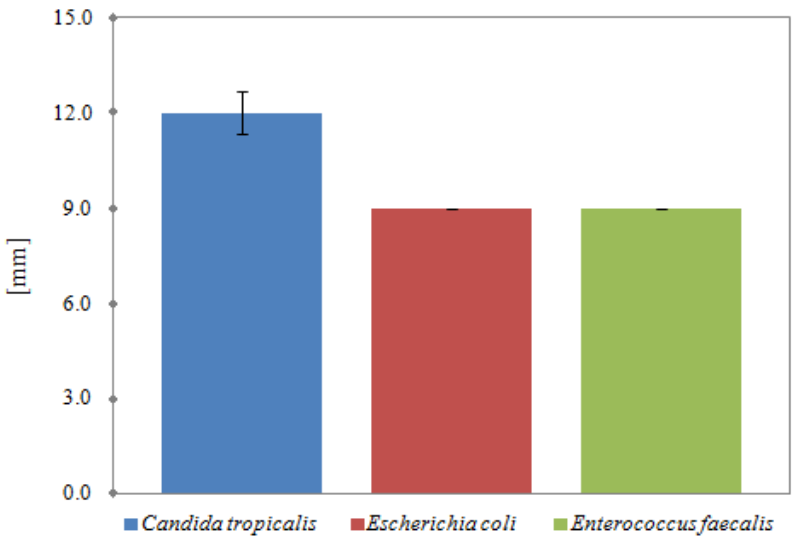
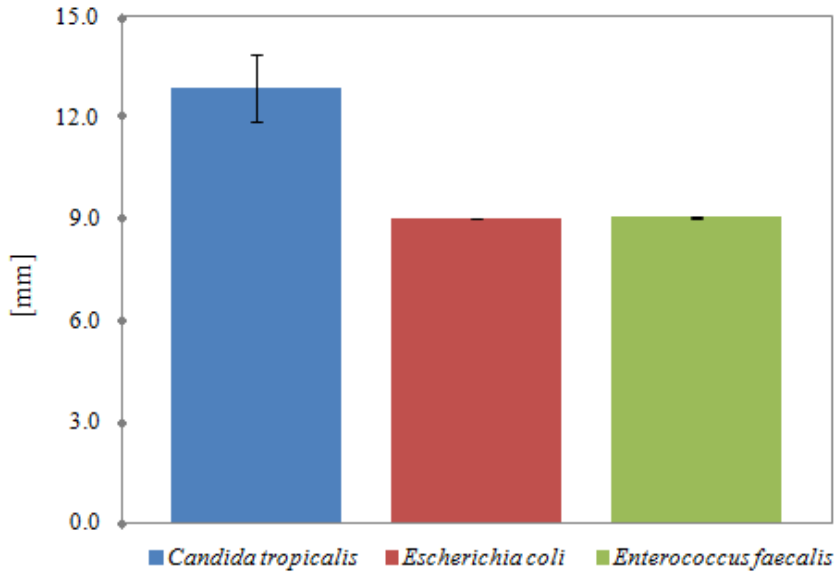


Figure 1 describes the antimicrobial effect of GSE on *Candida*, *Escherichia* and *Enterococcus*. The most evident inhibition can be seen on the blue field marking *Candida* yeast. There was no particular effect on *Escherichia* or *Enterococcus* (the diameter of zones was 9 mm). 9 samples were measured from every variant of microorganism (n=9).

Figure 2 Diameter of inhibitory zones in mm (on Y axis) according to microorganism (on X axis) after 48 hours



The progress of GSE activity on tested bacteria is depicted in Figure 2. Antimicrobial effects increased in both *Candida* and *Enterococcus*. There was no inhibition noted in the colony of *Escherichia*. 9 samples were measured from every variant of microorganism (n=9).

Health beneficial compounds can be found in different parts of the *Vitis vinifera* L. plant. Scientists proved the presence of antimicrobial properties in its leaves (Katalinic et al. 2013), anti-carcinogenic effects from its stems (Sahpazidou et al. 2014), and an antifungal effect from its pomace against *Botrytis cinerea* (Mendoza et al. 2013).

Recorded information in this study suggests that GSE has an antimicrobial effect. GSE in our study has a bigger inhibition effect in Gram-positive bacteria and yeast than in Gram-negative bacteria. There are many studies on food pathogens with similar results. GSE used in Spain also had better results on Gram-positive bacteria (Delgado Adámez et al. 2012), *Staphylococcus aureus*, and *Bacillus cereus*, than on *Escherichia coli*, *Pseudomonas aeruginosa*, and *Candida albicans* (Oliveira et al. 2013). To inhibit Gram-positive bacteria they used 850–1000ppm of GSE (Jayaprakasha et al. 2003).

A study of heated water soluble GSE (made of 'Ison' and 'Carlos' cultivars) showed the high potential of GSE to be a valuable natural preservative in beverages because of its antimicrobial activity against *Escherichia coli* (Kim et al. 2008). The results on *Escherichia coli* vastly differed from measurements in this paper. Such may be caused by different bacteria strains, or by the amount of antibacterial compounds in seeds used for the preparation of extracts. The properties of GSE could be enhanced in the future by using different types of solvents during extract production. Higher phenolic content could be obtained with ethyl acetate-water solvent (Jayaprakasha et al. 2001).

Studies testing GSE activity against pathogens do not end at common food borne organisms. A Kuwaiti study shows that 3 mg/ml of proanthocyanidin GSE (equivalent to 20.7 µg/ml flavonoid content) can be used for the inhibition of methicilin-resistant *Staphylococcus aureus* (Al-Habib et al. 2010). This proves the high utilisation of GSE in medicine.

Grape extract from skins and seeds of cultivar 'Arinto' was also tested against viruses. A Portuguese team of scientists found that this natural extract inhibits the replication of adenovirus type 5 irreversibly (Matias et al. 2010).

CONCLUSION

Extracts made from grape seeds were prepared and tested. Results varied according to each species. Higher antimicrobial activity was visible in colonies of *Candida* and *Enterococcus*. Suggested use of GSE against *Candida tropicalis* is 24 hours; against *Enterococcus faecalis* is 48 hours. This data shows that GSE can be used as an effective treatment against some pathogens. That is why it is important to utilise waste from the winemaking industry and spread information about this valuable commodity.

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THE USE OF HEMP AND COLOR WHEAT FLOUR AS BAKING INGREDIENTS

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Abstract: This paper deals with the application of flour, obtained by milling wheat varieties with colored pericarp (Rosso, Karkulka) and blue aleurone (Scorpion); and hemp products (hemp grits, hemp protein) for the production of bakery products. For testing purposes nine recipes ware created, 10% of hemp grits or 5% of hemp protein were added to the wheat flour. The influence of the recipes on the bakery product properties was evaluated by the bakery experiment - RMT test. The highest yield of the dough was measured in variants where hemp protein was added. The specific bread volume was higher in those variants which contained flour wheat from Scorpion variety (368 ml/100g). On average, the lowest loss at baking was determined after the addition of hemp protein. The addition of hemp grits supports the convexity of the baked goods. The use of this recipe also affected other sensory characteristics of the product associated with its consumption.

Key Words: color wheat, hemp grits, hemp protein, baking experiment, sensory analysis

INTRODUCTION

Currently there is a growing interest in the functional properties of food and also in the role of antioxidants, which are able to scavenger free radicals (Pasqualone et al. 2015). From this perspective, the anthocyanins and carotenoids are interesting. They are characterized by their antioxidant activity and are found in the caryopses of the color wheat (Janečková et al. 2015). A positive fact is, the inclusion of color wheat in the food, with a higher content of antioxidants in the diet may have beneficial effects on the human health (Knievel et al. 2009). There is a certain evolutionary theory, but it's effects are not yet supported by clinical tests (Martinek et al. 2010). It is true that the anthocyanins contribute to have better resistance against diseases (Havrlentová et al. 2014). Also, it is scientifically documented that they are physiologically active substances which promote health and reduces the risk of chronic diseases. The presence of these substances in the raw materials of the food, which include colored varieties of wheat, could significantly affect the nutritional value of the resulting food product (Chabinová et al. 2011). It has been demonstrated that the consumption of foods that contain anthocyanins reduces the risk of cardiovascular diseases and protect the body against oxidative stress. Also, they prevent DNA damage, the aggregation of thrombocytes and the oxidation of lipoproteins as well as possess anti-inflammatory effects (Hrnčířová 2011). The blue caryopsis differs from the purple caryopsis in the composition and content of individual anthocyanins; as well as the storage in different anatomical layers (Abdel-Aal and Hucl 2003). The same as the color wheat also cannabis could be used in the baking technology. The hemp seed contains essential amino acids and fatty acids. The seed also contains 25 to 30% of oil, 20–25% of protein, 20–30% of carbohydrate and 10–15% of fiber

approximately. Further, the hemp seeds contain a number of bioactive substances, such as flavones, polyphenols, proteins, albumin and edistin (Norajit et al. 2011); manganese, potassium, iron, zinc and magnesium (Cozea et al. 2016) and vitamins A, B, C and E (Pejcz et al. 2015). Also they contain a number of essential oils such as myrcene, trans – Caryophyllene, trans-β-Ocimene and α-humulene (Novak et al. 2000). Moreover, the hemp seeds lower the blood pressure and cholesterol and also strengthen the immune system (Pejcz et al. 2015). On the other hand, the hemp oil contains a high amount of linoleic acid. Furthermore, contains α-linolenic and oleic acids (Galasso et al. 2016) with the ratio of linoleic acid and α-linolenic of 3 : 1 (Leizer et al. 2000).

By the addition of hemp flour the amount of proteins could be increased in those products that have less, such as for example the rice (Norajit et al. 2011).The advantage of both materials, mentioned above, is that they could be processed and modified for use in bakery production. Their combination could contribute to the creation of new, nutritive and better balanced food products.

MATERIAL AND METHODS

Wheat flour was used in every recipe. The flour was obtained by milling the purple varieties of Karkulka and Rosso, and the blue aleurone (Scorpion variety). Into the preparation of the dough a percentage of hemp semolina and hemp protein were added. The representations of the individual components are shown in the Table 1.

Table 1 Overview of the variants

Variety	Combination*	Wheat flour [g]	Hemp grits [g]	Hemp protein [g]
Scorpion	Flour	500	-	-
Rosso		500	-	-
Karkulka		500		-
Scorpion	Flour + grits	450	50	-
Rosso		450	50	-
Karkulka		450	50	-
Scorpion	Flour + protein	475	-	25
Rosso		475	-	25
Karkulka		475	-	25

**Note: for each variant was also used: an addition of salt – 7.5 g, sugar – 5 g, oil – 5 g and yeast – 25 g.*

Preparation of dough and baking process

The dough was prepared by mixing all raw materials at once. It was kneaded in a dough-kneader for about one minute. It was raised in a proofer at 32 ± 1 °C and humidity of 80 ± 5% for 20 minutes. After the removal from the proofer, the dough was rested for 10 minutes and weighted. Then it was shaped into the desired pieces weighing 80 g and it was allowed to rise again at 32 ± 1 °C and humidity of 80 ± 5%, for 25 minutes. Before loading the pieces into the oven, they were sprinkled it with water, and baked at 230 °C to 240 °C in a laboratory oven with a proofer. At the beginning of the baking, the oven was steamed with 50 ml of water. The baking time was 20 minutes.

During the baking experiment the yield of the dough (%), the baking losses (%), the height of the bread (%), the specific bread volume (ml/100 g) and the convexity of the bread characterized by a proportional number was evaluated.

Sensory analysis

An hour after baking the sensory analysis, by a team of sensory evaluators (n = 10), was carried out. During the sensory analysis the shape, color of the crust, aroma, flexibility of the crumb, color of the crumb, easiness of biting, sensation after chewing, consistency, moisture of the crumb and taste were evaluated. The sensory evaluation was made by unstructured graphic scales, which had a range of 100 mm, ten millimeters in the scale correspond to one point.

Evaluation of the results

The statistical evaluation of the identified data was performed using Microsoft Excel and Statistica 12. The one-way ANOVA method was used, which is used for the evaluation of the analysis of variance. The average and the standard deviations of the observed data were calculated and the significant differences were determined for each characteristic.

RESULTS AND DISCUSION

The baker's experiment

Flours from different types of color wheat: Karkulka, Rosso and Scorpion were tested in this experiment. The Karkulka variety has a significant content of substances in the caryopses layers as well in the endosperm, which could be used in the food industry. Also, it has a high protein content and a very high gluten content (Rückschloss et al. 2014). The Rosso wheat variety is also characterized by having valuable technological parameters, which increases significantly its use in bakery products (Hřivna et al. 2014). Generally, the blue grained wheat has higher levels of anthocyanins than the purple wheat pericarp (Martinek et al. 2012). Also from this perspective it could be considered that the growing of the Scorpion wheat as very appropriate. Moreover, with the exception of the crop weight, it has very satisfactory crop attributes (Hřivna et al. 2014). It is also assumed that the flour of blue grained varieties has more antioxidants, because the antioxidants are not in the pericarp but are located closer to the endosperm, in the aleurone layer.

Table 2 Results of the baking experiment

Variety	Yield of the dough (%)	Baking losses (%)	Yield of the bread (%)	Specific bread volume (ml/100g)	Ratio number
1	164.58	13.24	142.78	368.00	0.46
2	165.76	12.07	145.76	316.00	0.56
3	165.24	13.81	142.43	288.00	0.62
4	158.54	13.07	137.82	316.00	0.61
5	159.92	10.27	143.49	308.00	0.75
6	159.42	9.72	143.92	302.00	0.66
7	166.08	11.45	147.06	328.00	0.47
8	167.10	10.17	150.11	308.00	0.60
9	165.94	10.35	148.76	308.00	0.53

Var 1, 4, 7 – flour from the Scorpion variety, var. 2, 5, 8 – flour from the Rosso variety, var. 3, 6, 9- flour from the Karkulka variety, var. 4, 5, 6– addition of 10% of hemp grits, 7, 8, 9 addition of 5% of hemp protein.

The highest yield of the dough was observed when using the Rosso wheat variety, but there are not considerable differences among the other types of flour. It could be considered positive that the addition of hemp protein increased the yield in all variants. The increase was from 0.7 to 1.5% more than those, which contained only flour. On the other hand, the addition of hemp semolina decreased significantly the yield of the dough. Also a high proportion of oil in puffed cornmeal snacks (Pejcz et al. 2015) limited the water intake of the dough and reduced its yield (Table 2). It is also important to take into consideration that the yield of the dough depends on the water loss during the baking. According to Dvořáková et al. (2005), these losses are in the range between 15 ± 5%. This was confirmed by this research. The lowest loss during the baking was determined in the recipe that contained Karkulka flour and hemp grits (var. 6). Furthermore, the highest water loss was detected in the var. 3, where the recipe had only wheat flour. The highest yield of bread was obtained in those varieties which contained hemp protein and used all kinds of flours (var. 7 to 9). The flour from the wheat variety Scorpion, scored the highest specific volume. The bakery products made from this type of flour score the lowest convexity of the pastries, often characterized by the ratio number or index gluten behavior. The highest ratio number was determined by the recipe which contained Rosso wheat flour and hemp grits.

Sensory analysis

The influence of the recipe was analyzed during the sensory evaluation of the products. The most important parameter, in terms of sensory evaluation, is the shape of the product. Often it decides the consumer's interest in the product, which is mainly influenced by the convexity of the baked good and its volume. In some of the measurements carried out in this study, this characteristic was shown through the scores. The best score for the shape of the product was achieved by the sample 3 and 5 (Table 3), although the sample 5 scored the highest ratio number.

Moreover, the color of the crust depends on the composition of the dough and on the baking mode. The non-enzymatic browning, which is the result of a chemical reaction between amino acids and reduced sugars, followed by the caramelization of the sugars and the color of the ingredients, could significantly influence this characteristic. The typical color was only found, where the dough was made only of flour or it contained hemp grits. The addition of hemp ingredients, especially hemp protein, has a negative influence on the color. Further, the worse evaluated color was on those samples which contained a high amount of hemp flour as well as in the studies of Apostol et al. (2015) and Pejcz et al. (2015), who mentions that the color of the crust was worse on those samples which contain hemp as ingredient.

Also, the formulation has a significant influence on the aroma ($p > 0.95$) of the product. In this study was found that the Scorpion wheat flour has a significant effect t on the aroma. In general, the additions of hemp ingredients have a negative effect on the aroma of the products.

The springiness of the crumb was evaluated evidently ($p > 0.95$) as the best (7.9) for those samples which used flour from the Scorpion wheat variety. Moreover, the addition of hemp ingredients has a bad effect on this characteristic independently on the type of flour. A similar conclusion was reached by Apostol et al (2015). Mainly, the technical parameters of the used wheat flour were manifested. The results (of this study) are related with the specific volume of the bread. The highest value was obtained in those variants which contained milled grain wheat from the Scorpion variety (Table 2).

The color of the crumb was highest scored (8.2) for the products with wheat flour from Scorpion variety (var. 1). It was not possible then the influence of the pigments, located at the aleurone (Martínek et al. 2012).

A positive aspect to consider is that there were not found any significant differences related with the consumption of the product through the easiness of biting in the mouth. Similar results were obtained on the consistency and moisture of the crumb (Table 3).

Figure 1 Appearance and shape of the best rated product

Variety 1 *Variety 2*

Furthermore, the taste might be affected by the technology and the formulation of the product. The influence of the technology is related with the baking process or the damage of the raw materials; which could also manifest as a weak flavor (Příhoda 2012). The best score in the taste was achieved with those variants where only flour was used. The addition of hemp grits has influence on the taste more than the hemp protein. The best overall impression was achieved by variants 1 and 2 (Figure 1). The added hemp ingredient was evaluated as the worse on the overall impression. The negative influence of the added hemp ingredients on pastries were mentioned by Apostol et al. (2015), who recommend as ideal recipe that, from the sensory analysis perspective, the addition of 5% of hemp flour. Similar results were obtained by Pejcz et al. (2015).

Table 3 The results of the sensory analysis

Var.	Form	Color - crust	Aroma	Springiness - crumb	Color - crust	Easiness of biting	Sensation after chewing	Consist -ency	Moisture - crumb	Taste	Overall impression
1	6.1^{a}	6.5^{a}	7.8^{b}	7.9^{c}	8.2^{a}	6.8^{a}	6.6^{a}	6.6^{a}	7.0^{a}	7.5^{b}	7.5^{a}
2	7.2^{ab}	7.5^{a}	6.5^{ab}	7.1^{bc}	7.7^{a}	6.9^{a}	6.8^{a}	6.4^{a}	7.2^{a}	7.5^{b}	7.5^{a}
3	7.9^{b}	7.0^{a}	6.2^{ab}	6.6^{abc}	7.7^{a}	6.6^{a}	6.8^{a}	6.7^{a}	7.7^{a}	6.8^{ab}	7.1^{a}
4	6.7^{ab}	7.4^{a}	6.2^{ab}	7.0^{bc}	8.1^{a}	6.4^{a}	5.9^{a}	6.4^{a}	7.2^{a}	5.6^{a}	6.4^{a}
5	7.8^{b}	6.7^{a}	5.6^{ab}	6.6^{abc}	7.8^{a}	7.3^{a}	6.3^{a}	6.9	7.9^{a}	5.9^{ab}	6.4^{a}
6	7.5^{a}	6.6^{a}	5.5^{ab}	5.9^{ab}	7.9^{a}	7.0^{a}	6.1^{a}	6.0^{a}	7.5^{a}	6.4^{ab}	6.9^{a}
7	6.2^{ab}	5.9^{a}	5.9^{ab}	6.8^{bc}	7.2^{a}	7.8^{a}	6.6^{a}	6.5^{a}	7.8^{a}	6.8^{ab}	6.3^{a}
8	7.3^{ab}	6.4^{a}	5.9^{ab}	6.2^{ab}	7.3^{a}	7.6^{a}	7.1^{a}	6.5^{a}	7.7^{a}	6.8^{ab}	6.9^{a}
9	7.0^{ab}	5.7^{a}	6.2^{ab}	5.1^{a}	7.1^{a}	6.6	6.4^{a}	5.6^{a}	7.3^{a}	6.7^{ab}	6.7^{a}

Note: The average of the samples it does not have a significant different (p > 0.95), if there is the same superscript/

CONCLUSION

The results of the research and the sensory evaluation of the bakery products showed that the application of flour from colored wheat might be a possible to improve the bakery products nutritional quality. By the use of hemp products in the recipe, they contribute to increase the diversity of bakery products. Along this study it was possible to confirm the value of the purple wheat (Karkulka and Rosso varieties) and blue aleurone (Scorpion) as flour. Also, the use of these wheat varieties depends on their baking quality. Because of this, it is possible to achieve satisfactory values in all the studied parameters. Through this research it could be concluded that the best flour is that which is obtained from Scorpion variety. Also, it was positively evaluated the use of cannabis products, which increased the nutritional value of the bread. On average, the lowest baking loss was achieved after the addition of hemp protein. Also, the addition of grits promoted the convexity of the baked goods. The flour obtained by the milling of colored wheat grains, enriched with an acceptable quantity of cannabis products, contributes to the expansion of functional foods in bakery products.

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EFFECT OF STORAGE REGIME ON TEXTURE AND OTHER SENSORY PROPERTIES OF CHOCOLATE

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Abstract: As part of the experiment, we have studied the effect of different storage conditions chocolate in a normal household. Freshly produced chocolate was stored in five temperature regimes, namely frozen -18 ºC, 6 °C, 12 °C, 20 °C, and 30 °C. After one month of storage, the samples were removed from individual regimes and immediately subjected to sensory analysis supplemented by the evaluation of textural properties on the TIRA test device. The significantly lowest values ($p > 0.95$) of hardness were determined for samples stored at 30 °C (2.5–5.5 N). The greatest hardness of the chocolate had frozen samples (52.2–75.9 N). When stored at temperatures 6 °C or 12 °C, the products did not significantly differ in terms of hardness. At the room temperature of 20 °C, conclusive softening already took place (19.3–29.6 N). Higher storage temperature (30 ºC) significantly ($p > 0.95$) reduced the colour evaluation, while glossiness was the significantly better ($p > 0.95$) than samples at 30 °C, 6 °C and frozen, but did not different compared to 12 °C. Higher temperatures (30 ºC) deteriorated breakage and aroma. Temperature of 30 °C significantly ($p > 0.95$) decreased the hardness at the time of consumption and increased the stickiness of chocolate.

Key Words: chocolate, storage, changes in texture, sensory properties

INTRODUCTION

Quality chocolate has a completely homogeneous structure, fine melting flavour, hard consistency, shell-like fracture, and shiny surface. Quality of chocolate products is affected by the entire manufacturing process, recipe, raw materials used, and storage conditions. All these aspects act on the rheological, physical, and sensory properties, and thus determine the final quality of the products (Afoakwa 2010).

In chocolate processing, for obtaining high quality products an important role is played by the composition of matter and especially the crystallization of cocoa butter. For chocolate production, the desired crystalline form is the V (β) which dominates in a well-tempered chocolate (Quast et al. 2013, Fernandes et al. 2013). The crystallization conditions determine not only the crystal form of cocoa butter and arrangement of the crystalline lattice, but are the main factors that determine the rheological and textural properties of chocolate (Afoakwa et al. 2008).

Quality ingredients and properly implemented tempering do indeed significantly affect the sensory and textural properties of chocolate. However, also crucial are the conditions in which we store it, particularly the temperature regime, and also the conditions during consumption.

One of the important sensory characteristics is the hardness of chocolate. This is influenced not only by the recipe composition and tempering technology (Afoakwa 2010), but also by the temperature conditions during storage. And these conditions can significantly influence the overall perception during its consumption. Unsuitability of storage temperature, especially high temperature is reflected in soft texture and the appearance of fat blooms (Debaste et al. 2008, Afoakwa et al. 2007).

High temperatures during storage of chocolate products promote migration of fat through the matrix of chocolate particles and consequently lead to its recrystallization on the surface. Dull surface appearance due to blooming occurs due to diffusion of light by agglomerates of fat crystals that protrude from the surface of chocolate (Aguilera et al. 2004, Lohman and Hartel 1994). Other defects associated

with fat migration include softening of chocolate layers, hardening of fillings in filled chocolates and desserts, as well as an overall sensory deterioration of products (Svanberg et al. 2011).

In our experiment, we have focused on monitoring the impact of temperature of storage and temperature during consumption of chocolate on texture and other sensory properties.

MATERIALS AND METHODS

Materials

We have analysed the chocolate product *Boci* - milk chocolate (cocoa solids 35%) with pieces of biscuit (10%) and apricots (3.5%).

Samples were stored for one month in five temperature regimes, namely frozen (-18 ºC), 6 °C, 12 °C, 20 °C, and 30 °C. Storage temperatures were selected so as to mimic normal procedures of storing in homes. Here the consumer has the option to keep the chocolate in a freezer, in a refrigerator at t = 6 °C to 12 °C, at room temperature of 20 °C, or nonstandard elevated temperature such as in overheated room, orcontact with a source heat, such as sunlight. For the experiment, we have used refrigerators with freezers with temperature control, air-conditioned warehouses, and a thermostat. At the beginning of the experiment after 30 days storage, we have carried out physical analysis of the product texture by a texturometer, and a sensory analysis.

Texture analysis

For texture measurements, we have used universal instrument intended for measurement of physical characteristics, namely the TIRA test (type 27025) from Germany.To test chocolate products we have used a penetration test with a probe in the shape of a knife.The selected criteria for penetration tests of chocolate products via a pressure test included the length of knife blade 10 mm, $v_1 = 40$ mm/min (test speed).

Sensory analysis

Immediately after the removal from the respective storage regime, we have carried out sensory analysis using experienced sensory evaluators (n = 10). The parameters evaluated in chocolate included colour, odour, glossiness, fat bloom on the top and bottom sides of the product, fracture, hardness on bite, consistency, homogeneity, adhesion - stickiness in mouth, melting in mouth, flavour, and finally the overall impression. For graphic representation of results, we have used unstructured scales, 100 mm long, where 1 mm scale corresponded to 1 point.

Statistical data processing

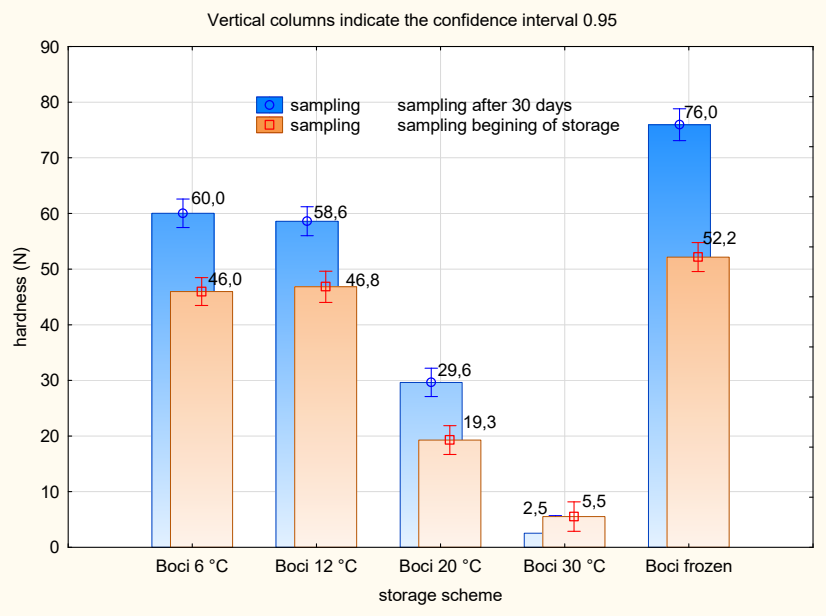
The collected data were processed using MS EXCEL. Statistical analysis of data obtained was performed via statistical program STATISTICA Version 12–ANOVA, namely analysis of variance with interactions, tested at a significance level of P = 0.05.

RESULTS AND DISCUSSION

Texture analysis

Textural characteristics of the stored products are evaluated as a force in Newtons that must be applied to get the penetration body through the chocolate sample. The measured quantity then characterizes the strength or hardness of the product. The results in Figure 1 clearly show the influence of the temperature regime used in storage on the hardness of analysed chocolate samples. Significantly lowest values (p = 0.95) of hardness were determined for samples stored at 30 °C. The frozen samples had the greatest hardness. When stored at temperatures of 6 °C to 12 °C, the samples were not significantly different.The room temperature of 20 °C has already caused a significantsoftening. Except for the highest storage temperature, at all other storage temperature regimes, the storage time positively and significantly affected their hardness. We can evaluate the long-term storage of chocolate at temperatures above 20 °C as critical. Similar experiences were presented by Ali et al. (2001).

Figure 1 Effect of temperature on the hardness of stored products



Legend: Conclusive of differences is defined error bars

Rating of sensory properties

Measuring of colour changes

Colour, glossiness, shape of chocolate, and its surface texture are among the basic features characterizing the look and decisive influence on consumer interest about a given product (Simonot and Elias 2002). Colour perception may be largely influenced. These attributes result from complex interactions of the incident light, optical properties, and human perception (Afoakwa 2010). In the case of our assessment, colour received a significantly worse scores ($p > 0.95$) in products stored at 30 °C. Higher storage temperatures caused a lighter product colour (Table 1).

Table 1 Results of sensory analysis after 30 days storage

Storage regime	Colour	Glossiness	Fat bloom upper side	Fat bloom lower side	Fracture	Aroma
Frozen	6.9^{b}	3.7^{a}	9.3^{a}	9.6^{b}	7.9^{b}	8.2^{a}
6 °C	7.0^{b}	4.1^{a}	9.6^{a}	8.31^{ab}	7.9^{b}	8.3^{a}
12 °C	7.6^{b}	7.1^{bc}	9.5^{a}	7.56^{a}	7.8^{b}	8.3^{a}
20 °C	7.3^{b}	8.5^{cd}	9.6^{a}	7.87^{a}	7.2^{ab}	7.9^{a}
30 °C	4.9^{a}	5.4^{ab}	9.5^{a}	7.2^{a}	5.5^{a}	7.5^{a}

Legend: Averages of the individual variants are not significantly different ($p > 0.95$), if they have an identical superscript.

Table 1 Results of sensory analysis after 30 days storage (continued)

Storage regime	Hardness in bite	Consistency of fracture	Stickiness on palate	Melting in mouth	Taste	Overall impression
Frozen	7.3^{b}	6.5^{a}	7.3^{b}	7.1^{a}	6.3^{a}	6.1^{a}
6 °C	6.9^{b}	6.7^{a}	7.2^{b}	7.2^{a}	6.7^{a}	6.3^{a}
12 °C	7.0^{b}	6.7^{a}	6.9^{b}	7.0^{a}	6.5^{a}	6.0^{a}
20 °C	6.0^{b}	6.7^{a}	7.0^{b}	6.9^{a}	6.5^{a}	6.1^{a}
30 °C	2.5^{a}	6.2^{a}	5.1^{a}	5.7^{a}	5.9^{a}	4.8^{a}

Legend: Averages of the individual variants are not significantly different ($p > 0.95$), if they have an identical superscript.

Similarly Briones and Aguilera (2005) indicate that chocolate products that are stored at higher temperatures experience yellowing, which is associated with the development of fat bloom. We can therefore assume that there was influence of fat bloom. Aguilera et al. (2004) stated that the bloom on

chocolate is produced by action of high temperatures and includes a gradual discoloration, loss of glossiness, and causes grey surface appearance of chocolate. In their study Bui and Coad (2014) also recorded colour changes among the experimental and control samples during storage at 30 °C. They found out that during storage at higher temperatures, there was a lighting of products increasing with the storage time.

More results support it as well. Higher temperature deviations from the room temperature of 20 °C also adversely affect ($p > 0.95$) the product glossiness. In chocolate stored at 30 °C, the glossiness was limited due to colour change, and its lightening, which as mentioned above, was probably due to fat bloom. As reported by Ali et al. (2001), migration of fats with lower melting point towards the surface of chocolate may at higher temperatures, such as 30 °C, occur prominently. Conversely, temperatures around 18 °C can be considered non-problematic from the point of view of fat bloom and not threatening for glossiness. The glossiness of chocolate at 20 °C was the best from all temperatures, but it was not significantly different from the temperature of 6 °C and 12 °C. Freezing of the product or storing it at 6 °C or 12 °C ensures the preservation of its quality and freshness, as stated by Machálková et al. (2015). The problem occurs when the product is consumed immediately upon removal from storage and is not subjected to stabilization at 20 °C ± 2 °C, as stated by Afoakwa (2010). Due to micro condensation, the product loses its glossiness. This result was statistically significant ($p > 0.95$) in both storage regimes (frozen, 6 °C) . Colder products were better evaluates in assessing of fat bloom lower side. Samples at -18 °C had significantly better evaluation of fat bloom of lower side, but not statistically different from samples at 6°C. The dull fat bloom after one month storage was probably helped by the fact that the chocolate was without filling. There could thus not be a significant migration of fat from the filling to the surface of the product. Differences in the triacylglycerol composition between the soft fillings, such as nut paste or peanut butter, and the chocolate, leadto the migration of fat towards the product surface. This manifests itself in an undesirable softening of the chocolate layer by diluting the solid fat content (cocoa butter), hardening of the filling, and recrystallization of the cocoa butter on the chocolate surface, which is reflected by visible fat bloom (Nöbel et al. 2009).

Although the fat bloom was not observed on the upper side of the products, the lower side had noticeable statistically differences ($p > 0.95$). The worst state was determined in the warmest storage regime. The dull surface appearance caused by fat bloom occurs due to diffusion of light by agglomerates of fat crystals that protrude from the surface of chocolate (Lohman and Hartel 1994).

Higher storage temperature (30 °C) than at lower temperatures (12 °C, 6 °C, -18 °C) significantly affected ($p > 0.95$) the fracture of products. Warmer products were soft and this negatively reflected on their textural characteristics. Products stored at 12 °C and below, were better ranked than chocolate stored at 30 °C (Table 1). They had even better texture properties that were also visible on a shell-like fracture which is typical for high-quality chocolate. Likewise, the smell of these products was more intense, which was reflected on the favourable outcome of sensory evaluation.

The results by Mexis et al. (2010) suggest that changes in texture, accompanied by a change in colour due to fat bloom, indicate that there was also softening of the chocolate product.

This was also reflected in our experiments not only in assessing fracture, but also in evaluating hardness of the product that is best evaluated “on bite”. The storage temperature of 30 °C had a statistically significant ($p > 0.95$) negative impact on this parameter. Chocolate was characterized by minimal resistance to pressure exerted upon its chewingin the mouth. This condition very closely corresponded with the results assessed by texturometer (TIRA test). Consistency on fracture was not significantly affected. Products stored at high temperatures have been rated lower than those stored at lower temperatures.

Storage temperature 30 °C has a statistically significant ($p > 0.95$) effect on stickiness of products, worsened their melting in mouth, and negatively affected the taste as well. These factors were negatively reflected in the overall evaluation of products, which was unfavourable for storage regime with the highest temperature (30 °C). We can consider as positive that the other temperature regimes did not manifest themselves significantly ($p > 0.95$) when assessing the overall impression.

CONCLUSIONS

Storage conditions for chocolate can be very diverse. Chocolate is not always stored in satisfactory conditions. Due to its composition, what can most affect its quality are temperature and humidity of the environment. In our observation, we have focused on the influence of storage temperature on its textural and sensory characteristics after being removed from various types of storage regimes with the immediate assessment of the sensory quality. The produced results have shown that the storage temperature is very important for the sensory quality of chocolate. The most suitable temperature for the subsequent immediate consumption seems to be 12 °C, or the room temperature of 20 °C. Higher temperatures adversely affect both the texture and other sensory properties of chocolate. Higher temperature promotes change in the colour of chocolate and deteriorates its glossiness. This largely corresponds with the formation of fat bloom, and the chocolate becomes soft and sticky. For chocolate that is frozen or stored in the temperature regime of 6 °C, which corresponds with conventional refrigerators, it is necessary to wait prior to consumption until temperatures equalize and if possible not to allow occurrence of condensation of the product. Especially glossiness of chocolate in immediate consumption is negatively affected.

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DETERMINATION OF HEAVY METALS IN FISH PRODUCTS

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Abstract: Cadmium, lead and mercury contents in fish were studied. Fresh and frozen fish from 17 FAO localities were bought in Czech markets. Atomic absorption spectrometry technique was used for determination of cadmium, lead and mercury concentration. Ten samples exceeded the maximum permissible limit for mercury (0.5 mg/kg or 1 mg/kg for selected fish species) and three fish samples exceeded the maximum limit for cadmium (0.05 mg/kg) set by Commission Regulation (EU) No1881/2006. The limit of lead concentration (0.3 mg/kg) was not exceeded in any fish sample. This study shows that fish samples of marlin (*Tetrapturus albidus*) and swordfish (*Xiphias gladius*) are one of the most contaminated fish which can pose a great risk for human health after consumption. Because mercury and cadmium contents in some samples were higher than maximum limits recommended by FAO/WHO, our research led to withdraw of some batches of the fish from the Czech markets.

Key Words: mercury, cadmium, lead, fish

INTRODUCTION

Heavy metals such as cadmium, lead and mercury are significant environmental contaminants. In the Czech Republic, as in other developed parts of the world, there is an increased risk of heavy metals (Hg, Cd, Pb etc.) in the environment. Currently, the heavy metal compounds released into the aquatic ecosystem mainly from anthropogenic sources, i.e. as a result of industrial, agricultural and mining activities (CCME 2000, Stancheva et al. 2013). The contamination of the environment by heavy metals reduces the hygienic quality of foodstuffs (freshwater and especially marine species of fish) obtained from aquatic organisms (Atobatele 2014).

Maximum levels for cadmium, lead and mercury in foodstuffs are in the Czech Republic set by the framework EU legislation, namely by the Commission Regulation No1881/2006. A tolerable Weekly Intake (TWI) established by the international scientific committee FAO/WHO for mercury, cadmium, and lead is 1.3 μg/kg Hg body weight (b. w.), 7 μg/kg Cd b. w. and 25 μg/kg Pb b. w., respectively (Commission Regulation No1881/2006).

Because a healthy lifestyle and unconventional food is currently interesting for many people, a lot of specialty stores devoted to the sale of seafood are established in landlocked states. The same situation can be observed in the Czech Republic. In recent years, there is an expansion of shops selling fresh seafood. This led us to monitoring of the trade network in the Czech Republic, and particularly to assess of the risks associated with the consumption of contaminated fish.

MATERIAL AND METHODS

Collection of samples

From September 2015 to June 2016 33 species of fish samples from 17 FAO localities were purchased in Brno City (Czech Republic) markets. A minimum of three samples per batch were

purchased. Total amount of bought and analysed fish samples was 159, contaminated fish species were purchased several times from the other batches for monitoring of contamination. Samples were transported to the laboratory and stored in -20 °C until the time of analysis. Frozen samples were slowly thawed and 0.1–0.6 g were weighed for individual analysis. Analysed fish species are presented in Table 1.

Table 1 Overview of fish species subjected to analysis

English name	Latin name	English name	Latin name
Alaska Pollock	Theragra chalcogramma	Golden redfish	Sebastes marinus
Angler	Lophius piscatorius	Greater argentine	Argentina silus
Argentine hake	Merluccius hubbsi	Greenland halibut	Reinhardtius hippoglossoides
Asian sea bass	Lates calcarifer	Indo-Pacific sailfish	Istiophorus platypterus
Atlantic herring	Clupea harengus	Nile perch	Lates niloticus
Atlantic mackerel	Scomber scombrus	Nile tilapia	Oreochromis niloticus
Atlantic salmon	Salmo salar	Pink salmon	Oncorhynchu sgorbuscha
Blue shark	Prionace glauca	Ray	Raja
Bogue	Boops boops	Red mullet	Mullus barbatus
Dusky grouper	Epinephelus marginatus	Salmon trout	Oncorhynchus mykiss
Escolar	Lepidocybium flavobrunneum	Senegalese hake	Merluccius senegalensis
European anchovy	Engraulis encrasicolus	South Pacific hake	Merluccius Gayi
European pilchard	Sardina pilchardus	Swordfish	Xiphias gladius
European seabass	Dicentrarchus labrax	Tub gurnard	Triglia lucerna
Flatfish	Pleuronectiformes	White marlin	Tetrapturus albidus
Flathead grey mullet	Mugil cephalus	Yellowfin tuna	Thunnus albacares
Gilthead bream	Sparus aurata		

Determination of cadmium and lead in fish samples

A microwave digestion (Ethos ONE, Milestone, Italy) was used for decomposition of fish samples. The 10 ml HNO_3 (1 : 1) was added to 600 ± 0.1 mg of fresh fish muscle tissue and it was decomposed in the microwave oven at 210 °C (1000 W) for 30 min.

Electrothermal atomic absorption spectrometer (Series AA 280, Agilent Technologies, United States, equipped with Zeeman correction) was used under the optimized conditions for determination of Cd (228.8 nm) and Pb (283.3 nm) in mineralized fish samples. The standards 1 g/l of Cd and Pb (Merck, Germany) were used for calibration. Temperature of pyrolysis was 500 °C for Cd and 1000 °C for Pb, atomisation temperature was 1800 °C for Cd and 2100 °C for Pb. $Pd/Mg(NO_3)_2$ was used as modifier. The limits of detection (LOD) for cadmium and lead determination were 0.12 and 3.11 μg/kg, respectively.

Determination of total mercury content in fish samples

An AMA 254 advanced mercury analyzer (Altec, Prague, Czech Republic) was used for the determination of total Hg concentration by direct analysis of fish samples. 0.1 g of fish sample was inserted into pre-cleaned combustion boats and loaded into the AMA 254 analyser. During analysis the sample was dried at 120 °C for 90 s and thermally decomposed at 550 °C for 180 s under an oxygen flow. Selectively trapped mercury was subsequently released from the gold amalgamator by a brief heat-up and finally quantified (measuring cycle, 60 s) as Hg^0 by the cold-vapor AAS technique at 253.65 nm. The limit of detection (LOD) for mercury determination was 0.1 μg/kg.

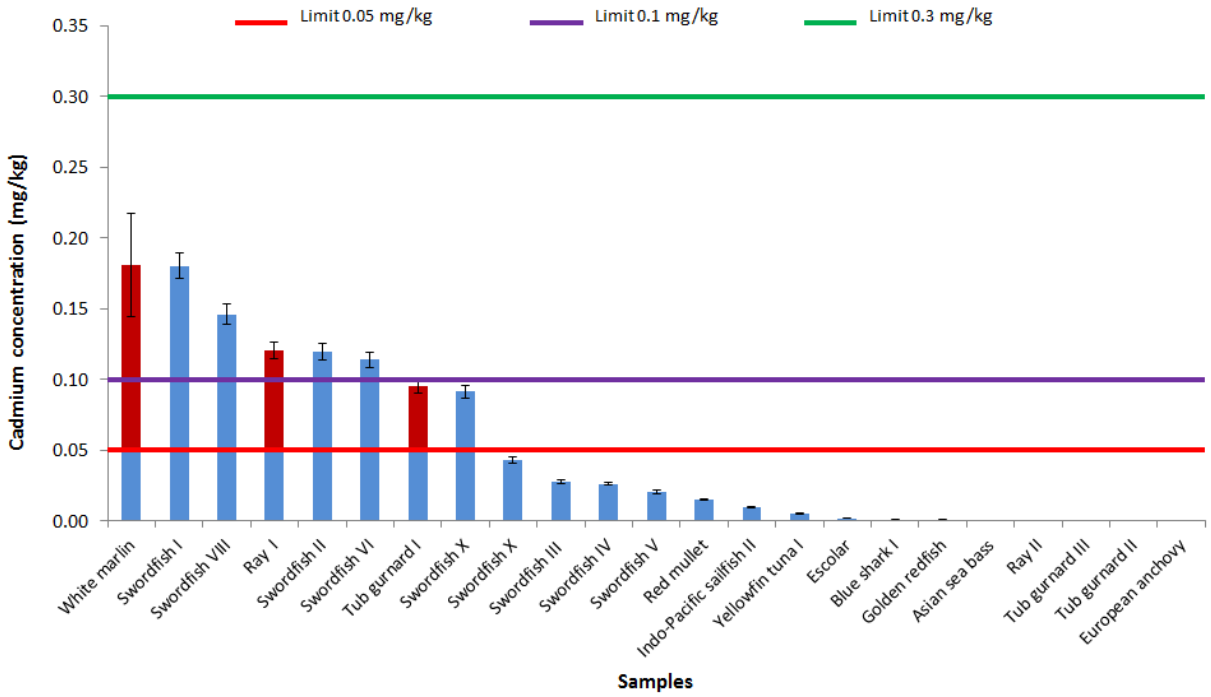
RESULTS AND DISCUSSION

Content of cadmium in fish samples

Cadmium concentration in analysed fish muscle tissues and cadmium limits (Commission Regulation (EU) No1881/2006) are shown in Figure 1. Three limits for cadmium are highlighted in this

figure for defined fish species. Limit 0.3 mg/kg is set only for swordfish, limit 0.1 mg/kg is for 11 selected fish species according to this Regulation and limit 0.05 mg/kg is set for all other fish species.

Figure 1 Cadmium content in fish samples



Cadmium was detected only in the samples mentioned in Figure 1. The cadmium concentration in the other fish samples was below LOD (0.12 μg/kg). The highest concentration of cadmium (0.1809 ± 0.0362 mg/kg) was determined in the muscle tissue of white marlin (*Tetrapturus albidus*).

Only three fish samples exceeded the cadmium limit – tub gurnard (0.0950 ± 0.0047 mg/kg), ray (0.1203 ± 0.0060 mg/kg) and white marlin (0.1809 ± 0.0362 mg/kg). After repeated purchase of fish, the limit was not exceeded as is shown in Figure 1 (Ray II, Tub gurnard II and III). According to the international scientific committee FAO/WHO calculation of risk for human health, 70 kg man can eat 27 portions (100 g) of analysed white marlin for reaching cadmium Tolerable Weekly Intake (TWI).

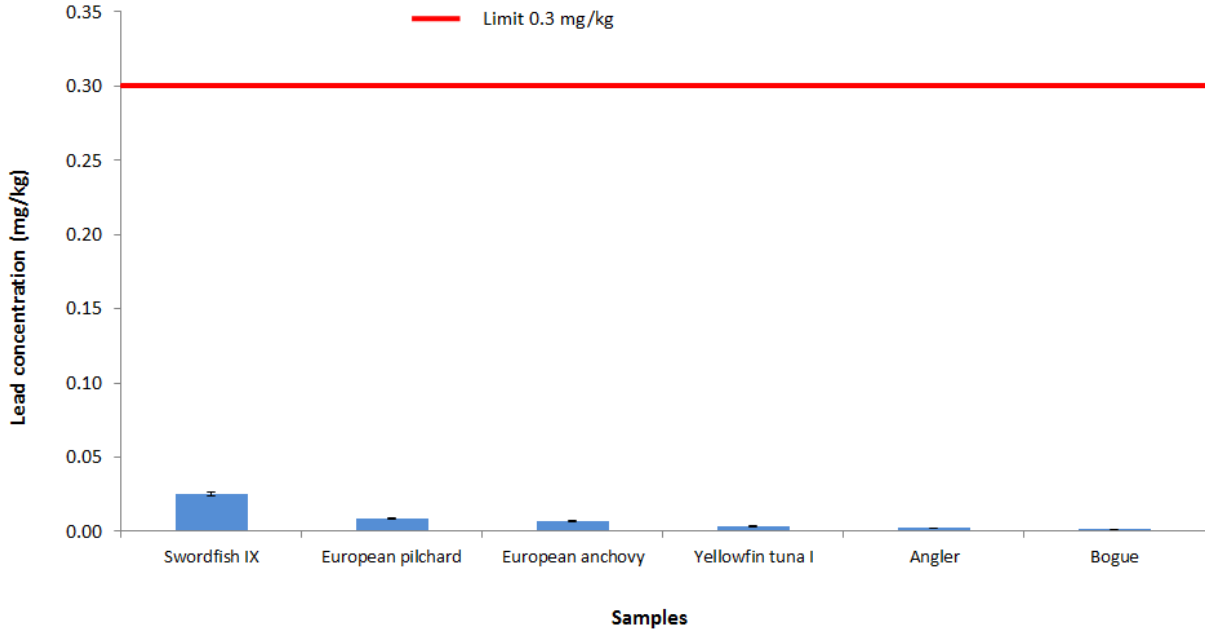
Jinadasa et al. (2014) determined the highest cadmium content in swordfish (0.087 mg/kg), which belongs to a group of billfish (together with marlin and sailfish) (Rodrigues and Amorim 2016). Although Indo-Pacific sailfish (*Istiophorus platypterus*) belongs to billfish, but there we didn't detect an over limit content of cadmium in our samples. The same situation was observed in the case of mercury content in Indo-pacific sailfish.

Content of lead in fish samples

The concentration of lead in analysed fish samples was very low (see Figure 2). The highest concentration of lead (0.0250 ± 0.0013 mg/kg) was determined in the muscle tissue of swordfish (*Xiphias gladius*). 70 kg man can eat 700 portions (100 g) of analysed swordfish for reaching lead Tolerable Weekly Intake (TWI). Only samples with lead concentration higher than LOD (3.11 μg/kg) are presented in Figure 2.

Fish species showed lower lead contents compared to Cd and total Hg. Zaza et al. (2015) reported higher lead content in the European seabass (0.1670 ± 0.0580 mg/kg), but in our samples of this fish, concentration of lead was below LOD. Squadrone et al. (2016) reached the same findings as we obtained.

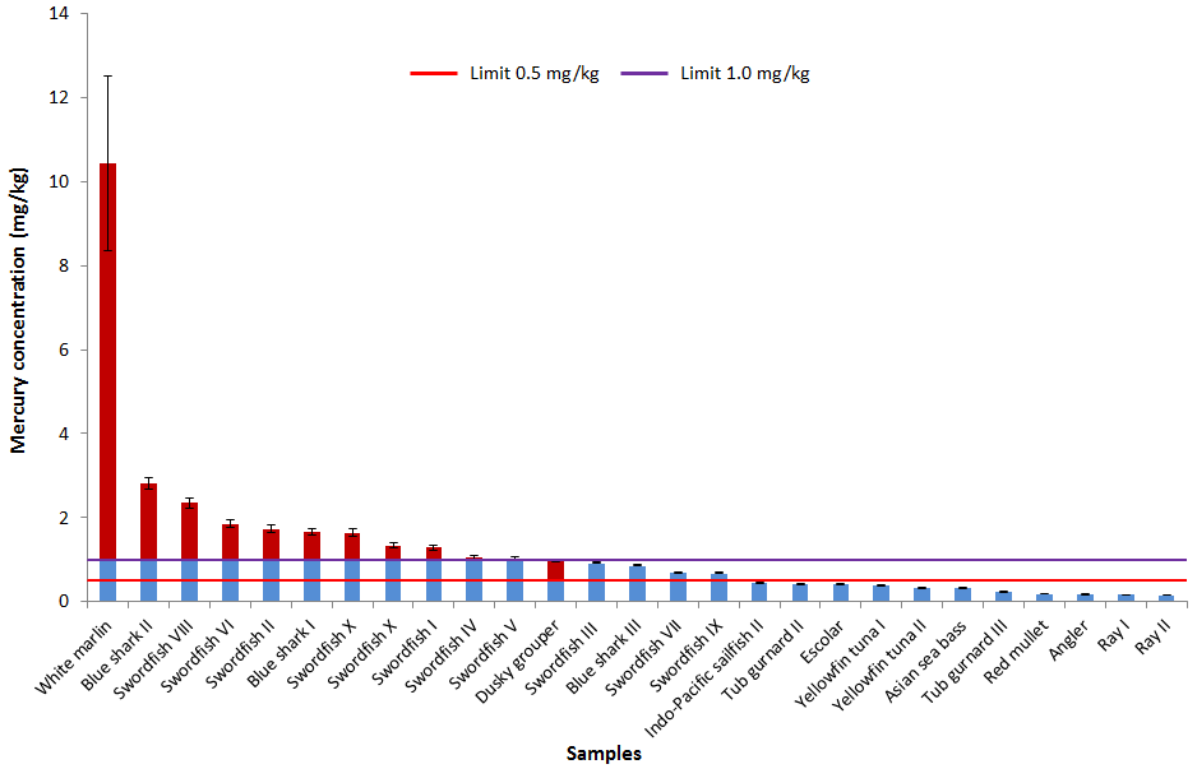
Figure 2 Lead content in fish samples



Content of mercury in fish samples

The Figure 3 shows the total mercury concentrations in muscle tissue of analysed fish samples.

Figure 3 Mercury content in fish samples



Mercury was detected in all analysed fish samples. Only samples with mercury content higher than 0.15 mg/kg are presented in Figure 2. The highest concentration of the mercury (10.42 ± 2.08 mg/kg) was determined in the muscle tissue of white marlin (*Tetrapturus albidus*), while the lowest concentration (0.0031 ± 0.0002 mg/kg) was determined in Nile tilapia (*Oreochromis niloticus*). TWI is reached by eating of only 0.09 portion (the portion is 100 g) of analysed white marlin by 70 kg man. This is only 9 g of the fish weekly.

Ten fish samples exceeded the maximum permissible limit for mercury set by Commission Regulation (EU) No1881/2006. The sample of dusky grouper (*Epinephelus marginatus*) exceeded the lower mercury limit (0.5 mg/kg), while the higher limit for selected fish species (1.0 mg/kg) was exceeded in the case of swordfish (*Xiphias gladius*), blue shark (*Prionace glauca*) and white marlin (*Tetrapturus albidus*). Bergés-Tiznado et al. (2015) reported similar results (0.5600 ± 0.0400 mg Hg/kg) for sailfish.

Rodriques and Amorim (2016) published a review about mercury levels in marlin and swordfish. In this review, Shomura and Craig (1972) presented that the highest content of mercury were found in marlin, specifically from the area of Hawaii, which belongs to the fishing area FAO 77 (Pacific, Eastern Central). Our samples of marlin came from the fishing area FAO 87 (Pacific, Southeast) which is in close proximity to FAO 77.

CONCLUSION

As a consequence of shops selling fresh seafood expansion, the ordinary consumers can easily buy unusual fish species (for our geographical area) and they are often unaware of the risks associated with consumption of these fish. Therefore thorough monitoring of heavy metals concentrations in fish which are imported from abroad and are widely available on the market of landlocked countries is necessary.

Since 2004, the European system RASFF (Rapid Alert System for Food and Feed) has recorded in the Czech Republic 14 cases of above-limit content of heavy metals in fish where six cases were related to Hg, six cases to Cd and two cases to both elements. Two reports have already been submitted by the Czech Republic in 2016. Our research has led to reporting of 8 cases of above-limit content of heavy metals in fish, which were submitted into the European system RASFF The last case which we reported was over the limit of cadmium content in swordfish submitted in February 2016, which was followed by the withdrawal from the markets in EU.

As our study confirmed, predatory fish species (such as marlin, shark or swordfish), which are on the top of the food chain, pose a greater risk for consumer due to the cumulative property of heavy metals in organisms.

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THE INFLUENCE OF ASCORBIC ACID ON SENSORY AND ANALYTICAL PARAMETERS OF WHITE WINES

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Abstract: Ascorbic acid is used as a supplement to sulphur dioxide in winemaking. Its main advantage lies in quick oxidation under conditions of white wine. This fact is based on the ability of ascorbic acid to protect the oxidizable components of wine, including phenolic and taste substances. This research was focused on the effect of ascorbic acid on sensory and analytical parameters of white wines during production technology. Our study compares various doses of ascorbic acid (10 g/hl and 15 g/hl) and sulphur dioxide (40 mg/l), which were added to the mash of two varieties: Rhine Riesling and Grüner Veltliner. The experiment examines the amount of reductones, the amount of free and total sulphur dioxide and the effect on analytical and sensory component of resulting wines. The values were determined using titration method with standard solution of iodine. On the basis of the results obtained we can conclude that the best option appears to be the variant where ascorbic acid is combined with sulphur dioxide. On the contrary, the variant using only ascorbic acid is completely inappropriate for production of white wines.

Key Words: ascorbic acid, sulphur dioxide, oxidation, mash, must

INTRODUCTION

Wine is a product that has belonged to human life for centuries. Its production has began to modernize massively in the course of time. But there still is one substance which is needed in the production of wine - sulphur dioxide. The main role of sulphur dioxide as an antioxidant is to prevent formation of oxidation products. These products are formed by metal-mediated oxidation of phenolic compounds with molecular oxygen. (Danilewicz 2011). Higher amount, however, negatively affects human health. Therefore new substances that are capable of substituting sulphur dioxide have been searched for these days. Ascorbic acid that is vitamin C is one of such substances. Ascorbic acid is used as a supplement to sulphur dioxide in winemaking. Quick oxidation under conditions of white wine belongs to its main advantage. Ascorbic acid is able to protect the oxidizable components of wine, including phenolic and flavour substances (Deutsch 1997). When ascorbic acid is not present, oxygen enters wine and subsequently reacts with metal ions. This results in formation of hydrogen peroxide and ortho-chinon compounds (Buetnner et al. 2009).

Our aim is, however, making wine without faults and diseases. Sulphur dioxide, often abbreviated as sulphite, is the most important chemical substance that prevents oxidation of wine. In addition to the antioxidant effects, it also has antimicrobial effects. However, the negative impact of SO_2 on human health has led to restrictions in winemaking by the World Health Organization (WHO) and the International Organization of Vine and Wine (OIV) (Li et al. 2008). Apart from that, its excessive use can lead to deterioration of qualitative parameters in musts and wines. Ascorbic acid is sparsely used as antioxidant in winemaking, especially in white wines. Other more extensive studies confirm that ascorbic acid can have, including antioxidant properties, as well for-oxidizing properties. That situation depends on conditions and amount of ascorbic acid in wine (Barill et al. 2016). A lot of recent researches suggest that if ascorbic acid is used in combination with sulphur dioxide, it can then lead to faster consumption of SO_2 and subsequent oxidation of the product (Bradshaw et al. 2001). Therefore, it is necessary to find a balance in the use of sulphur dioxide and ascorbic acid. Using ascorbic acid directly into the grape mash and must appears to be one of several possibilities to solve the problem.

MATERIAL AND METHODS

Varieties selected: The experiment that is part of this thesis was carried out in the Znojmo wine region. That is why the varieties were chosen typical for the area: Rhine Riesling and Grüner Veltliner. At the same time, these varieties have very good sensory results, if they are treated with ascorbic acid (fruitiness, freshness).

Each variety (50 l of mash) was divided into a sealable container with a capacity of 70 l. Three variants and a check variant were prepared for individual dosage of ascorbic acid and SO_2. The check variant, where just a dose of SO_2 in the amount of 40 mg/l was homogenized, was the first sample. The first variant contained medium recommended dose of ascorbic acid (10 g/hl), the second variant got medium recommended dose of ascorbic acid and a dose of SO_2 (10 g/hl and 40 mg/l), and, finally, the third variant contained maximum recommended dose of ascorbic acid (15 g/hl). After necessary homogenization of the substances given, containers were closed and the substances were put on 12 hour maceration. These microsamples were pressed on a classic wooden vertical press. Gravity clarification for 24 hours was the next step. Fermentation itself was carried out in 30 l glass containers and it lasted for 17–20 days. After fermentation SO_2 was added to all variants in the amount 50 mg/l and it was bottled due to sensory analysis.

The first sampling was conducted after homogenization of active substances into mash before maceration. The second sampling was conducted 12 hours after maceration and pressing was done. The third sampling was carried out during final fermentation of vinified samples and the last sampling was done after sulphur dioxide was added when the fermentation had ended. The samples were carefully labeled after each sampling and were immediately frozen to prevent unwanted oxidation. After sampling, all the necessary frozen samples were taken to the lab, where there were analyzed.

The following parameters were determined in the course of analytical analysis: free SO_2, total SO_2 and reductons. All of these parameters were measured using a titration method.

RESULTS AND DISCUSSION

The aim of our experiment is to compare respective variants and combinations of dosages of ascorbic acid and sulphur dioxide so that we can provide the best variant for wine-making practice.

For better orientation, various stages of sampling and variants were abbreviated:

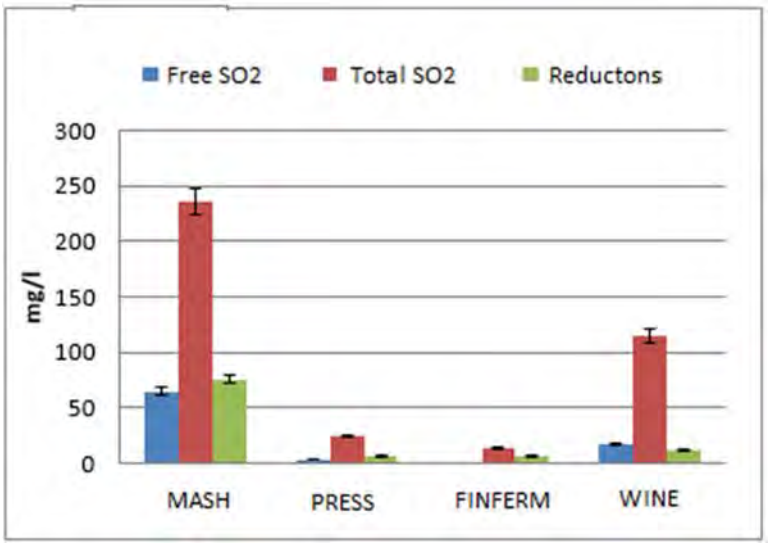
Samples (stages):

- MASH - mash stage.
- PRESS - a stage after pressing.
- FINFERM - a stage of must undergoing the final phase of fermentation.
- WINE - a stage of finished wine.

Variants (dose):

- SO2 - check variant; addition of SO_2 (40 mg/l free SO_2).
- SAA - variant 1; addition of medium dose of ascorbic acid (10 g/hl).
- SO2SAA - variant 2; addition of sulphur dioxide and medium dose of ascorbic acid (combination of 40 mg/l free SO_2 and 10 g/hl ascorbic acid).
- VAA - variant 3; addition of high doses of ascorbic acid (15 g/hl).

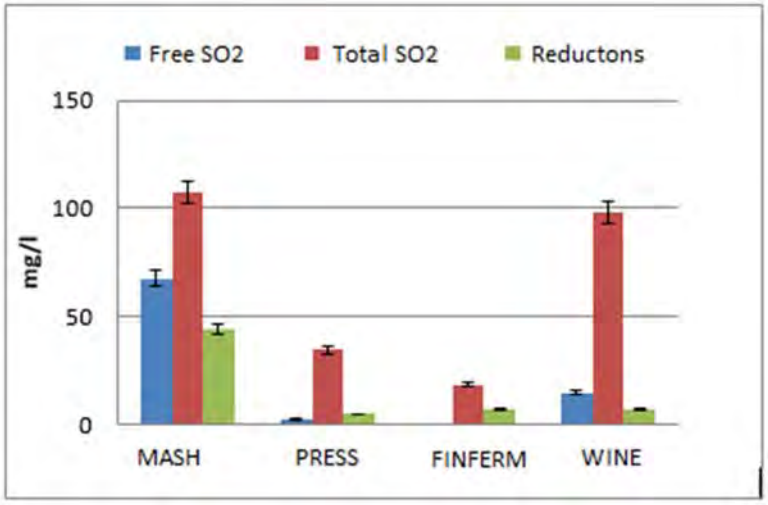
Figure 1 SO2SAA variant is the best variant for Rhine Riesling. It contains a dose of 40 mg/l of free SO_2 and 10 g/hl of ascorbic acid.



For variety Riesling is clearly evident influence of the year 2014. Specifically, this variety was heavily damaged by fungal diseases, especially fungal diseases *Botrytis cinerea*. Botrytis-affected grapes contain oxidoreductase, which is known as laccase. Unlike tyrosinase, this enzyme is stable at must pH values and is more resistant to sulphur dioxide. Adding ascorbic acid allows to completely prevent or limit oxidation of musts catalyzed by tyrosinase or laccase (Peng et al. 1998). Values of free sulfur dioxide and reductones but after twelve hours maceration ranged from 4–6 mg/l. These values are insufficient to protect the mash and must from unwanted oxidation. Most protected was the mash immediately after homogenization of active substances that have not already been oxidized.

The combined variant (SO2SAA), where there is a dose of sulphur dioxide and medium dose of ascorbic acid, has the highest value of reductones. The combined dose of sulphur dioxide and ascorbic acid was able to protect Rhine Riesling product against oxidation most effectively.

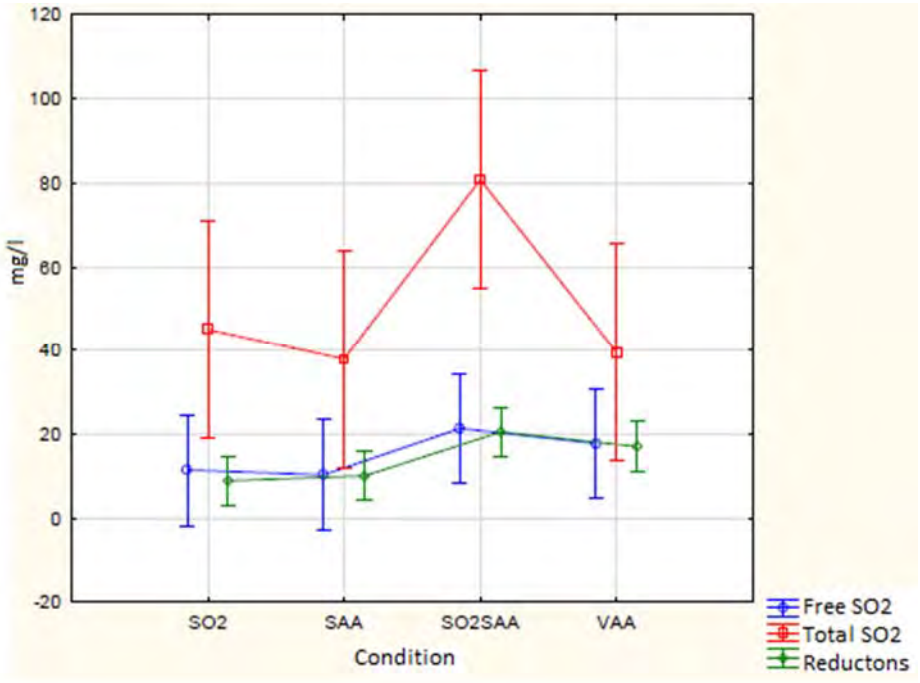
Figure 2 The best variant for Grüner Veltliner contains a dose of 40 mg/l of free SO_2 and 10 g/hl of ascorbic acid. This is SO2SAA variant.



2014 vintage did not influence health condition of grapes of Grüner Veltliner as it did with the previous variety. Nevertheless, SO_2 and reductones in the must quickly oxidized. Values of free sulphur dioxide and reductones ranged from 2 to 8 mg/l in the stage after maceration. The protective role of sulphur dioxide is only effective if there is appropriate concentration of it. Such a concentration for white wine (pH 3.0–3.3) is set at 10 mg/l. This concentration is considered as critical level before the

onset of undesirable features (Rankine 2004).Such a value is not sufficient for proper protection of must from oxidizing. Interesting values were recorded at the stage of must undergoing the final phase of fermentation. The value of reductones for all variants was higher than at the stage after pressing the must. The value ranges from 7 to 10 mg/l.

Figure 4 Total values of all varieties and variants



This general chart clearly shows that SO2SAA was the best variant, i.e. the variant with a dose of sulphur dioxide and medium dose of ascorbic acid. Figure 4 includes Rhein Reisling and Gruner Veltliner.

Figure 5 Total results for all stages of winemaking

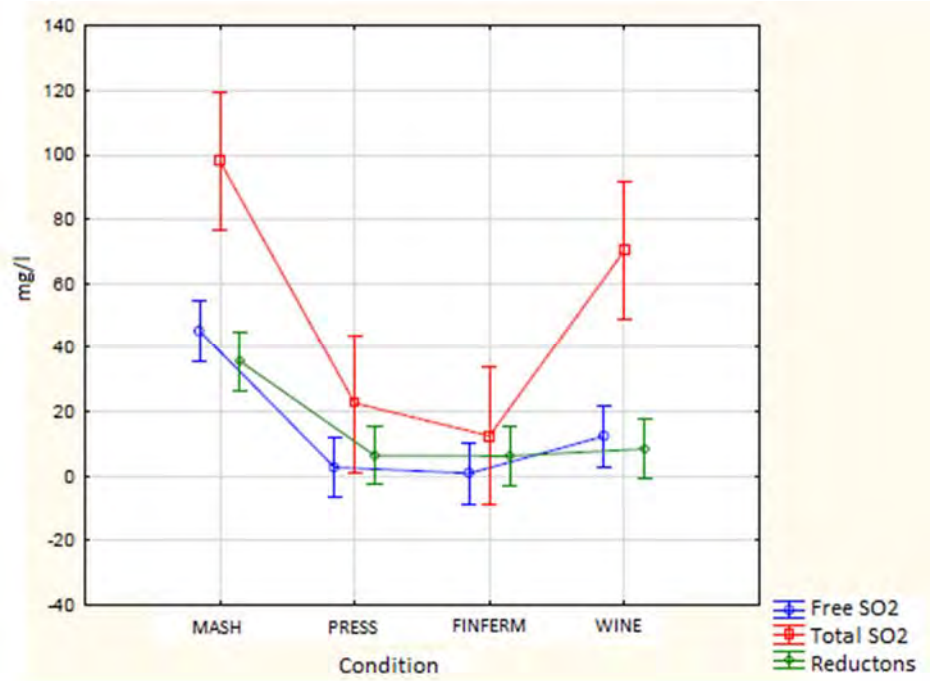


Chart 5 shows the overall results at all stages of winemaking. . This chart includes all varieties and variations. The results can be seen clearly insufficient protection of the mash after maceration.

The results are significantly affected by a problematic 2014 vintage. The experiment clearly showed that chosen doses of active substances are insufficient for such a difficult vintage. The dose of 30 mg/l of free sulphur dioxide into the mash is insufficient with heavily infected grapes. Therefore, it is necessary to increase the dose of SO_2 in order to effectively protect the mash and must in such vintages. To inhibit tyrosinase, it is necessary to add 50 mg/l of sulphur dioxide into the must. (Ribereau-Gayon et al. 2006). The protection using ascorbic acid seemed to be the most efficient right along with sulphur dioxide. The product was only little protected against unwanted oxidation processes when omitting sulphur dioxide even though minimum air got in.

CONCLUSION

Our experiment conducted in 2014 pointed out to the fact that ascorbic acid is a substance effectively acting as an antioxidant in wine production technology. On the basis of our experiment we have come to an apparent conclusion that a dose of ascorbic acid on its own is not able to protect the mash. On the contrary, it can even threaten the quality of wine. For effective protection of mash and resulting wine it is necessary to appropriately supplement ascorbic acid with sulphur dioxide. This very variant combining sulphur dioxide and ascorbic acid has been evaluated as the best for both varieties. It is the only way to ensure correct mechanism of wines, musts or mash protection against oxidative processes. Combining ascorbic acid and SO_2 makes it possible to achieve lower doses of sulphur dioxide and make thus wine less harmful to health. However, it is important to find balance between health safety and technological purity of the product produced.

ACKNOWLEDGEMENTS

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EVALUATION OF CHANGES IN THE COMPOSITION OF APPLES DURING STORAGE BY NIR SPECTROSCOPY

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Abstract: As part of the experiment, fruit varieties of Idared, Desert and Rubinola were stored under a normal atmosphere in a cold room at 4 °C; the pieces of fruit counted 25 per variety. The fruit was stored for 90 days; every 10 days, the spectra were measured using near-infrared ("NIR") spectroscopy. The stored fruit was evaluated for the content of titratable acidity, soluble solids, polyphenols and ascorbic acid. The spectra of the pieces of fruit were evaluated using NIR spectroscopy based on developed models of calibration. The calibration models consisted of 188 pieces of fruit of Rubinola, Desert and Idared varieties. The aim of the study was to evaluate changes in the material composition of apples during the period of storage using NIR spectroscopy. For all of the three varieties the content of studied substances changed in a very similar manner during the storing period; the content of ascorbic acid was the only exception in that it increased for Rubinola during the time and gradually reduced for both the Desert and the Idared varieties. Idared and Desert had approximately the same content of ascorbic acid at the beginning of storing (about 45 mg/kg). During the storage period the ascorbic acid content decreased almost identically for both of the varieties to reach a final value of about 30 mg/kg.

Key Words: fruit, calibration model, polyphenols, titratable acidity, ascorbic acid

INTRODUCTION

Fruits and vegetables make up a substantial part of a well-balanced diet. At least five servings of fresh fruits and vegetables is recommended per person and day; when served fresh, the food maintains the greatest possible amount of biologically active substances (Vaughan and Geissler 2009).

Apples contain a large quantity of substances beneficial for health in low concentrations that have synergistic effects. These include polyphenols, flavonoids, fibre, linolenic acid, D-limonene, epigallocatechin, gallic acid, isoflavones, vitamins (A, B, C and E), calcium, selenium, potassium, and glutathione (Karakaya and Kavas 1999).

(Růžičková et al. 2006) observed the possibility of application of near-infrared spectroscopy for monitoring of internal changes in stored apples inficated by Gloeosporium album. The discriminate analysis was applied to separate into clusters. This method was used for distinguishing infected and non-infected apples of both cultivars.

The recent years have seen the development of using NIR spectroscopy - a method enabling one to measure the quality of the horticultural products without causing destruction of the fruit (Bobelyn et al. 2009).

The method is also widely used as an alternative to the one referred to as "wet chemistry". The advantage of NIR spectroscopy is the speed of determination, the accuracy and the simplicity (Liu 2011). When determining the content of components in the samples, however, it is necessary to perform an accurate calibration of the NIR spectrometer using an appropriate set of calibration standards of known composition as well as appropriate analytical methods known as the reference methods. (Büning -Pfaue 2003).

Near-infrared spectrometry is a method of molecular spectroscopy which uses the spectral region of near-infrared radiation, i.e. the wavelength range of 800 to 2500 nm, or wave numbers of 12,500 to 4,000 cm^{-1} (Nicolai et al. 2007).

MATERIAL AND METHODS

Fruit material and parameters

The experiment evaluated varieties of apples (Rubinola, Idared, and Desert) grown on the university grounds of the Faculty of Horticulture, Lednice. Fruit varieties of Idared, Desert and Rubinola were stored under a normal atmosphere in a cold room at 4 °C; the pieces of fruit counted 25 per variety.

The fruit was stored for 90 days; every 10 days, the spectra were measured using near-infrared ("NIR") spectroscopy. Each of the pieces of fruit was first measured from three sides by Antaris II (NIR spectrophotometer); there were a total of 50 scans. Each evaluation made use of the average spectre. The stored fruit was evaluated for the content of titratable acidity, soluble solids, polyphenols and ascorbic acid. The spectra of the fruit were evaluated based on the developed models of calibration of NIR spectroscopy. The calibration models consisted of 188 pieces of fruit of Rubinola, Desert and Idared varieties.

NIR spectoscopy

NIR spectra were measured after the reflectance of the radiation from the sample. Measurements were performed in the apparatus Antaris II (Nicolet). NIR spectra were measured using the OMNIC software package (ThermoFisher Scientific Inc.). Each fruit specimen was measured from three sides and a mean spectrum was thereafter used for calibration by averaging with the TQ Analyst software (Thermo Fisher Scientific Inc.), which can be used for the development of both quantitative and qualitative methods of molecular spectroscopy. Calibration models were developed by averages of the PLS method that allows compression of extensive matrices of spectral variables into a much smaller number of the so-called PLS factors. Distinction was 16 cm^{-1}. The calibration values were obtained through analysing the fruit during both the maturation and storage periods. It is always the dependence between inserted data which were measured analyticaly and calculated data which are the NIR data.

Determination of Titratable Acids

Total acids were assessed by a pH meter ino Lab 7310 with a combined electrode by alcalimetric titration using the 0.1 mol/l NaOH (up to pH 8.1). Contents of acids were expressed as content of malic acid (%).

Determination of Ascorbic Acid Content

A sample of 10 g was homogenised with oxalic acid (10 ml) in a mortar. At first, the homogenate was filtered through a filter paper and thereafter through a nylon filter (mesh size 22 μm) into a brown vial. The prepared sample was thereafter loaded into the chromatographic column. Conditions during measuring: column: prevail 5 μm organic acid 110A HPLC Column 250 × 4.6 mm, flow rate of the mobile phase 25 mM KH_2PO_4 1 ml/min, wavelength 210 nm, and temperature 30 °C.

Determination of Soluble Solids

Soluble solids content (SSC) was expressed by the index of refraction (°Bx) measured by Abbe refractometer.

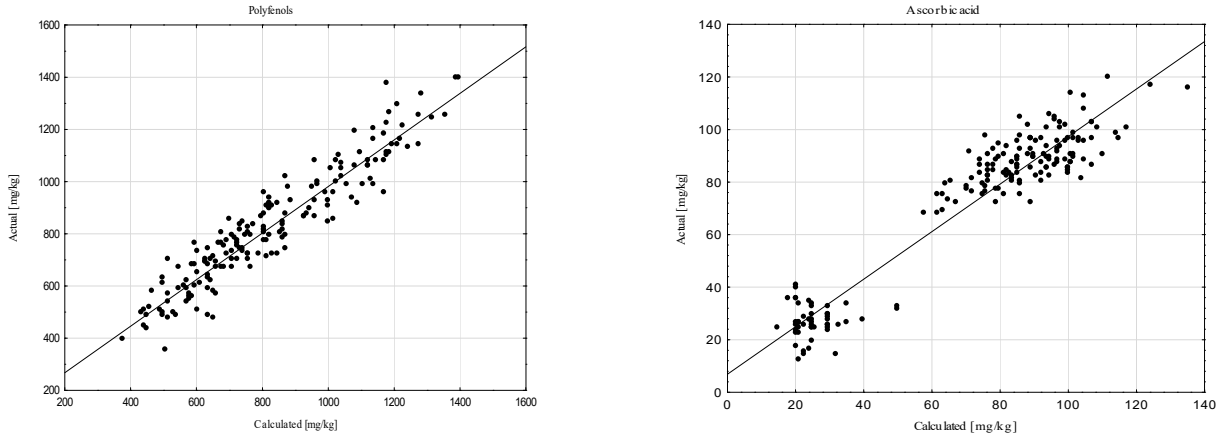
Determination of Total Polyphenols

Each fruit specimen was homogenised in a mixer. After the end of mixing, the juice was squeezed and centrifuged. Thereafter 0.5 ml of apple juice were pipetted into a 50 ml volumetric flask, diluted with 20 ml of distilled water and mixed with 1 ml of Folin-Ciocalteau reagent. After three min, 5 ml of 20% solution of Na_2CO_3 were added, the volumetric flask was filled up to the gauge line with distilled water and mixed again. Absorbance values were measured 30 min later in the spectrophotometer (Specord 50 plus) using a 10 mm cuvette at the wavelength of 700 nm and the results were compared with values determined in a blind experiment. The assessed content of total polyphenols was then converted to fresh matter of plant material and expressed as mg of gallic acid per kg fresh weight (mg/kg). Each fruit was measured three times.

RESULTS AND DISCUSSION

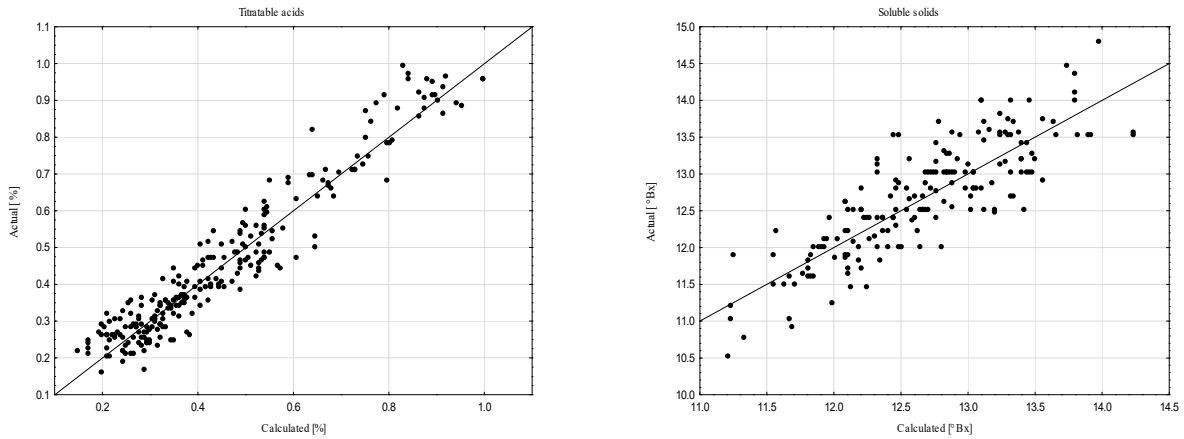
The spectra of the stored fruit were evaluated based on the models of calibration of NIR spectroscopy. The calibration models of total polyphenols, titratable acidity, soluble solids and ascorbic acid consisted of 188 pieces of fruits of Rubinola, Desert, and Idared varieties.

Figure 1 A calibration graph of total polyphenols and ascorbic acid by NIR spectroscopy.



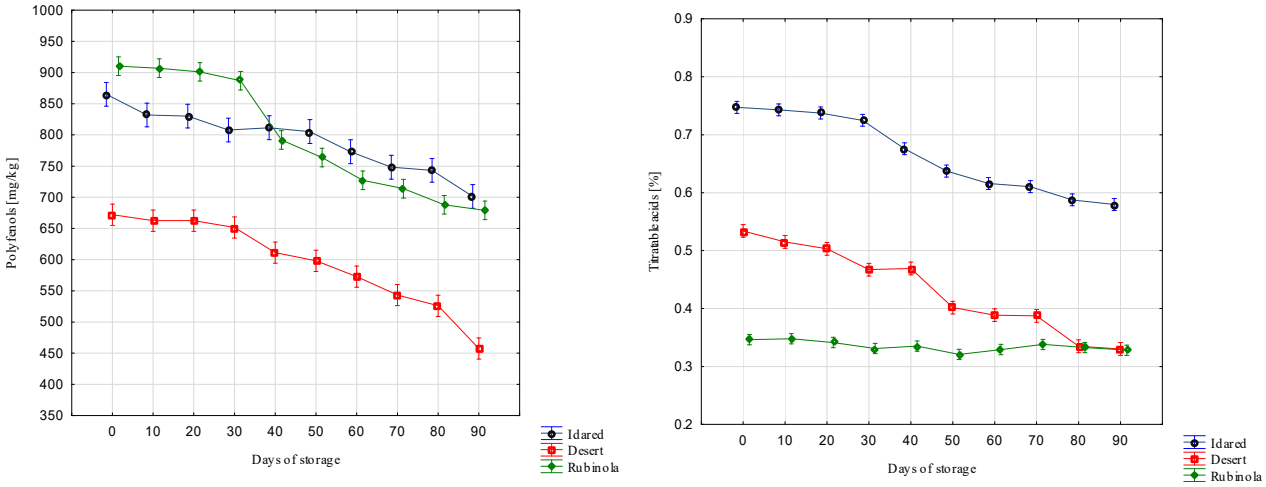
Sample calibration graphs that were used for evaluating the stored fruit. The developed calibration model of total polyphenols (Figure 1) ranges from 350 to 1,400 mg/kg. The calibration error is 90 mg/kg. The correlation coefficient equals 0.94. The developed calibration model of ascorbic acid (Figure 1) ranges from 12 to 120 mg/kg. The calibration error is 9 mg/kg and the correlation coefficient equals 0.95. The calibration values were obtained through analysing the fruit during both the maturation and storage periods.

Figure 2 A calibration graph of titratable acids and soluble solids by NIR spectroscopy.



The calibration model of titratable acidity (Figure 2) ranges from 0.15 to 0.99%. The calibration error is 0.037% and the correlation coefficient equals 0.96. The calibration model of soluble solids (Figure 2) ranges from 10.5 to 14.6 °Bx. The calibration error is 0.39 °Bx. The correlation coefficient equals 0.92. The calibration values were obtained through analysing the fruit during both the maturation and storage periods. The accuracy of the models developed was verified by analysing ten control samples. Deviations between the NIR method and the reference methods were consistent with calibration errors of the models developed.

Figure 3 Changes in the total content of polyphenols and titratable acids during the storage period.



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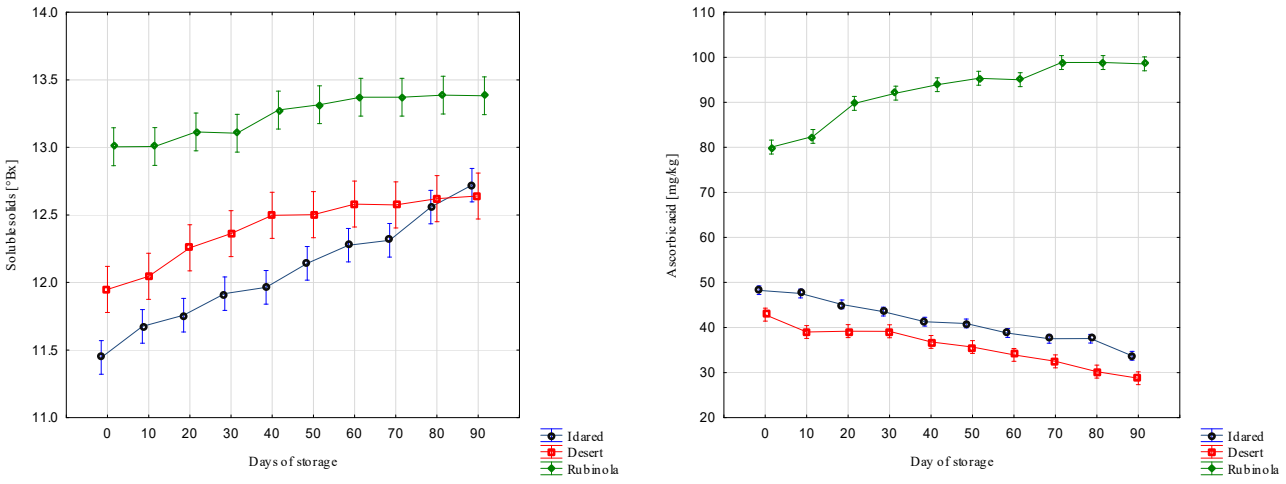
For each of the three varieties the highest polyphenol content was measured immediately after harvest (Figure 3). For Rubinola and Idared, the content was similar (around 900 mg/kg). During the storage period, both the values of and changes in the polyphenol content were very similar. After 90 days of storage the content of polyphenols was almost identical for both of the varieties (700 mg/kg). Desert was initially lower in terms of polyphenol content (670 mg/kg); after 90 days of storage the content decreased to 450 mg/kg. For Rubinola there were statistically significant differences in terms of polyphenol content between the first 30 days and the last 30 days of storage. For Desert the same occurred between the day 0 to day 60 period and the day 80 to day 90 period. For Idared, the statistically significant differences occurred between the day 0 to day 50 period and the day 60 to day 90 period. During the storage period, the acidity gradually decreased for Idared and Desert (Figure 3). The highest acidity was found for Idared throughout the storage period when the fruit contained 0.75% acidity at the beginning of the time. While Desert contained 0.53% acidity in the beginning of the storage period, after 90 days the content reduced to a quantity consistent with Rubinola. For Idared, statistically significant differences in acidity are between the day 0 to day 30 and day 60 to day 90 periods. For Desert it occurred between the day 0 to day 20, day 50 to day 70 and day 80 to day 90 periods. For Rubinola there were no significant differences in acidity during the storage period.

Figure 4 Changes in the total content of soluble solids and ascorbic acid during the storage period.



Soluble solids gradually increased for all the three varieties during the storage period (Figure 4). The highest values were found for Rubinola for which 13 °Bx was measured immediately after harvest. Extending the storage period did not increase that very much. At the beginning of storing, the lowest

values of soluble solids were found for Idared (11.4 °Bx). After 90 days of storage the content increased to 12.7 °Bx. Any statistically significant difference in terms of soluble solids was found for Idared only; it occurred between the day 0 to day 40 and day 60 to day 90 periods.

Idared and Desert had approximately the same content of ascorbic acid at the beginning of storing (about 45 mg/kg). During the storage period the ascorbic acid content decreased almost identically for both of the varieties to reach a final value of about 30 mg/kg (Figure 4). In contrast, the Rubinola fruit contained 80 mg/kg of ascorbic acid in the beginning of the storage period. The quantity further increased during the storage period to reach as much as 100 mg/kg. In terms of ascorbic acid content, there are statistically significant differences between the first 20 days and the last 30 days of storing for Idared. For the ascorbic acid content of Desert, there are statistically significant differences between the day 0 to day 40 and the day 60 to day 90 periods, while for Rubinola statistically significant differences were seen to occur between the first 10 days and the remainder of the days of storage.

Ventura et al. (1998) monitored in their study the content of soluble solids in the samples of apples after 5 months of storing. Two varieties of apple, Golden Delicious and Jonagold, were harvested in September and October and stored for five months at 2 °C; the pieces of fruit counted 340. Final analysis was conducted in April. The apples were first probed using NIR spectrophotometer, followed by analysis of soluble solids using refractometer. The values of soluble solids ranged from 11.7 to 14.2 °Bx. The same range was found for the varieties within the present study in terms of soluble solids.

Pissard et al. (2013) analysed the content of ascorbic acid, soluble solids and polyphenols in the fruit of 37 varieties of apple. The studied samples of apple counted 1,474 for ascorbic acid content; 2,646 for polyphenol content; and 1,875 for the content of soluble solids. The number of samples used for developing a calibration model was 800 for ascorbic acid; 2,000 for polyphenols; and 1,000 for soluble solids. The presence of substances in the analysed samples of fruit ranged from 2.7 to 750 mg/kg for ascorbic acid; 276 to 7,600 mg/kg for polyphenols; and 7.2 to 20.9 °Bx for soluble solids.

Liu and Ying (2005) studied the potential of quick measurements of soluble solids and titratable acidity in apple fruit using NIR spectroscopy. The experiment used a total of 333 fruit of the Fuji variety, of which 235 pieces were used for calibration and 98 for validating the model. The calibration model developed for soluble solids had a correlation coefficient of 0.97 and a calibration error of 0.48 °Bx. The calibration model developed for titratable acidity had a correlation coefficient of 0.73 and a calibration error of 0.0041 °Bx. The calibration model for soluble solids of Rubinola, Idared and Desert had a correlation coefficient of 0.92 and a calibration error of 0.39 °Bx. For titratable acidity, the calibration model had a correlation coefficient of 0.96 and a calibration error of 0.037 °Bx.

Vrhovsek et al. (2004) analysed a total of eight varieties of apple in his study ('Braeburn', 'Fuji', 'Golden Delicious', 'Granny Smith', 'Morgenduft', 'Renetta', 'Royal Gala' and 'Red Delicious') for the content of ascorbic acid and total polyphenols. A total of 41 pieces of fruit were collected between August and November. Ascorbic acid content ranged from 4 to 81 mg/kg. The lowest and the highest value were found for Royal Gala and Braeburn, respectively. The latter variety had a comparable content with Rubinola, which in the early stages of maturation featured ascorbic acid content of about 80 mg/kg.

CONCLUSION

The study aimed at identifying changes in the content of total polyphenols, ascorbic acid, titratable acidity and soluble solids during the storage period of apple fruit. Evaluation of changes in material components was carried out using NIR spectroscopy calibration models. For the stored fruits of all the three varieties there was a decrease in polyphenol content by about 200 mg/kg over the storage period. The lowest polyphenol content throughout the storage was seen in the Desert variety with the fruit containing 450 mg/kg polyphenols in the late stage of storing, while the value for Rubinola and Idared was about 700 mg/kg in the same period Idared and Desert had approximately the same content of ascorbic acid at the beginning of storage (around 45 mg/kg). During the period of storage the content in both varieties decreased almost identically to the resulting level of around 30 mg/kg. In contrast, Rubinola was found to contain much more ascorbic acid (80 mg/kg) which further increased during the storing period to reach as much as 100 mg/kg.

During the period of storage, a rather significant decrease in titratable acidity was seen in Desert and Idared (by 0.2% approximately). For Rubinola the acidity content did not change significantly,

moving around 0.35% throughout the storage period (90 days). Soluble solids gradually increased for all the three varieties during the storage period. Rubinola exhibited the highest values, found to have 13 °Bx through measurements; any further storage did not increase the content very much. Idared was recorded to have the highest increase when the content grew from 11.5 °Bx to 12.7 °Bx after 90 days of storing.

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ANALYSIS OF RED CURRANT (*RIBES RUBRUM*) AND RED GOOSEBERRY (*RIBES UVA-CRISPA*) VARIETIES BY INDUCTIVELY COUPLED PLASMA ATOMIC EMISSION SPECTROSCOPY

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Abstract: Berries are highly valued fruits containing many organic compounds with significant health benefits. This work is focused on determination of elemental composition of different red currant and gooseberry varieties, which is less known. An acid digestion method was used to decompose fruit samples and a method for determination of 9 nutritionaly important elements by inductively coupled plasma optical emission spectrometry was optimized. From the analysed varieties ´Jesan´ and ´Rubigo´red currants and ´Hinnonmaki Rot´ and ´Krasnoslawjanskij´red gooseberries were evaluated as the best for the use in food industry in terms of elemental composition.

Key Words: currant, gooseberry, fruit, elemental composition

INTRODUCTION

Currants and gooseberries are known in Europe since the 14th century. They were consumed either fresh or as juices and jams, or as a fermented fruit beverages. Currant is a small shrub belonging to the family of Grossulariaceae, of the genus Ribes. The currant bush grows up to a height of 1–1.5 meters. Its leaves are yellowish-green in color and are arranged spirally on the stems in bunches of five. During each season, the shrub bears pendulous chain of small berries. The fruit has size of about 1 cm in diameter with a glossy skin and a persistent calyx at the apex, and containing 3–10 tiny seeds. Depending on the berries colour, red, white and black currants varieties can be distinguished (Strik 2003, Djordjevic et al. 2014). Gooseberries are deciduous shrubs, fast growing under optimum conditions to 1 m tall and 1.5 m wide. The leaves are alternate, single, deeply lobed, and glossy dark green. The fruit borne singly or in pairs at the axils, is a berry with many minute seeds at the center. A gooseberry may be green, white, yellow, or shades of red from pink to purple to almost black. Fruits of the European gooseberry may be very large but usually they are up to 3 cm long, less in width (Strik 2003).

Growing of currants and gooseberries has been recently extensively on the decline mainly due to a massive expansion of *Podosphaera mors-uvae* fungus, the most important disease in gooseberry and currant. Within the modern trend of consuming foods with a high content of biologically active substances the cultivation of new varieties of currants and gooseberries resistant to pests and diseases becomes again very significant. In 2005 there were nearly 40 000 hectares of orchards growing gooseberry and 163 000 hectares growing currants worldwide.

Berries are good source of many nutritionally significant compounds. Whereas the organic composition of the currant and gooseberry is subject of numerous publications (Benvenuti et al. 2004, Pantelidis et al. 2007, Da Silva Pinto et al. 2010, Vagiri et al. 2013) elemental composition of different currant and gooseberry varieties is less known. Elemental analysis of food matrices can be performed by various spectroscopic techniques. One of the often used techniques is atomic emission spectroscopy,

mainly with inductively coupled plasma. The main analytical advantages of the ICP–OES over other spectroscopic techniques are the capability for efficient and reproducible vaporization, atomization and excitation of wide range of elements in various sample matrices, minimum spectral interferences and simultaneous determination of all metallic elements and some metalloids and non-metals (Ebdon et al. 1998).

This work is focused on the elemental analysis of six different varieties of red currant and gooseberries. These varieties were grown at Research and Breeding Institute of Pomology Holovousy and they are resistant for most diseases typical for currants and gooseberries. As the elemental composition of fruit is one of the important nutrition parameter used in food industry, the results of this study may help to choose the best fruit cultivar in restored breeding programme of currant and gooseberry in the Czech Republic or in other countries.

MATERIAL AND METHODS

The ICP–OES instrument was calibrated by certified solutions of metals of interest (1 g/l, Astasol, Analytika, Czech Republic). For the decomposition of fruit samples nitric acid (Analpure, Analytika) was used. In all analyses only ultrapure water prepared by ELGA station (Veolia Watter systems Ltd., UK) was used. To verify the proper function of ICP–OES instrument the quality control material METRANAL no.3 containing different metals in strawberry leaves matrix (Analytika) was used. All of the analyses were performed on an ICP–OES (Ultima 2, Horiba Jobin Yvon, France) equipped with Mainhard type nebuliser and cyclonic spray chamber. An analytical balance AND HA-202M (A&D Company, Japan) was used to weight samples. Samples were shaken on GFL 3006 shaker (Gesselschaft für Laboratortechnik GmbH, Germany) and heated on Gerhardt heating plate (Gerhardt Bonn, Germany). All of the currant and gooseberry varieties were grown in the Czech Republic at the Research and Breeding Institute of Pomology Holovousy Ltd. (50 22'29" N, 15 34'38" E, 321m alt.) in the experimental orchard. The soil type in the experimental orchard is heavy loamy clay soil with a minimum thickness of 60–80 cm. The bedrock consists of clay stone. Average annual temperature of the locality is 8.14 °C. Average annual rainfall is 655 mm and average rainfall during vegetation period is 379 mm. All plants were 3 years old and no pesticides were used during the cultivation. The samples came from the harvests in 2013 and 2014. All fruits were harvested at full maturity, stored under -18 °C prior analysis and analyzed as soon as possible. Analyzed red currant varieties were: ´Detvan´, ´Jesan´, Junnifer´, ´Losan´, ´Rovada´, ´Rubigo´ and analyzed gooseberry varieties were: ´Alan´, ´Hinnonmaki Rot´, ´Karát´, ´Karmen´, ´Krasnoslawjanskij´,´Remarka´.

Approximately 10 grams of fruit was decomposed by 20 ml of concentrated nitric acid. After 24 h of nitric acid addition and continuous sample shaking the sample was heated for 60 min until the complete decomposition. After cooling down, the samples were transferred into the 100 ml volumetric flasks and filled up with water. Each of the fruit variety was decomposed and analysed three times. The quality control material samples were prepared by the same way as the fruit samples. All concentrations were expressed as the average. The standard deviation was less than 10 % for all analysed samples. The concentrations in mg/kg of fresh weight were calculated as $c_m = c_s .V / m$, where c_m is the concentration of element of interest in mg/kg, c_s is the concentration of element of the interest in the analysed solution (mg/l), V is the volume of analysed solution (l) and m is the weight of the sample used for the analysis (kg). Obtained data were further analyzed with the XLStat and Microsoft Excel software. Testing for significance of mean effects and interactions on all variables was calculated using ANOVA analysis of variance and Tukey´s test. Statistical significance was set at $P = 0.05$.

RESULTS AND DISCUSSION

Before the analysis ICP–OES operating conditions were optimized by analysis of a standard solution containing 10 mg/l of each element of the interest. The standard matrix was modified to be the same as in the analysed samples. The optimal wavelengths were chosen in order to achieve the sufficient sensitivity and the least interference and they are presented in Table 1. The optimal power to plasma was 1200 W and peristaltic pump rotation 20 rpm. The optimal nebuliser pressure was estimated by measuring the ratio of the intensities of the magnesium 280 and 285 nm spectral lines and by measuring the ratio of signal and background (SBR). The results are presented in Figure 1 and it can be seen that

the optimal pressure was 0.29 MPa. The plasma gas flow was adjusted according to the manufacturer's recommendations to 13 l/min. The optimal flow of additional (auxiliary) gas was different for minor (0.2 l/min) and major elements (0.8 l/min) as it is illustrated in Figure 2. The alkali and alkaline-earth metals have low ionization energy and they emit radiation already in the initial radiation zone which is located under the edge of the ICP torch. Addition of auxiliary gas lifts the plasma above the injector tube and it allows better monitoring of the radiation emission. After the ICP–OES conditions have been optimized, the correct settings of the ICP–OES instrument were verified by analysis of quality control material. The recoveries ranged from 92 to 105% (Table 1) which is acceptable. Results from the elemental analysis of red currant cultivars are summarized in Table 2. Large differences were observed in metal contents in currant. While zinc, copper and manganese were present in quantities less than 1 mg/kg, sodium, iron and magnesium up to 50 mg/kg and calcium with phosphorous up to 700 mg/kg. All currants were an excellent source of potassium (up to 1800 mg/kg). The difference between mineral content of different currant varieties was statistically significant ($p < 0.05$) which can be related to different pomological characteristic of analysed cultivars. From the nutritional point of view the ´Jesan´ and ´Rubigo´ varieties were evaluated as the best as they contained significantly higher concentrations of all major elements. In an average it can be concluded that consumption of 100 g of red currants examinated in this study cover about 1–5% of the recommended dietary allowance of potassium, calcium, phosphorous, sodium, iron, manganese, magnesium, copper and zinc for woman and men (Driskell 2009).

Table 1 ICP–OES chosen wavelengths and results from the analysis of QCM material

	ICP-OES wavelenght	QCM certified Value	Measured value	Recovery
Zn	206.191	27.1 ± 1.8	24.9 ± 1.4	92
P	213.618	n.a.	*	*
Mn	257.610	187 ± 18	179 ± 15	96
Fe	259.940	912 ± 90	872 ± 43	96
Mg	285.213	4210 ± 420	4191 ± 249	99
Cu	324.750	8.68 ± 0.76	8.14 ± 0.55	94
Ca	422.673	n.a.	*	*
Na	588.900	n.a.	*	*
K	766.490	21200 ± 2100	22319 ± 1712	105

Legend: n.a. – not available

Results from the elemental analysis of red gooseberry cultivars are summarized in Table 3. The fruits of red gooseberry contained highest concentration of phosphorous, potassium, calcium and magnesium while other elements concentration was comparable to concentrationfound in red currants varieties. The difference between mineral content of differentgooseberry varieties was statistically significant ($p < 0.05$). In terms of the total mineral content it can be concluded that ´Hinnonmaki Rot´ and ´Krasnoslawjanskij´ varieties are the most promising for use in the food industry. Consumption of 100 g of these gooseberry varieties can cover up to 8% of the recommended dietary allowance of minerals for woman and men (Driskell 2009)

The comparison of red currant and gooseberry mineral composition with mineral composition of other small fruit species like strawberry, raspberry, elderberry, coenelian cherry and sea buckthorn is presented in Table 4 (Strik 2007, Hegedus et al. 2008, Kalyoncu et al. 2009, Charlrbois et al. 2010, Bal et al. 2011,Vagiri et al. 2013, Cetkovska et al. 2015, Divis et al. 2015). It can be seen that only cornelian cherry contains significantly higher concentration of major elements, however in general it can be said that the nutritional value of red currant and gooseberry is comparable with other small fruits.

Figure 1 Effect of the additional gas flow to the intensity of measured signal (I). Additional gas flow increases from 0.1 to 0.8 l/min

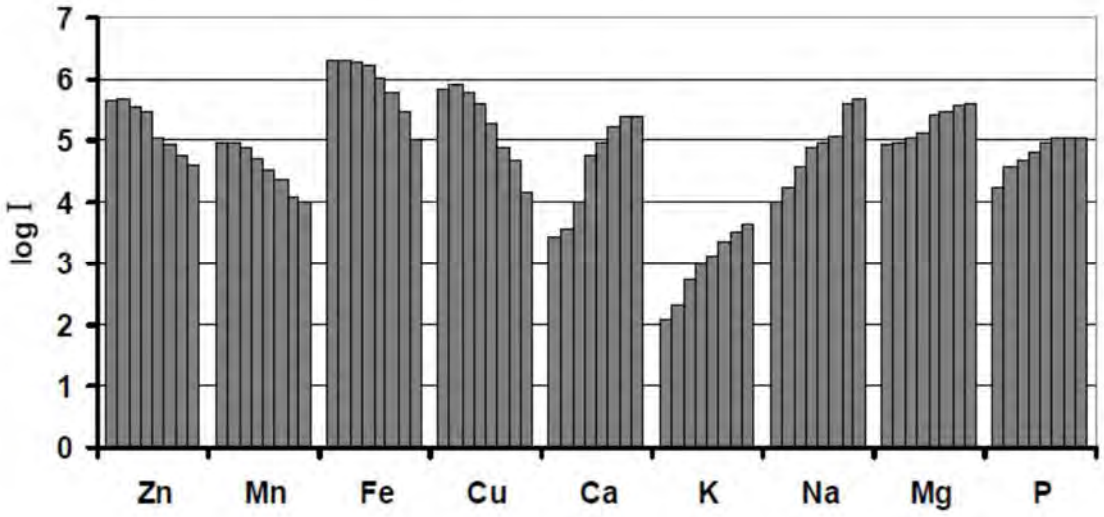
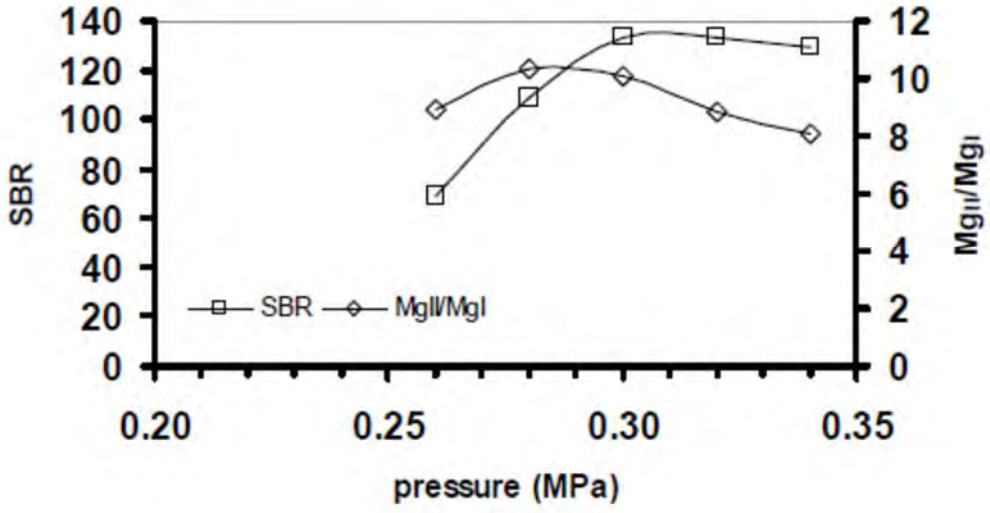


Figure 2 Effect of the pressure applied on the nebuliser



Legend: SBR – signal to background ratio

The comparison of red currant and gooseberry mineral composition with mineral composition of other small fruit species like strawberry, raspberry, elderberry, coenelian cherry and sea buckthorn is presented in Table 4 (Strik 2007, Hegedus et al. 2008, Kalyoncu et al. 2009, Charlrbois et al. 2010, Bal et al. 2011,Vagiri et al. 2013, Cetkovska et al. 2015, Divis et al. 2015). It can be seen that only cornelian cherry contains significantly higher concentration of major elements, however in general it can be said that the nutritional value of red currant and gooseberry is comparable with other small fruits.

As it was mentioned above only few studies was published before dealing with elemental analysis of currant and gooseberry. Hegedus et al. (2008) analysed ´Detvan´,´Jonkheer van Tets´ and ´Rondom´ red currant varieties grown in Hungary, while Nour et. al analysed ´Abundent´, ´Houghton Castle´ and ´Rosu Timpuriu´ red currants varieties grown in Romania. In comparison with this study, red currant varieties grown in Hungaria and Romania contained higher concentration of major elements, mainly calcium, potassium and magnesium. Other elements concentration was comparable with results published in this study. The differences in elemental composition can be caused by different soil type used for the cultivation and by different climatic conditions in each region. The measured elemental

composition of gooseberry can be compared only with the results published by United States Department of Agriculture (USDA 2014). The data published by USDA are consistent with the results measured in this work and varies relatively little.

Table 2 Elemental composition of different red currant varieties. Average value in mg/kg from two seasons. Values in the same line with different letters are significantly different at p<0.05.

	´Detvan´	´Jesan´	´Junnifer´	´Losan´	´Rovada´	´Rubigo´
Zn	1.09^{a}	0.86^{b}	0.61^{c}	1.11^{a}	0.83^{b}	0.58^{c}
P	156^{cd}	252^{a}	206^{bc}	176^{c}	229^{b}	262^{a}
Mn	0.29^{bc}	0.34^{b}	0.21^{c}	0.41^{a}	0.22^{c}	0.33^{b}
Fe	2.8^{c}	4.1^{ab}	3.2^{bc}	4.9^{a}	2.9^{c}	3.8^{b}
Mg	30^{a}	27^{b}	27^{b}	25^{c}	21^{d}	28^{b}
Cu	0.75^{b}	0.79^{b}	0.78^{b}	0.72^{b}	0.87^{a}	0.93^{a}
Ca	91^{c}	147^{a}	92^{c}	115^{b}	148^{a}	151^{a}
Na	43^{a}	46^{a}	38^{b}	26^{c}	21^{d}	28^{c}
K	1298^{cd}	1469^{b}	1272^{d}	1291^{cd}	1340^{c}	1782^{a}

Table 3 Elemental composition of different red gooseberry varieties. Average value in mg/kg from two seasons. Values in the same line with different letters are significantly different at p<0.05.

	´Alan´	´Hinnonmaki Rot´	´Karát´	´Karmen´	´Krasnoslawjanskij´	´Remarka´
Zn	0.75^{c}	2.65^{a}	0.70^{c}	1.65^{b}	2.65^{a}	0.80^{c}
P	305^{c}	327^{c}	205^{d}	441^{b}	568^{a}	173^{d}
Mn	0.65^{cd}	0.73^{c}	0.51^{d}	1.23^{b}	1.55^{a}	0.43^{de}
Fe	4.7^{c}	9.1^{b}	3.2^{d}	7.5^{b}	11.2^{a}	3.9^{d}
Mg	79^{bc}	84^{b}	55^{c}	108^{b}	149^{a}	42^{c}
Cu	0.40^{bc}	0.30^{c}	0.31^{c}	0.48^{b}	0.61^{a}	0.31^{c}
Ca	323^{c}	335^{bc}	223^{d}	380^{b}	660^{a}	134^{c}
Na	24^{a}	11^{c}	16^{b}	10^{c}	20^{ab}	10^{c}
K	1023^{c}	2825^{a}	899^{d}	1992^{b}	2909^{a}	944^{cd}

Table 4 Elemental composition of different small fruits in mg/kg

	Strawberry	Raspberry	Elderberry	Blueberry	Cornelian cherry	Sea buckthorn
Zn	0.8–1.1	2.7–4.0	5.1–11.3	1.6	0.5–4.4	n.a.
P	210–241	290–351	390–1131	120	606	82–206
Mn	2.8–3.4	1.6–7.0	0.6–9.5	3.4	0.7–1.9	n.a.
Fe	2.8–9.5	5.2–7.0	16–30	2.8	0.5–1.8	4–15
Mg	121–154	176–222	50–739	60	72–715	40–240
Cu	0.5	0.9–1.1	1.1–2.0	0.6	0.5–3.6	n.a.
Ca	160–312	219–250	380–1528	60	517–1560	64–256
Na	10–26	10–51	60–146	10	n.a.	7–125
K	1405–1530	1510–1718	2800–5494	770	4225–14301	62–806

CONCLUSION

An inductively coupled plasma optical emission spectrometry was preferably applied to get information about elemental composition of 6 different red currant and gooseberry varieties. It was demonstrated, that minor and major elements is better to analyze separately in two steps because they require different settings of spectrometer. Despite of different settings for minor and major elements an inductively coupled plasma optical emission spectrometry has still many advantages over other spectroscopic methods. This study confirmed that red currant and gooseberry are good sources of minor and major metals. From the analyzed varieties ´Jesan´ and ´Rubigo´ red currants and ´Hinnonmaki Rot´ and ´Krasnoslawjanskij´ red gooseberries showed favorable elemental composition and they can be recommended for breeding programme of currants and gooseberries in the Czech Republic.

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THE STRENGTH MONITORING OF COCONUTS BY THE ACOUSTIC EMISSION METHOD

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Abstract: The article deals with monitoring of the strength of coconut shells by way of the acoustic emission method. The purpose of this research is the process of formation and diffusion of micro fissures. These micro fissures rise by weighting of coconut shell samples through the use of compression force between two platens. The aim of the research is to focused on the possibilities of the acoustic emission usage for maximal coconuts strength prediction. In addition, the experimental measurement is focused on suitable placement and gripping of acoustic emission sensor.

Key Words: coconut, Acoustic Emission, Coconut shell

INTRODUCTION

Botanically speaking, a coconut is a fibrous one-seeded drupe, also known as a dry drupe. However, when using loose definitions, the coconut can be all three: a fruit, a nut, and a seed (Prades 2012).

At one time scientists identified over 60 species of Cocos palm. Today, the coconut is a monotypic with one species, nucifera. However, there are over 80 varieties of coconut palms, which are defined by characteristics such as dwarf and tall. It takes 11–12 months for the coconut to mature (Ohler 1999).

Some scientists like to refer to the coconut as a water dispersal fruit and seed. A seed is the reproductive unit of a flowering plant. From a reproductive point of view, a seed has the “baby” plant inside, with two basic parts: the embryo root (hypocotyl) and the embryo leaves (epicotyl). In the coconut’s case, if you look at one end of the coconut, you’ll see three pores. The coconut seed germinates and a shoot emerges from one of the pores. In addition to the “baby” plant in the seed, there is the food to kick off its life called the endosperm. The endosperm is what makes up most of the seed and, in the coconut’s case, is the yummy white stuff we eat. The word coconut itself can also be confusing because the word nut is contained in the word. A nut can be defined as a one - seeded fruit. With that loose definition, a coconut can also be a nut. However, a coconut is not a true nut. A true nut, such as the acorn, are indehiscent or do not open at maturity to release its seeds. The seeds are released when the fruit wall decays or are digested by an animal (Woodroof 1979).

Coconuts are renowned for their hard shells, which are vital to ensure their seeds successfully germinate. Coconut palms can grow 30 metres high, meaning that when the ripe fruits fall to the ground, their walls have to withstand the impact to stop them from splitting open. To protect the internal seed, the coconut has a complex structure of three layers: the outer brown, leathery exocarp, a fibrous mesocarp and a tough inner endocarp surrounding the pulp, which contains the developing seedling (Wood 2016).

Within the endocarp layer, which consists mainly of highly lignified, or woody, stone cells - the vessels that make up the coconut's vascular system have a distinct, ladder-like design, which is thought to help withstand bending forces. Each cell is surrounded by several woody rings, joined together by parallel bridges. The endocarp seems to dissipate energy via crack deflection. This means that any newly developed cracks created by the impact don't run directly through the hard shell. It is thought that the

angle of the vascular bundles, which are designed to transport water and minerals, helps to divert' the trajectory of the cracks. The longer a crack has to travel within the endocarp, the more likely it is that it will stop before it reaches the other side. The angle to the vascular bundles could be applied to the arrangement of textile fibres within concrete, the experts believe (Wood 2016).

MATERIAL AND METHODS

Samples of coconuts came from Ivory Coast, exactly the Nucifera type. The measuring was designed with 10 samples of coconuts and focused on monitoring of weight parameters, length proportions and wideness of coconut and fatness of coconut shell (see Figure 1). The weight of all samples was specified by use of digital scale and the other parameters - length, wideness and thickness were specified by calliper.

Table 1 Measured values (author)

Sample	Weight [g]	a [mm]	b [mm]	c [mm]	Max. load [N]	RMS [mV]
1	333	157	134	3.75	4289.3	1240
2	438	165	145	4.28	4450.2	1413
3	386	155	136	4.06	4319.8	1264
4	441	172	140	5.22	5566.3	1639
5	430	167	147	3.18	3892.3	1010
6	395	158	147	2.34	2497.9	815
7	541	177	159	4.96	5463.2	1632
8	472	157	159	2.40	2718.9	951
9	379	159	143	3.83	4294.2	1252
10	322	159	127	4.38	4695.1	1503

The universal instrument was used for measuring of the physical parameters by means of the tensile machine ZDM 5/51 (see Figure 2). The instrument provides for measuring different materials throughout tension, compression and bending. As the main method was chosen layers' compression. The coconut was compressed until the point of coconut shell disruption. The parameters of measuring are displayed in Table 2. The pressure force is rising during weighting until the point of coconut shell disruption. The pressure force F depends on displacement of x mainly in linear direction until the point of coconut shell disruption. The value of force – Fc, the point of coconut shell disruption, models fracture force and accordant the displacement xc. Except absolute value of this deformation is used also measuring fracture force (Braga et al. 1999).

$$R^2 = 1 - \frac{\sum(y_i - \hat{y}_i)^2}{\sum(y_i - \hat{y})^2}$$

The value of R^2 varies between 0 and 1; a value of $R^2 = 0.957$ indicates that 96% of the total variability in the response variable is accounted for by the predictor variables. However, a large value of R^2 does not necessarily mean that the model fits the data well. Thus, a more detailed analysis is needed to ensure that the model can satisfactorily be used to describe the observed data and predict the response for another set of data different from the one used to generate the model.

Figure 1 Scheme of the experimental sample proportions (see Table 1)

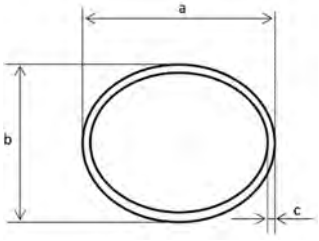
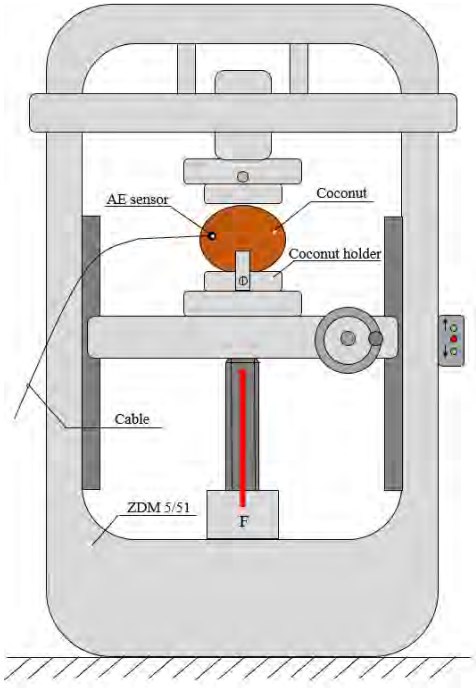


Figure 2 Scheme of tensile machine ZDM 5/51



The assessment of coconut strength during the process of compression between two straight layers.

The coconut is horizontally places between two straight layers. The lower layer is firm fixed. Upper layer is moving by given speed generally by 1–1000 mm/min and is connected with dynamometer, that provide temporal subservience of the force F which is affecting shell.

Table 2 Parameters of measuring (author)

Test type:	Pressure
Crosshead velocity:	10 mm/min
End threshold:	Decrease in strength 40%

Legend: Load Capacity – The maximum load for which the equipment is designed by the manufacturer. Test type – The type of testing is tensile or compressive. Crosshead velocity – The crosshead velocity is defined as change of displacement per time interval. End threshold – Test is terminated when force decreases to value entered (40%).

Acoustic emission system

The acoustic emission means physical effect during that is possible to observe acoustic signals broadcasted by the mechanical, heat or chemical subjected by the solid and it also includes diagnostic method based on this effect. The acoustic emission is performed in the source of acoustic emission during the energy disengaging caused by inner and outer powers. The acoustic emission formation is generated by nonreversible dislocated and degradation processes in the material microstructure and macrostructure, also by cavity processes in the hydro dynamical systems, by the turbulence during the pipeline liquid fading, dielectric degeneration etc. Energy is transformed to the mechanical tension impulse. This impulse is dilating throw the material such as elastic tension longitudinal or transverse wave (Dostal et al. 2012).

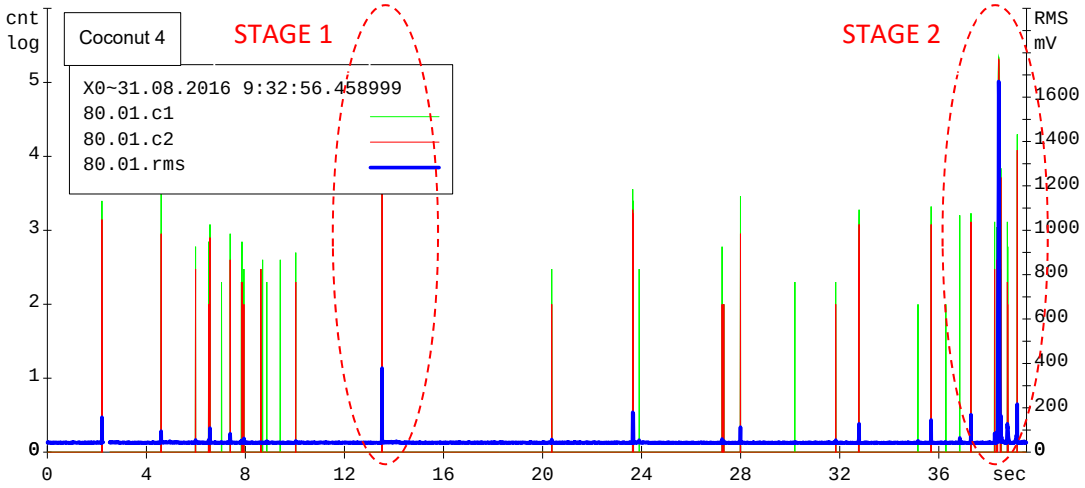
The sensor IDK-09 was used in this research. The reason is the suitable sensitivity of it. It is common to place the sensor on the top of the tested sample during the monitoring of quality defined samples. The acoustic emission sensor was fixed by the specific elastic rubber rings on coconut shell.

RESULTS AND DISCUSSION

There were 10 verification measures within the experimental measuring. Each of the measuring included continual acoustic emission scanning see in enclosed literature (Dostal et al. 2012). Gained

data were tested by the regressive analysis. The result of the experiment is that the maximal weight stress and the signal force are affected by the thickness of the coconut shell.

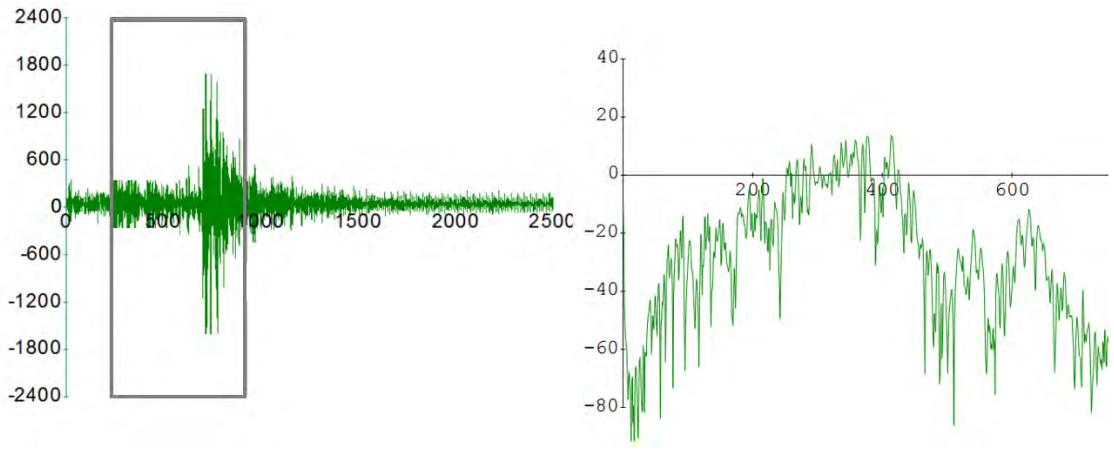
Figure 3 Sample 4, RMS 1639 mV stage



For this measurement with using the specific sensor IDK-09 and amplifying 48dB was necessary to use HW measuring interval 7 ms is depicted in x-axis. This was set by the software and could not be less. System does not allow to decrease it. This interval is the lowest suitable value what can be chosen for those purpose in described setting. The reason is that the system was made for different purpose.

The Figure 3 of sample depicts the whole measure logging of the acoustic emission signal during the burdening of the sample 4 on the maximal tolerable force 5566.3 N. We can divide the logging into three phases. The first stadium takes the time period 0–20 sec. We can observe the logging of the gradual weighting of tested sample. There is no formation of micro cracks. The second part of measuring in the time period 20–37 sec shows the creation and diffusion of micro cracks. Finally, the third part involves the time period since 37 sec. There is the linking of the particular micro cracks which leads to creation of the major arm link. The maximal RMS value is 1639 mV which is the notation of the creation of the plastic deformation. The maximal RMS value is specified in the following Figure 6.

Figure 4 Frequency analysis – power spectrum density (PSD) is taken from sample 4 STAGE 2



There is the representative example of the frequency analysis - power spectrum density (PSD) in the Figure 4. The power spectrum density (PSD) takes about 400 kHz at all samples. The PSD shows the typical frequency analysis.

Figure 5 Frequency analysis – power spectrum density (PSD) is taken from sample 4 STAGE 1

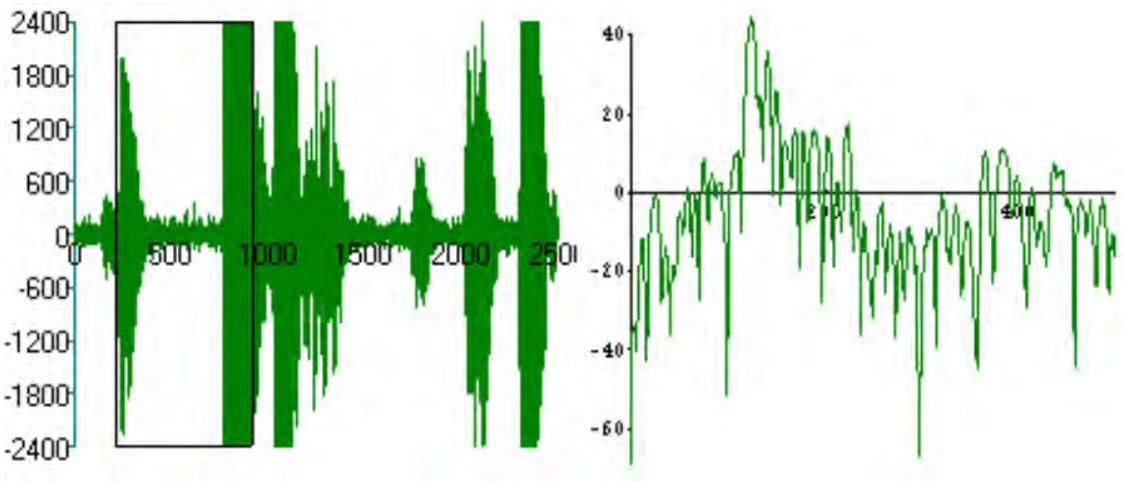
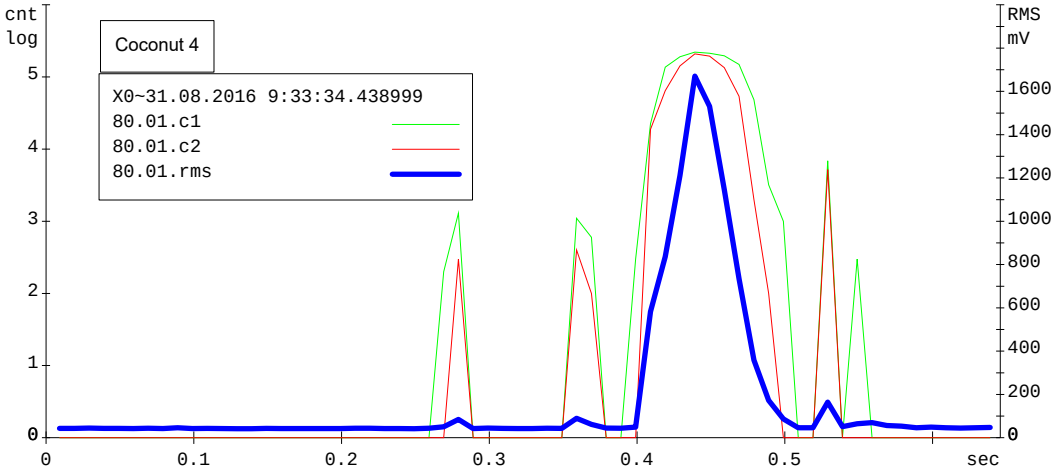
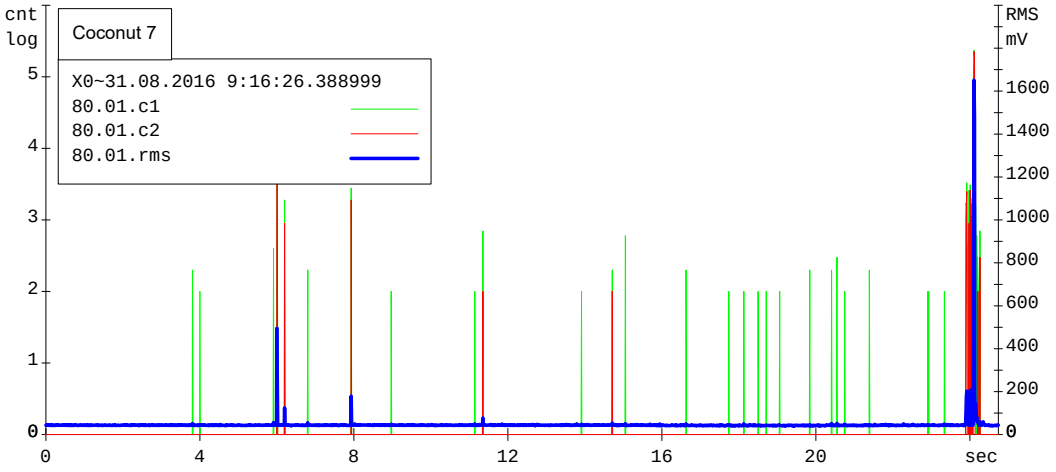


Figure 6 Sample 4, RMS 1639 mV, approached maximum peak



There are two collated samples – the sample 4 and the sample 7 – as the example of the comparison to the measuring results of the acoustic emission signal. The sample 7 indicates similar results as the maximal weighting, see Figure 7. There is similar process of the acoustic emission signal during the creation of the plastic deformation through the weighting about 5463.2 N and the RMS value is 1632 mV.

Figure 7 Sample 7, RMS 1632 mV



One of the defined claims was to predict the moment of creation coconut shell micro fissures during the weighting with the aid of the acoustic emission apparat. The micro fissures prediction must

be exempted because of constitution and structure of the shell. The acoustic emission question linked to coconut shells was also solved by (Wang et al. 2006) and (Braga et al. 1999).

The results of each measuring can be different from each other because of the heterogeneous structure. Many of scientific publications were focused on this question (Woodroof 1979). To make future measuring it is necessary to be engaged in question of the homogeneity of coconut shell, for example to divide the age of coconut to several groups and to assign specific system of data evaluation.

CONCLUSION

There was described the use of acoustic emission during testing quality of coconut shell in this paper. By means of this non-destructive testing method was visualized the signal for better understanding of degradation process of coconut shell by pressure. There was description of the basic research in this work. The system of assessment of degradation was made for engineering applications.

The method of acoustic emission within the research of the compressive strength can bring new improvements how to comprehend specific construction of these shells. It is suitable to evolve specific waveguide for better acoustic emission signal collection for the further research. The coconut shell strength is one of the most important factors affecting the quality off coconut. The technological process needs a feedback for quality control, grading, manipulation, storing and transporting. Coconut succumb to irreversible degradation processes and to inception of fissures by all these factors and that used to be dangerous to health. It is very important to monitor lifecycle of groceries by different ways and instruments.

This experiment was used to configure the methodology and preparation for application of acoustic emission and applied this process for the monitoring of coconut shell strength. It is obvious that submitted methodology can be applied as the one of the measurable parameters for evaluating of different agricultural products strength consistence.

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CONTENT OF PROTEINS, LIPIDS AND SACCHARIDES IN EGG YOLK AND ALBUMEN OF DIFFERENT HEN BREEDS

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Abstract: The first aim of this study was to determine the amount of proteins, lipids and saccharides in the hen egg yolk and albumen of different hen breeds. The second aim was to determine the correlation between amount of analysed components (proteins, saccharides and lipid) in hen albumen and amount of analysed components (proteins, saccharides and lipid) in hen yolk, during storage time. Hen eggs were stored at 6 °C for 57 days and were sampled in two periods (29 June 2015, 23 February 2016). Two different breeds were used for this study – Hybrid Isa Brown (36rd week of lay) for first period and Hybrid Hisex Brown (13rd week of lay) for second period. Total of 180 eggs were analysed. Biuret agent was used for determine of proteins in hen yolk and albumen. Concentrated sulfuric acid and 5% phenol were used for determination of saccharides in hen yolk and albumen. The amount of proteins and saccharides was determined by the UV-VIS absorption spectrometer (Helios Epsilon, Thermo Scientific). For determination of lipids saponification value was used. The differences in amounts of proteins, lipids and saccharides were not found between Hybrid Isa Brown and Hybrid Hisex Brown. Correlation was not found between amounts of proteins, lipids and saccharides in hen yolk and hen albumen for storage time.

Key Words: proteins, lipids, saccharides, UV-VIS spectrometry, hen yolk, hen albumen

INTRODUCTION

Humans have utilized hen eggs as a nutritional food since ancient times (Yamamoto et al. 1997). Hen egg is the source of proteins known for their nutrition, biological and technological potential. (Machado et al. 2007) besides vitamins from the vitamin B complex (thiamine, riboflavin, niacin, pyridoxine and cyanocobalamin), they contain liposoluble vitamins (A, D, E and K). Further, egg contains minerals (iron, calcium, potassium, sodium, phosphorous and zinc). Hen egg is an important source of cholesterol and unsaturated fatty acids, mainly oleic acid (Kritchevsky 2004, Horbanczuk et al. 1999). In many publications the amount of proteins, saccharides and lipid in egg yolk and albumen was observed. (Aquino et al. 2010, Horbanczuk et al. 1999, Kritchevsky et al. 2004, Machado et al. 2007, Segura-Campos et al. 2013)

Analysis of proteins, lipids and saccharides are important for food industry and human nutrition. Content of proteins, lipids and saccharides in hen egg differs from breed hen age, storage, etc. (Janairo et al. 2011, Okutucu et al. 2007).

Modern instrumental methods such as mass spectrometry, absorption spectroscopy, chromatography etc. are used for determination of proteins, lipids and saccharides, but these methods are expensive, difficult for manipulation and time-challenging. Traditional spectrophotometric and titration methods are cheap, fast, easy-working and the most common way to quantitate content of protein (Noble and Bailey 2009).

The first goal of this study was to determine the content of proteins, lipids and saccharides in two different henbreeds – Hybrid Isa Brown and Hybrid Hisex Brown. Second goal of this study was to analyse the influence of storage time of hen eggs to the amount of proteins, lipids and saccharides in hen yolk and hen albumen.

MATERIAL AND METHODS

Hen eggs were analysed in two periods - 29 June 2015, 23 February 2016 - were stored at 6 °C for 57 days. Two different breeds were used for this study – Hybrid Isa Brown (36th week of lay) for first period and Hybrid Hisex Brown (13rd week of lay) for second period. Nine sampling days were for both periods. A total of 180 hen eggs were analysed. Hens were kept in cages.

Determination of proteins in hen yolk and hen albumen

Biuret agent was used for determination of proteins in egg yolk and egg albumen. Biuret agent = copper sulfate pentahydrate $CuSO_4.5\ H_2O$ (c = 13.0 mmol/l), potassium sodium tartrate $KNaC_4H_4O_6.4H_2O$ (c = 32.0 mmol/l), sodium hydroxide NaOH (c = 0.6 mol/l). Diluted sample was added into three tubes, further Biuret agent was added. The tubes were left 30 minutes at room temperature (~ 25 °C). After 30 minutes the absorbance was measured by the UV-VIS spectrometer Helios Epsilon (Thermo Scientific) at 540 nm. Blank (mineralized water in place of sample) was used for determination of proteins in hen yolk and hen albumen. The protein assays were always performed in triplicates for result verification. Calculation of the amount of proteins was based on linear regression equation obtained by evaluation of standard curves of bovine serum albumen.

Determination of saccharides in hen yolk and hen albumen

Sulfuric acid (96%) and phenol (5%) were used for determination of saccharides in egg yolk and egg albumen. Diluted sample was added into three tubes, further sulfuric acid and phenol were added. The tubes were left 30 minutes at room temperature (~ 25 °C). After 30 minutes the absorbance was measured by the UV-VIS spectrometer Helios Epsilon (Thermo Scientific) at 490 nm. Blank (mineralized water in place of sample) was used for determination of saccharides in hen yolk and hen albumen. Protein assays were always performed in triplicates for result verification. Calculation of the amounts of saccharides was based on linear regression equation obtained by evaluation of standard curves of standard solution of D-glucose.

Determination of lipids in hen yolk and hen albumen

Determination of saponification value was used for analysed amount of lipids in hen yolk and hen albumen. Sample was added into round bottom flask, further ethanolic solution of potassium hydroxide (0.5 mol/l) was added. This mixture was heated under reflux for 30 minutes. After 30 minutes phenolphthalein was added. Further this mixture was titrated (0.5 mol/l HCl) into the colorless. Blank (mineralized water in place of sample) was used for determination of saccharides in hen yolk and hen albumen. Protein assays were always performed in triplicates for result verification. Calculation of the amounts of lipids was based on saponification value (Eq. 1).

$$Saponification\ value = \frac{(a-b)\,.\,c_{HCl}\,.\,M_{KOH}}{m} \quad (1)$$

a consumption of HCl for blank (ml)

b consumption of HCl for sample (ml)

c_{HCl} concentration of HCl (mol/l)

M_{KOH} molar weight of KOH (g/mol)

m weight of sample for determination of saponification value (g)

Statistical analyses

Statistical analyses of proteins content in albumen eggs and yolk eggs were made using one-way analysis of variance (ANOVA) and statistical significance was declared when p value was equal to or less than 0.05.

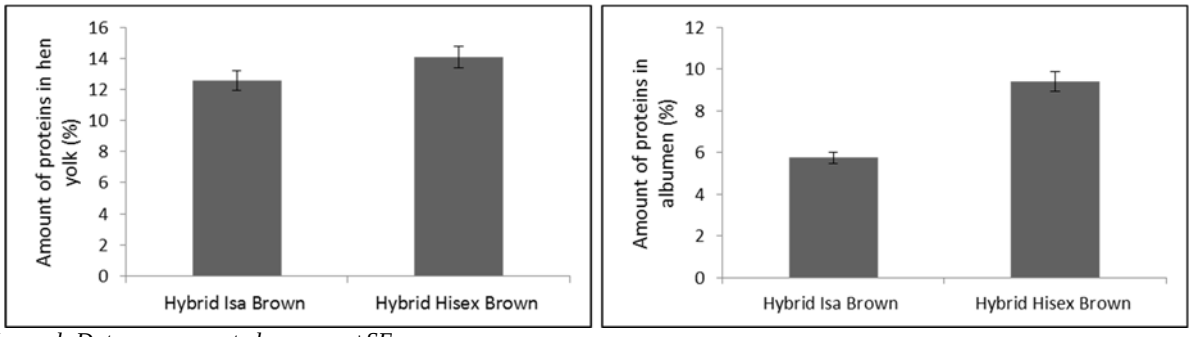
RESULTS AND DISCUSSION

A total of 180 hen eggs were analysed (90 from each period).

Determination of proteins in yolk and albumen for different hens breed

Amount of proteins in hen yolk and hen albumen are shown in Figure 1.

Figure 1 Amount of proteins in hen yolk and hen albumen for different hens breed



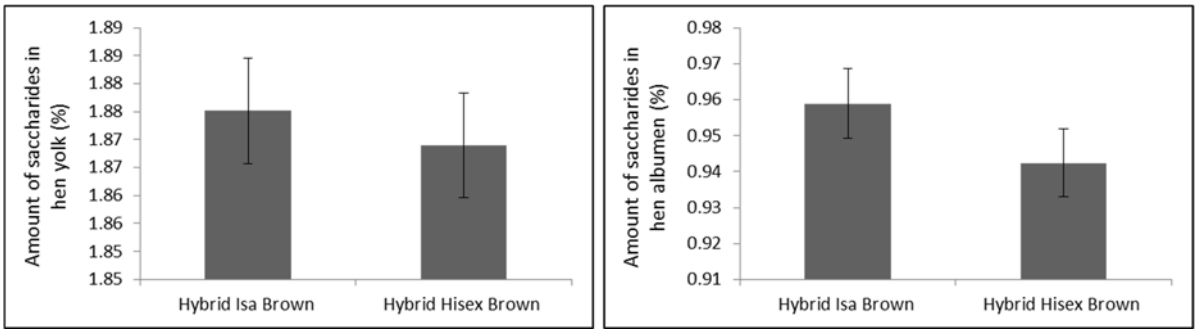
Legend: Data are presented as mean ±SE

The amount of proteins in hen yolk (12.60% for Hybrid Isa Brown, 14.07% mg/ml for Hybrid Hisex Brown) were not statistically different ($p<0.05$) between both breeds. The amount of proteins in hen albumen for Hybrid Hisex Brown (9.40%) were statistically higher ($p<0.05$) than these those in albumen eggs for Hybrid Isa Brown (5.75%). Higher amount of proteins was in hen yolk than in hen albumen in both breeds. Studies (Salakova 2014, Yamamoto et al. 1997, Segura-Campos et al. 2013) demonstrated higher content of proteins in yolk egg than in albumen egg.

Determination of saccharides in yolk and albumen for different hens breed

Amount of saccharides in hen yolk and hen albumen have been shown in Figure 2.

Figure 2 Amount of saccharides in hen yolk and hen albumen for different hens breed



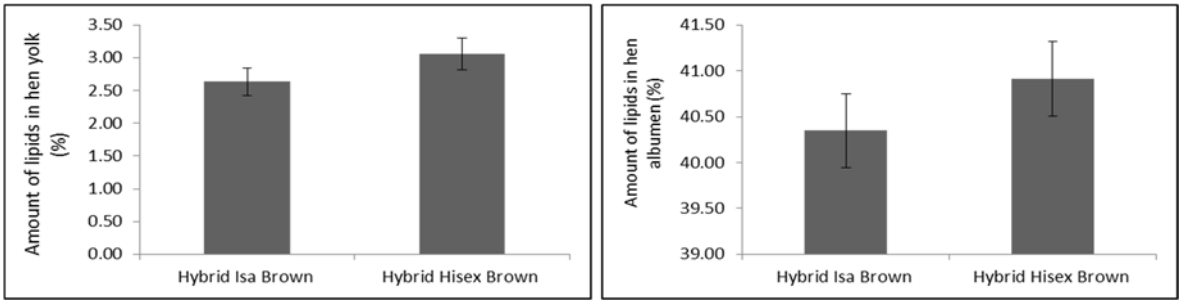
Legend: Data are presented as mean ±SE

The amount of saccharides in hen yolk (1.88% for Hybrid Isa Brown, 1.87% for Hybrid Hisex Brown) and hen albumen (0.96% for Hybrid Isa Brown, 0.94% for Hybrid Hisex Brown) were not statistically different *($p<0.05$)* between breeds. The same amount of saccharides may be caused by the storage time, because amount of saccharides was higher in yolk (1.89%) than in albumen (1.01%) for first day of experiment. On the other hand, amount of saccharides was higher in albumen (3.66%) than in yolk (1.50%) for last day of experiment. Salakova 2014 and Simenovova et al. 1999 presented higher amount of saccharides in yolk than2 in albumen.

Determination of lipids in yolk and albumen for different hen breeds

Amount of lipids in hen yolk and hen albumen are shown in Figure 3.

Figure 3 Amount of lipids in hen yolk and hen albumen for different hens breed



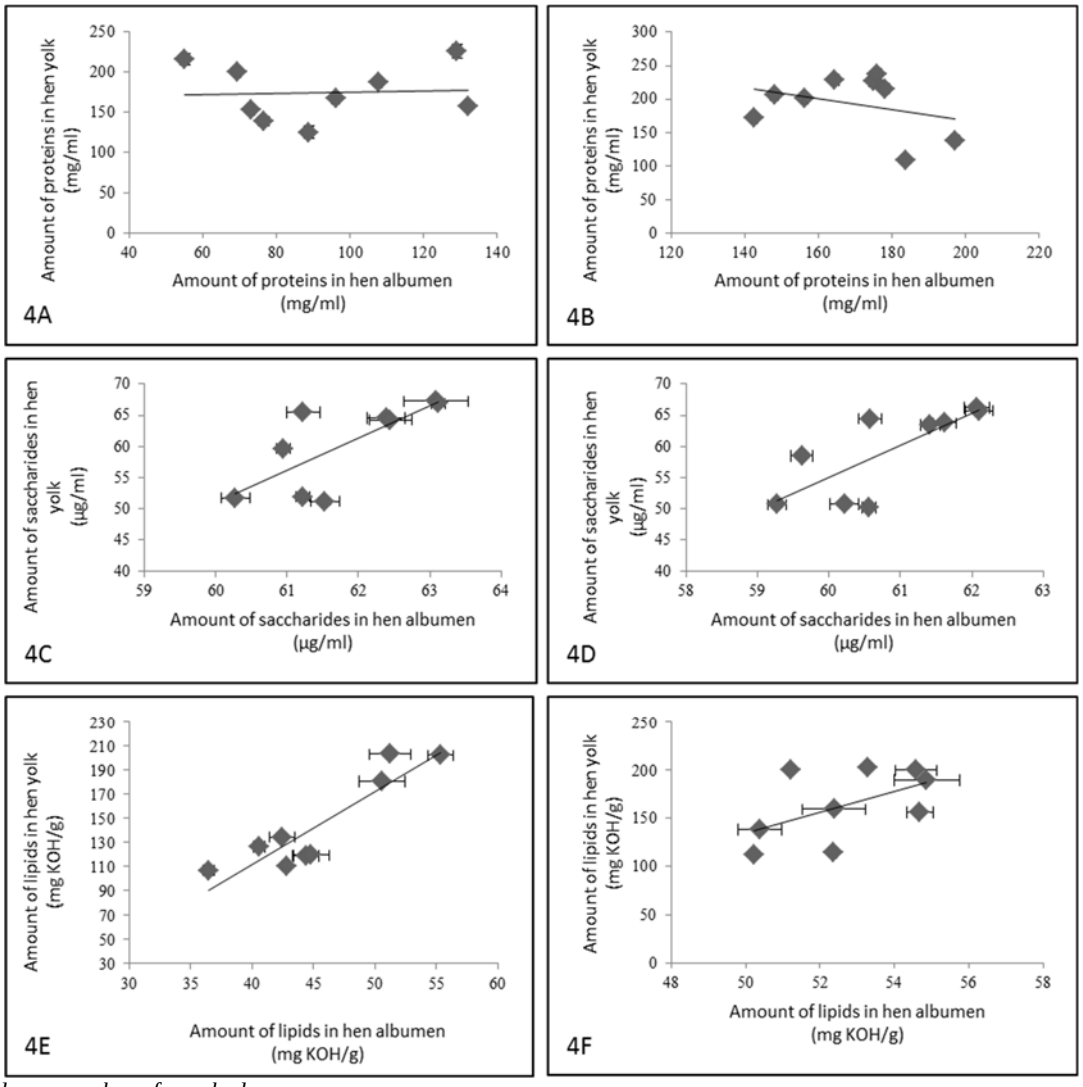
Legend:Data are presented as mean ±SE

The amount of lipids in hen yolk (40.35%for Hybrid Isa Brown, 40.91% for Hybrid Hisex Brown) and hen albumen (2.63% for Hybrid Isa Brown, 3.05% for Hybrid Hisex Brown) were not statistically different ($p<0.05$) between breeds. Higher amount of lipids was in hen yolk than in hen albumen. Aquino and Silva 2010 and Segura-Campos et al. 2013 presented the same results showing the higher amount of lipids in egg yolk than in egg albumen.

Amount of analysed f in hen yolk and hen albumen during storage time

Correlation was used for evaluation results between the amount components (proteins, lipids, saccharides) of the selected egg. Correlations a between amounts of analysed components (proteins, saccharides and lipids) are shown in Figure 4 (A, B, C, D, E, F)

Figure 4 Correlation between amount of proteins (A – Hybrid Isa Brown, B – Hybrid Hisex Brown), correlation between amount of saccharides (C – Hybrid Isa Brown, D – Hybrid Hisex Brown) and correlation between amount of lipids (E – Hybrid Isa Brown, F – Hybrid Hisex Brown) - n = 9



Legend: n = number of supply days

Pearson correlation coefficient was used for comparison correlation between amount of analysed components (proteins, saccharides and lipid) in hen albumen and amount of analysed components (protein, saccharides and lipid) in hen yolk during storage time.

Pearson correlation coefficient was 0.91 for amount of lipids in Hybrid Isa Brown eggs. For Hybrid Hisex Brown, Pearson correlation coefficient was 0.53. Pearson correlation coefficient was low for amount of saccharides in hen eggs (Hybrid Isa Brown r = 0.74, Hybrid Hisex Brown r = 0.76). For amount of lipids, Pearson correlation coefficient was lower than for amount of saccharides (Hybrid Isa Brown r = 0.06, Hybrid Hisex Brown r = -0.33). We can say that amount of analysed components in hen

yolk were not correlated with amount of analysed components in hen albumen. If amount of analysed folders was high in hen yolk, amount of analysed folders was low in hen albumen. Studies (Salakova 2014, Simeonovova et al. 1997, Aquino et al. 2010, Segura-Campos et al. 2013) presented that amount of analysed components (proteins, saccharides and lipids) in hen albumen decreases when amount of analysed components (proteins, saccharides and lipids) is increasing in hen yolk, and conversely.

CONCLUSION

Our results presented the differences in the amount of analysed components (proteins, saccharides and lipids) in hen yolk and hen albumen of different hen breeds (Hybrid Isa Brown and Hybrid Hisex Brown) and correlation between amount of analysed components (proteins, saccharides and lipid) in hen albumen and amount of analysed components (proteins, saccharides and lipid) in hen yolk during storage time. Differences in the amount of analysed components were not found between Hybrid Isa Brown and Hybrid Hisex Brown. Correlations were not found between amounts of analysed components in hen yolk and amount of analysed components in hen albumen. If amount of analysed components was higher in hen yolk, amount of analysed components was lower in hen albumen, and conversely.

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THE ACCEPTANCE OF INSECTS AS PART OF FOOD BY CONSUMERS IN THE CZECH REPUBLIC

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Abstract: Insects are considered a promising source of sustainably-produced protein-rich foods. Western culture attitude towards the value of edible insects dramatically changed in the past 20 years. Even in the Czech Republic the interest in Entomophagy is growing. The cultural centre in Mšec in the Czech Republic held public events. 164 visitors came within two days. Visitors heard a lecture about five breeding species of edible insects and then tasted the dishes with these insects. There were those species: *Tenebrio molitor*, *Zophobas atratus*, *Locusta migratoria*, *Apis mellifera*, *Gryllus assimillis*. Respondents evaluated the taste of insects, the smell, the crunchiness and softness of insects in food, the succulence of insects, the visibility of insect bodies in food, the difficulty of treatment and the difficulty of breeding. The research showed that respondents chose *Apis mellifera* and *Gryllus assimillis* as insects with the best flavours and aroma. Other results showed the suitability of individual insect species to Entomophagy needs in the Czech Republic. Research has also shown how to handle insects in the food that the consumer is the most accepted. Based on these results, we concluded that edible insects could be accepted by consumers as part of food in the Czech Republic.

Key Words: food, edible insect, consumers, entomophagy

INTRODUCTION

Climate change and shortage of water pose tremendous challenges for traditional farming, which can lead to a failure to meet the rising demand for farm animal protein. As a result, other sources of protein should be introduced to meet this rising demand, and in this regard, insects can play a significant role in terms of serving as a vital source of protein. Scientists have established insects to be, in principle, an excellent source of high quality protein that can be more sustainably produced than traditional livestock (van Huis 2013).

Yet most Western consumers are still not keen to adopt insects in their regular diets despite an awareness of the benefits (Verbeke 2015). Many people are reluctant to consume insects, because it is dirty, dangerous and causes fear. This is not true for most species of insects and even less for edible species. Some of the major groups of insects consumed, such as crickets or larvae of Lepidoptera and beetles feed exclusively vegetable matter or wood. From this perspective, they are "cleaner" and more hygienic than lobsters or crabs that feed on carrion (Mitsuhashi 2008). Refusing entomophagy by so-called culturally and economically advanced societies as something unhealthy or designed for primitive cultures rather indicates a misunderstanding of the issue (Premalatha et al. 2011).

When trying to get a new source of protein on the market, it is important to meet consumer expectations in terms of his habits, nutritional requirements and similarities with the food known to the consumer (Verkerk et al. 2007).

The aim of this study was to evaluate the attitude of consumers in the Czech Republic towards the edible insects.

MATERIAL AND METHODS

The description of event, where the survey was conducted

Mšec is a small town in central Bohemia. Public event took place over two days in November 2015. Lectures on the breeding and processing of edible insects were held in the cultural centre of the city. Each visitor listened to a lecture with examples of different types of insects.

Methods for obtaining information

Respondents heard a lecture about breeding insects also have a text with the description of the breed and photographs from farms. The breeder who answered the questions was present during the event. The breeder answered questions about the performance of individual species breeding. Personal relationship with each type and difficulty of handling is often decisive for beginners. Respondents tasted dishes with different kinds of edible insects in another room. These kinds of edible insects were used: *TM– Tenebrio molitor, ZA– Zophobas atratus, LM– Locusta migratoria, AM– Apis mellifera, GA– Gryllus assimillis.* Respondents completed a questionnaire regarding breeding and taste characteristics of each species of insects in the food. The questionnaire was completed by 164 respondents. Responses were evaluated for each type of insect. Semantic differential was prepared to see the results of all endpoints for each insect species.

Insect species used

Insect species used were Nymphs of migratory locust (*Locusta migratoria*), mealworm larvae (*Tenebrio molitor*), Giant mealworm beetle (*Zophobas atratus*), nymphs of field cricket (*Gryllus assimillis*), larvae and pupae of honey bee (*Apis mellifera*). Insects were purchased alive and stored in plastic boxes or frozen and stored in freezers. Bee brood was purchased from beekeepers already frozen in honeycombs.

Meals with edible insects

Another part of the event was tasting the meals that contained the species of edible insects. All the dishes were marked, which kind of insect has been used.

Examples of some of the recipes used

Papuan Crickets – 8 tablespoons butter, 1 cob with garlic cloves, 250 g of live crickets, salt, parsley, instant bouillon, rice. Cut the garlic into slices and slightly roasted in butter until brown. Add the crickets, salt and fry over low heat until crisp (4–5 minutes). Sprinkle with chopped parsley and add instant broth. Serve with steamed rice.

Cocktail Acachapoli – 250 g of locusts, juice of 2 lemons, salt, pepper, chopped 1 tsp chilli peppers. Locusts boil in salted water. When cooking, locusts turn red. Place them in a hot pan and leave them to dry. Then put them on a plate, drizzle with lemon, sprinkle with salt and pepper.

Questionnaire survey

Each insect species were evaluated separately. Respondents rated the insects on the seven-point scale (1–7), who expressed their personal opinion on the issue. The questionnaire was further supplemented with optional data – gender and age. 164 event participants completed the questionnaire. Respondents were aged 9–68 years.

Table 1 The list of questions in the questionnaire

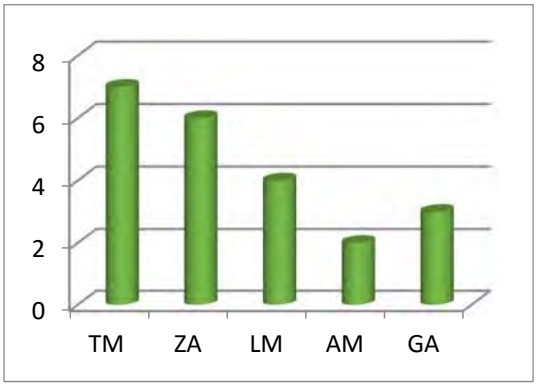
Rate it on a scale from 1 to 7. Nymphs of migratory locust (*Locusta migratoria*)								
	1	2	3	4	5	6	7	
Gross								Tasty
Stinky								Good smell
Not likeable								Likeable
Juicy								Dry
Must not see it								Whole bodies
Soft								Crispy
Difficult to process								Easy to process
Difficult breeding								Easy breeding

RESULTS AND DISCUSSION

Evaluation of edible insects by respondents

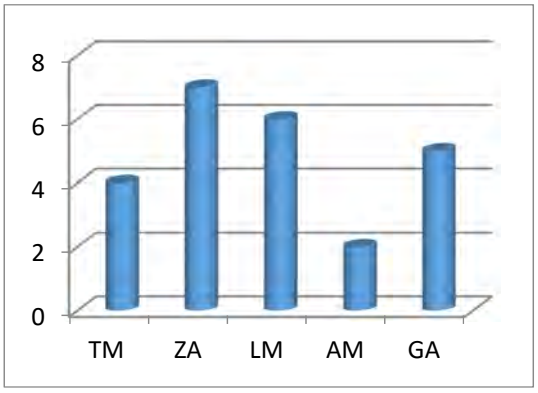
In total 164 answer sampling of respondents from five species of edible insects (*Tenebrio molitor, Zophobas atratus Locusta migratoria, Apis mellifera, Gryllus assimillis*) were evaluated: the easiest breeding insects, according to a survey, were *Locusta migratoria* and *Tenebrio molitor*, the worst was *Apis mellifera*. The easiest to process was *Zophobas atratus*, the most difficult was *Apis mellifera*. Respondents were least bothered eating the body of *Apis mellifera*, the most bothered them *Zophobas atratus*. The driest were *Apis mellifera* and *Locusta migratoria*, the juiciest *Gryllus assimillis*. *Apis mellifera* and *Gryllus assimillis* were very crispy in the meal, Zophobas *atratus* was soft. Respondents are most liked eating *Zophobas atratus* and *Locusta migratoria*; at least they liked *Gryllus assimillis*. *Apis mellifera*, *Gryllus assimillis* and *Zophobas atratus* had a good smell, *Zophobas atratus* was stinky. Respondents rated *Apis mellifera* and *Gryllus assimillis* as tasty, *Zophobas atratus* and *Locusta migratoria* as gross (see Figure 1–8).

Figure 1 The difficulty of breeding, Mšec, 2015



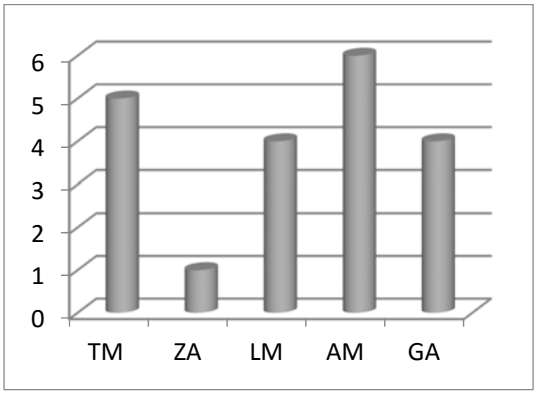
Legend: Difficult breeding=0, Easy breeding =7

Figure 2 The difficulty of treatment, Mšec, 2015



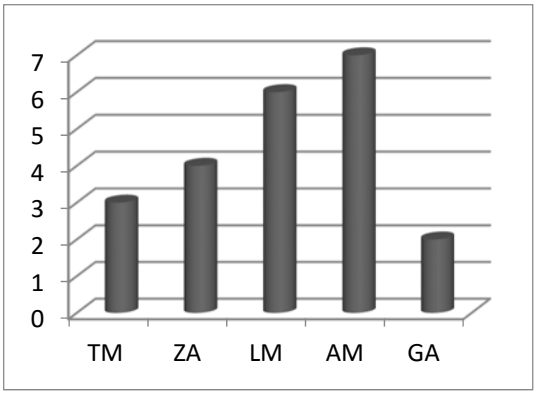
Legend: Difficult to process=0, Easy to process =7

Figure 3 The visibility of insect bodies, Mšec CR, 2015



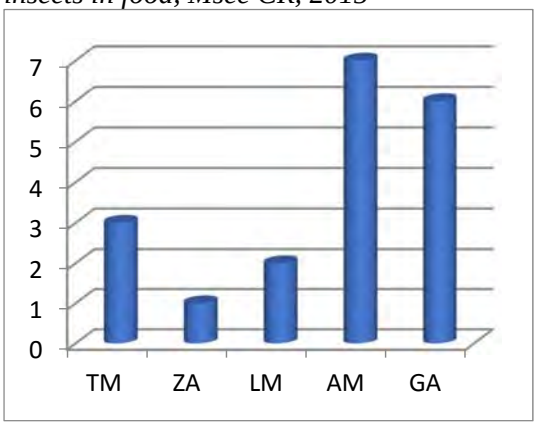
Legend: Must not see it = 0, Whole bodies =7

Figure 4 The succulence of insects in food, Mšec, CR, 2015



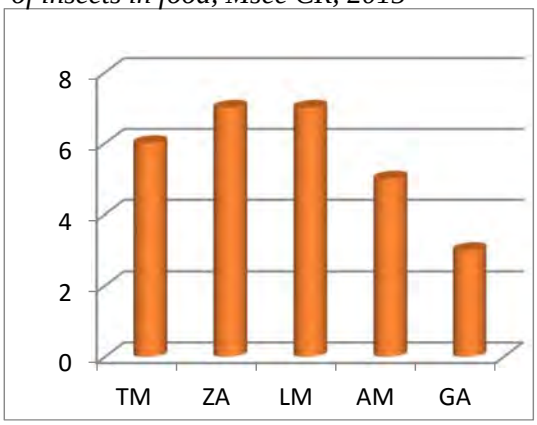
Legend: Juicy = 0, Dry = 7

Figure 5 The crunchiness and softness insects in food, Mšec CR, 2015



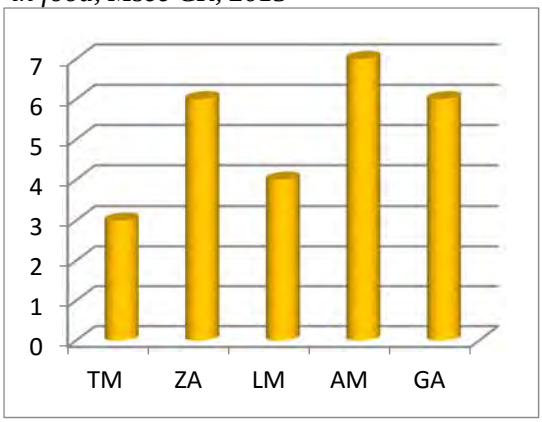
Legend: Soft = 0, Crispy = 7

Figure 6 The goodliness of insects in food, Mšec CR, 2015



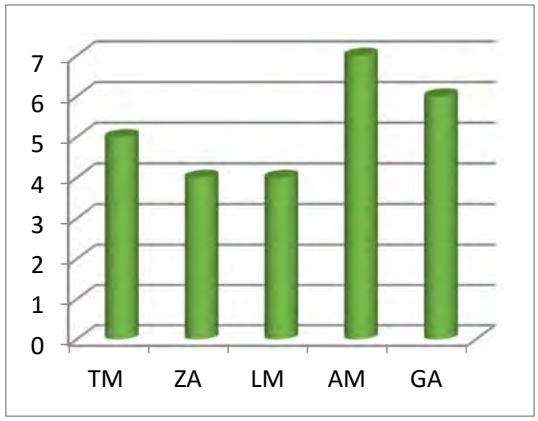
Legend: Not likeable = 0, Likeable = 7

Figure 7 The smell of insect in food, Mšec CR, 2015



Legend: Stinky = 0, Good smell = 7

Figure 8 The taste of insects in food, Mšec CR, 2015



Legend: Gross = 0, Tasty = 7

TM– Tenebrio molitor, ZA– Zophobas atratus, LM– Locusta migratoria, AM– Apis mellifera, GA– Gryllus assimillis

Evaluation of individual species of edible insects

Nymphs of migratory locust (*Locusta migratoria*) LM

This species was evaluated as the best by consumers, with the ratio of negative to positive ratings 1:7. The respondents positively evaluated the simplicity of the breeding process. This species is quite big, and therefore it is often required not to be seen in the food.

Larvae and pupae of honey bee (*Apis mellifera*) AM

The ratio of negative and positive characteristics was 2:6. The species reached the best values in taste, smell and appearance of the food. Visible larvae in food do not matter. The negative aspect is difficulty of obtaining.

Giant mealworm beetle larvae (*Zophobas atratus*) ZA

This species was evaluated rather positively with the ratio of 2:6. Ease of breeding and processing, crispness and aroma of the food were positively evaluated. The appearance of the food was rated negatively. Insects should not be visible in the foodstuff.

Mealworm larvae (*Tenebrio molitor*) TM

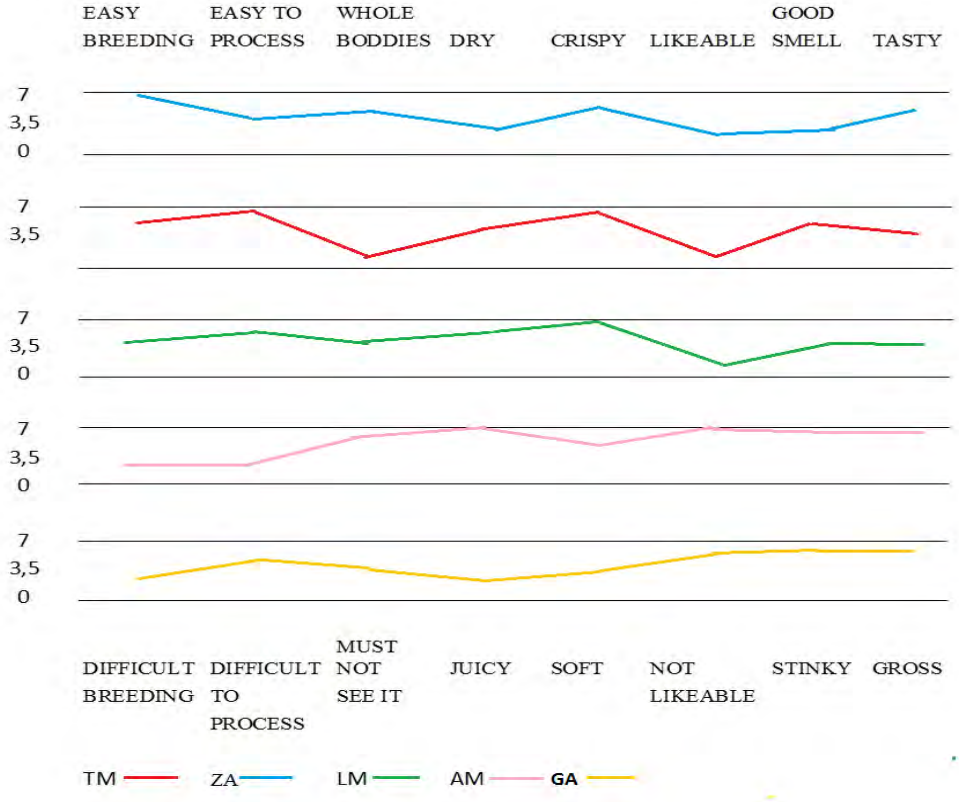
Positively rated species, with a ratio of negative to positive evaluations 3:5. Very positively evaluated were flavour, crispness and simplicity of breeding.

Nymphs field cricket (*Gryllus assimillis*) GA

Rated rather positively with the ratio of 3:5. Taste was evaluated positively. Soft body bothered some respondents. Mainly the difficulty of breeding was assessed negatively.

Evaluation of individual species of edible insects (see Figure 9).

Figure 9 Semantic differential comprehensive evaluation of individual insect species respondents, Mšec CR, 2015



CONCLUSION

The acceptance of some species of edible insects as part of the food in the Czech Republic is possible. Nymphs of migratory locust (*Locusta migratoria*), larvae and pupae of honey bee (*Apis mellifera*) and Giant mealworm beetle (*Zophobas atratus*) are among the most suitable species. Mealworm larvae (*Tenebrio molitor*) and nymphs of Field cricket (*Gryllus assimillis*) can be used as part of the food with some difficulties too.

ACKNOWLEDGEMENTS

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EFFECT OF STORAGE DURATION ON THE ANTIOXIDANT ACTIVITY OF THE HEN AND QUAIL EGGS USING ABTS METHOD

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Abstract: Eggs are a source of proteins and bioactive molecules, including antioxidants. The effect of storage time on the antioxidant activity in hen and quail egg components was chosen as an important parameter in this work. For the determination of antioxidant activity ABTS method was used. Our results showed higher antioxidant activity in yolk in both kinds of eggs and quail eggs showed higher activity in comparison with hen eggs. The obtained results also suggested that the total antioxidant capacity was decreased during storage, which was probably caused by the loss of naturally occurring antioxidants or formation of new compounds with pro-oxidant activity during storage.

Key Words: hen egg, Japanese quail egg, antioxidant activity, ABTS

INTRODUCTION

The chemical and nutrient composition of egg is well known (Kovacs-Nolan et al. 2005, Seuss-Baum 2007, Li-Chan and Kim 2008). The nutritional composition of quail and hen eggs is similar, although quail eggs are highly prized for their composition, because they contain around 2.5 times more vitamins A, B_1 and B_2 (Baumgartner and Hetenyi 2001). Concurrently, literature sources suggest that quail eggs have a slightly higher proportion of protein, carbohydrates, minerals and water (Shanaway 1994, Oliveira et al. 2009).

The eggs are perceived as important food product due to multifunctional properties and their nutritional, biological and technological potential. In addition to the nutritional value, biological molecules in eggs are a source of proteins and bioactive molecules, including antioxidants (Yu et al. 2011), which have different activities and they can render important health benefits (Miranda et al. 2015), but they are not generally considered as antioxidant diets. They are subjected to various processing and storage conditions before consumption, which may influence the antioxidant capacity of egg components. The effect of egg processing and storage conditions on the overall antioxidant activity play important role.

An antioxidant is defined as any substance that slows down, changes or removes oxidative damage to a target molecule (Halliwell 2007), directly acts as reactive oxygen species (ROS) (Ngo et al. 2011) or indirectly acts to regulate antioxidant barricade or inhibit ROS production (Khlebnikov et al. 2007). Consumption of antioxidants through diet is thought to be important in reducing oxidative damage (Valko et al. 2007, Halliwell 2012). These antioxidants play a critical role in protecting cellular components from potentially damaging ROS and maintaining homeostasis and cellular functions.

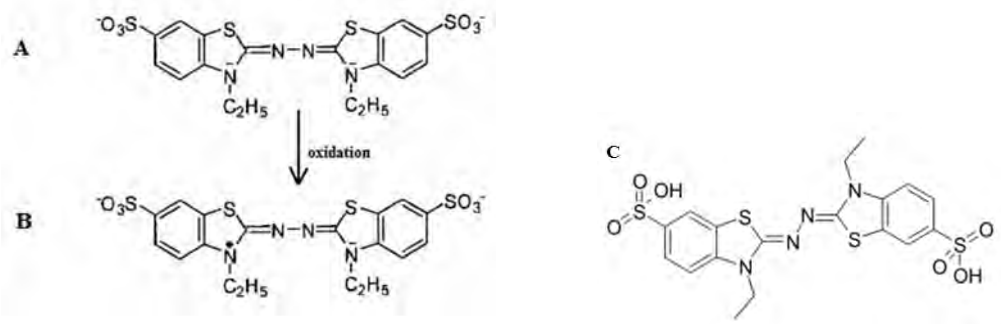
The ability of an antioxidant to inhibit the oxidative degradation of various compounds is defined as antioxidant activity (to prevent lipid peroxidation etc.) (Surai 2007). Egg storage is associated with lipid peroxidation within egg membranes, particularly those containing high levels of polyunsaturated fatty acids (Kodovska 2013).

In recent years many various methods for determination of antioxidant activity have been developed in chemical analysis and biological evaluation of antioxidant characteristics. The ABTS method is one of the most used methods for determining of total antioxidant activity. The sample is tested for the ability to extinguish a radical cation ($ABTS^{\bullet+}$) + ABTS (2,2'-azinobis (3-

ethylbenzothiazoline-6-sulfonic acid)), which is generated by oxidation of ABTS with potassium persulfate and it is reduced in the presence of hydrogen-donating antioxidants (Figure 1A–C). Radical $ABTS^{\bullet+}$ antioxidants that act as hydrogen donors, measured spectrophotometrically at a wavelength length of 734 nm on the basis of changes in the absorption spectrum. This method is very fast, simple and allows a various evaluation of the antioxidant activity of many substances and mixed samples (Paulova et al. 2004).

The main aim of this work is to study the effect of storage duration on the antioxidant activity of the egg components of hen and quail eggs.

Figure 1 A Molecular formula of ABTS, B Molecular formula of $ABTS^{\bullet+}$, C Formula of ABTS



MATERIAL AND METHODS

Hen and Japanese quail eggs

A total of 90 eggs obtained from hens and 180 Japanese quail eggs were used to investigate the effect of storage time on quality of eggs. One sample mixture obtained 10 pieces of eggs, which were analysed in triplicate. Therefore total was examined 81 analyses. Hens (ISA BROWN) and Japanese quails (Coturnix coturnix japonica) were kept in cage technology at a commercial breeding farm in the South Moravia region (Czech Republic).

The eggs were tested fresh and after storage for 1, 2, 8, 14, 21, 28, 42 and 56 days. Eggs were stored using refrigeration at 4 °C. All chemicals were purchased from Sigma-Aldrich (USA).

Sample preparation

The albumen and yolk were separated, after they were blended for 10 minutes to get homogeneous mixtures and after that they were used for determination of antioxidant activity. The melange was prepared by mixing whole eggs samples and blended for 10 minutes.

Antioxidant activity determination

ABTS 2,2′-Azinobis-(3-ethylbenzthiazoline-6-sulfonate acid) assay was based on the method of Re et al. (1999) with a slight modification. ABTS radical cation ($ABTS^{\bullet+}$) was produced by the reaction between 3.5 mmol/l ABTS solution and 0.06 mmol/l potassium persulfate ($K_2S_2O_8$). The solutions were mixed in a ratio 50 : 1 (ABTS : $K_2S_2O_8$) and the mixture was allowed to stand in the dark at room temperature for 16 h before use. After this time, the mixture was mixed with freshly prepared acetate buffer (pH 4.3) in a ratio 39 : 1 (buffer : ABTS). 2 ml of the prepared mixture and 25 ml of tested sample (albumen/melange/yolk) were pipetted into the tubes. The acetate buffer was used as a blank.

The absorbance of the mixture was measured at 734 nm after 30 minutes of incubation at room temperature. All determinations were carried out in triplicate.

Statistical analyses

Statistical analyses of antioxidant activity in hen and quail eggs were made using one-way analyses of variance (ANOVA) and statistical significance was declared when p value was equal to or less than 0.05.

RESULTS AND DISCUSSION

Calibration curve of gallic acid

The absorbance was compared with a standard curve of prepared gallic acid solutions (0.01, 0.02, 0.03, 0.04, 0.05, 0.06 μmol/l) and expressed as % of gallic acid.

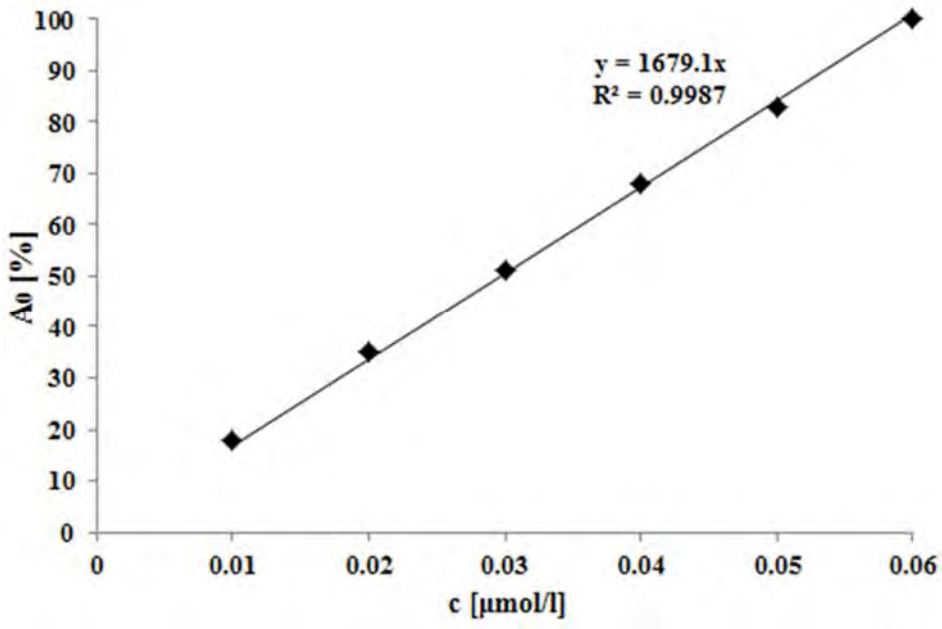
An absorbance was calculated by the formula:

$$A\ (\%) = A_0 - (A_1 / A_0) \cdot 100 \quad ,$$

where A_0 is the absorbance of the prepared mixture, and A_1 is the absorbance of the extract measured after 30 minutes.

The resulting dependence of the loss of absorbance A_0 *versus* concentration of gallic acid is shown in Figure 2 using a calibration curve of gallic acid.

Figure 2 Calibration curve of gallic acid



Antioxidant activity in hen and quail eggs

Figures 3 and 4 show antioxidant activity of hen and quail eggs during 56 days of storage. The higher antioxidant activity was observed in yolk compared with albumen ($p < 0.05$) in both kinds of eggs, which could be caused by oxidation of egg lipids, located in yolk. Approximately 65% of yolk lipids are triglycerides, while phospholipids, cholesterol and carotenoids create 30% and 4%.

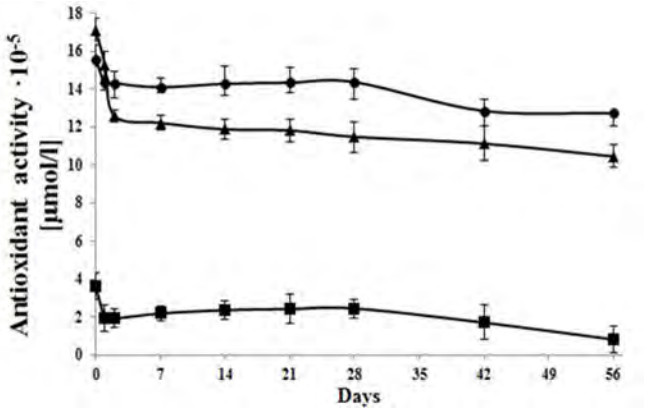
The aromatic amino acids and carotenoids contained in egg yolk are the major contributors to the antioxidant properties and probably due to these components the highest antioxidant activity in hen and quail yolk compared with albumen ($p < 0.05$) is shown in the figures 3 and 4. Our observation agrees with results of Remanan and Wu (2014), which suggest that higher antioxidant activity was determined for fresh egg yolk in comparison with fresh egg albumen and whole eggs.

The present study demonstrated that egg yolk antioxidant activity is stable during storage for all experiments. The results of Nimalaratne et al. (2016) showed that the antioxidant activity is stable during six weeks of simulated retail storage. In contrast, in the study of Barbosa et al. (2011), the total antioxidant activity of egg yolk decreased significantly after 14 days of storage at refrigeration temperature and after 7 days at room temperature.

It is known that the egg albumen protein hydrolysate has good antioxidant activity (Chen and Chi 2011).

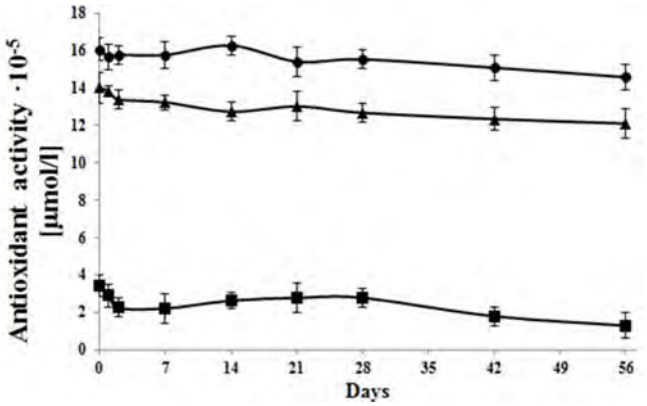
Obtained results suggest that storage can influence antioxidant activity, which decreased during storage after 28 days of experiment ($p > 0.05$), mainly for albumen components in both kinds of eggs. Similar results were observed in work of Xu et al. (2007), who tested antioxidant activity of hen egg ovalbumin in albumen.

Figure 3 Antioxidant activity of hen egg components (albumen, melange, yolk) during 56 days of storage



Legend: – Hen albumen, – Hen melange, – Hen yolk

Figure 4 Antioxidant activity of quail egg components (albumen, melange, yolk) during 56 days of storage



Legend: – Quail albumen, – Quail melange, – Quail yolk

Figure 4 shows that antioxidant activity of quail eggs is slightly higher ($p > 0.05$) in comparison with hen eggs (Figure 3), which could be caused by the fact that quail eggs have a slightly higher proportion of proteins, minerals and vitamin A, which may cause higher antioxidant activity (Shanaway 1994, Oliveira et al. 2009).

The bioactivity of egg antioxidants can be affected by food processing and storage. However, the antioxidant properties of egg may vary depending on several factors, for instance egg types, processing and storage conditions (Nimalaratne et al. 2016).

CONCLUSION

This study was focused on the effect of storage duration of the hen and quail eggs on the antioxidant activity using ABTS method. The antioxidant activity of egg components was measured and results of this study showed that higher antioxidant activity was observed in yolk compared with albumen ($p < 0.05$) in both kinds of eggs. The comparison of hen and quail eggs showed that higher activity was observed in quail eggs ($p > 0.05$). The antioxidant activity was decreased during storage ($p > 0.05$), which was probably caused the loss of naturally occurring antioxidants or formation of new compounds with pro-oxidant activity during storage experiment.

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ACOUSTIC EMISSION MONITORING OF CHICKEN FEMURS FIRMNESS IN BENDING TESTS

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Abstract: This paper describes standard testing of chicken broiler femoral bones strength with destructive breaking test and monitoring of structural stress with the acoustic emission (AE) signal in real time. Tested samples were assigned to four different groups according to nutrient levels in chicken feed, namely magnesium and calcium. Tested chickens were fast-growing hybrids ROSS 308, commonly used in poultry production. Objective of this experiment was assessment of AE recording in course of bending and breaking test, where the AE signal provides information on internal structural changes in material.

Key Words: acoustic emission, broiler, femur firmness, ROSS 308

INTRODUCTION

Bones of adolescent meat-type poultry exhibit a high incidence of problems that include weak skeletal structure, deformities, breakage, infections and osteoporosis-related mortality (Rath et al. 2000). Occurrence of these maladies is reflected in lameness, high mortality rate in adolescence and subsequently in decreased economic yields. Quality of bone structure is determined by nutrition, which is crucial in period of rapid adolescent growth. However, it is necessary to understand the physiology of bone development.

Bones consist of approximately 70% mineral, 20% organic matter and 10% water. Main structure is organic colagen matrix providing tensile strength and mineral hydroxyapatite providing compressional strength (Rath et al. 2000). Low metabolic uptake of phosphorus and/or calcium is related to thin bone structure prone to breaking. Experiment was aimed to assess the bone structural performance in conditions of decreased Ca and Mg nutrition.

Bones perform at their best subjected to static axial loads. Bending deformation occurs under fixation of both ends of bone, when force is applied perpendicular to length axis. Upper layers of bone are compacted and deformed with pressure, bottom layers are stretched and deformed with traction. Bones that are structurally conditioned to sustain the bending stress are hollow in core with ends extended into joints to spread the pressure onto larger area.

Acoustic emission (AE) is a physical phenomenon, where plastic deformation in material is accompanied with acoustic noise or cracking emitted inside the material (Cole et al. 2005). Due to ČSN EN 1330-9 technical standard nomenclature the term acoustic emission denotes the elastic tension waves generated by dynamic release of mechanical tension inside the material or in process causing the occurrence of elastic tension waves on surface of the object (Pazdera et al. 2004).

Entire process of emergence and detection of AE consists of subsequent phases: occurrence of AE, spread of tensile waves from source to sensor, detection of tensile waves with sensor, conversion to electric signal and finally assessment of resulting electric signal on measuring apparatus (Askeland et al. 2006).

MATERIAL AND METHODS

Femurs of ROSS 308 broiler hybrid were used in experiment; bones were prepared by de-boning the chicken thighs. Remnants of meat were scraped off with a sharp knife to use only a clean bone in experiment.

Tested samples were divided into four groups. Individual groups of chickens differ from each other in quantity of Mg (0–0.5 g) and Ca (6–9 g) in feed mix. In groups without addition of Mg the only source of Mg was in components of feed. Samples selected for experimental purposes are described in (Table 1) Samples were equipped with piezoelectric AE sensor to estimate the detailing characteristics.

Table 1 XEDO measuring aparatus configuration

Sample no.	Group	Cage/chicken ID	F [N]
1	Ca 6 g; Mg 0.5 g	2/541	265.98
2	Ca 6 g; Mg 0.5 g	10/645	317.26
3	Ca 6 g; Mg 0.5 g	10/572	248.99
4	Ca 6 g; Mg 0.5 g	10/596	216.84
5	Ca 9 g; Mg 0 g	11/595	217.65
6	Ca 6 g; Mg 0 g	13/510	232.53
7	Ca 9 g; Mg 0.5g Control	12/511	284.41

Universal measuring apparatus was employed to measure physical characteristics (TIRA test 27025, see Figure 1). The apparatus enables measuring of various materials for tensile, pressure and bending resistance. Bending test was selected for purpose of this experiment, where bone is burdened to the breaking point or to reduction of applied force by 30% (Table 2). Result of this test is a part of working diagram, which provides the data of breaking threshold in bending (Figure 2).

Figure 1 Tested sample equipped with piezoelectric AE sensor in bending test

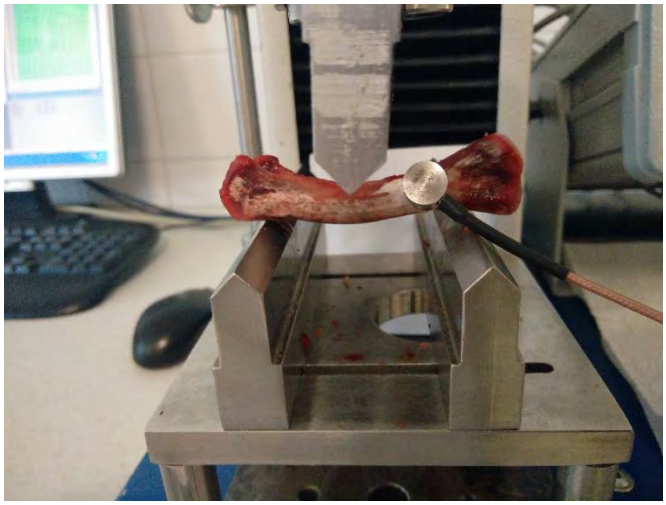


Table 2 TIRAtest 27025 universal measuring apparatus configuration

Parameter	Value
Force sensor:	1000 N
Test type:	Bending test
Velocity:	10 mm/min
Test end:	Force reduction by 30%

AE signals were recorded in course of test with piezoelectric sensor fixed with acoustic glue, which created a sound-carrying environment. It is necessary to carefully fix the AE sensors to the surface of tested sample to obtain quality data (Kopec 2008). Contact between the sensor and sample surface is realized through minimal contact areas on peaks of material microstructure. Majority of space under the

sensor surface is filled with air which has five orders of magnitude higher acoustic impedance compared to direct surface contact and therefore AE wave transfer is significantly reduced (Masmoudi et al. 2015). Primary function of bonding agent is to expel the air present between contact surfaces and thus enhance the signal transfer. AE measuring was conducted with Dakel XEDO measuring instrument.

In course of AE measuring the RMS (root mean square) parameter was observed. This parameter denotes so-called effective signal value. In case of alternating current the RMS is equal to direct current value, which would provide the equivalent mean electric power after application of resistance load. Unit of RMS is mV. This value responds to quantitative characteristics of measured AE events (Dostal et al. 2012).

RESULTS AND DISCUSSION

In course of single experimental measurement 20 repeating verification measurements were conducted. For better clarity only representative results are listed in graphs. When comparing the samples considering the bending stress it is clearly visible that results show high variability. Data from individual AE measurements are arranged and represented in form of graphs. The record consists of AE signal overshoot count. The record represents the basic sample characteristics. X axis represents the time course of test run, Y axis represents the AE intensity in logarithmic scale and its energy level (Counts). Red colour represents temporal relation of localized AE events count, green represents temporal relation of entire AE events count, blue curve shows the course of RMS effective signal values.

Preliminary results show variable performance of samples in comparison among individual samples and also among sample groups. In all graphs the initial increase in signal intensity is clearly visible in the beginning of a record, which probably represents the first micro fissures in course of force load. It is clear that collapse of sample integrity is accompanied with strong emission activity of AE sources and simultaneous fast collapse of structure in samples 11/595, 12/511, and 13/510. Contrary, the (Figure 3, 4, 5, 6) well document the final increase of AE RMS, which occurred previously and collapse of samples was accompanied with much lower RMS levels. Collapse of samples responds to finish of RMS curve, which indicates the stopping of measuring apparatus when achieving the maximum loading force.

Another phenomenon, which deserves attention in further course of this experiment is proper course of RMS in the test run.

Figure 2 Bending test results

Figure 3 AE record of specimen 2/541 bending test

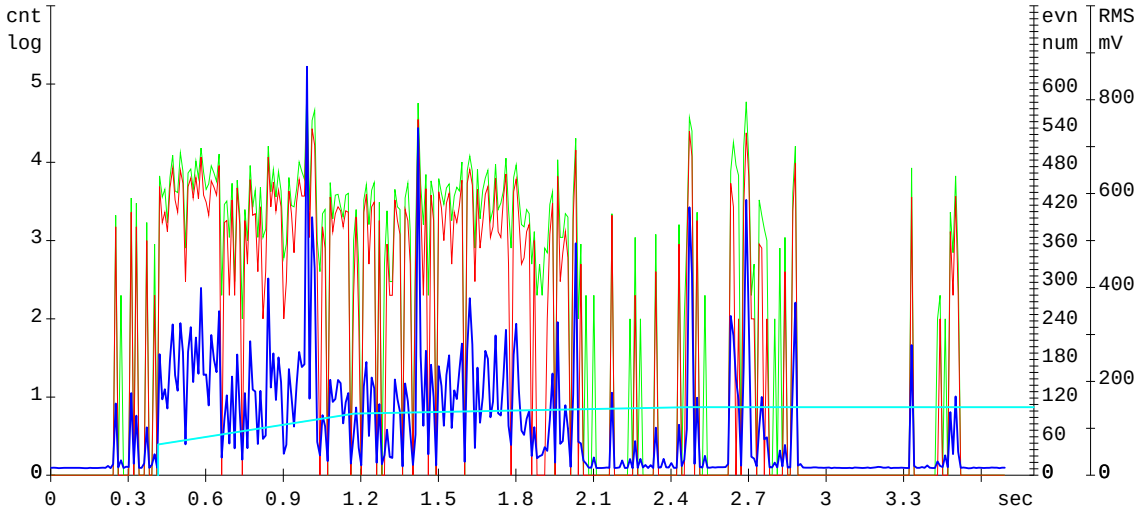


Figure 4 AE record of specimen 10/645 bending test

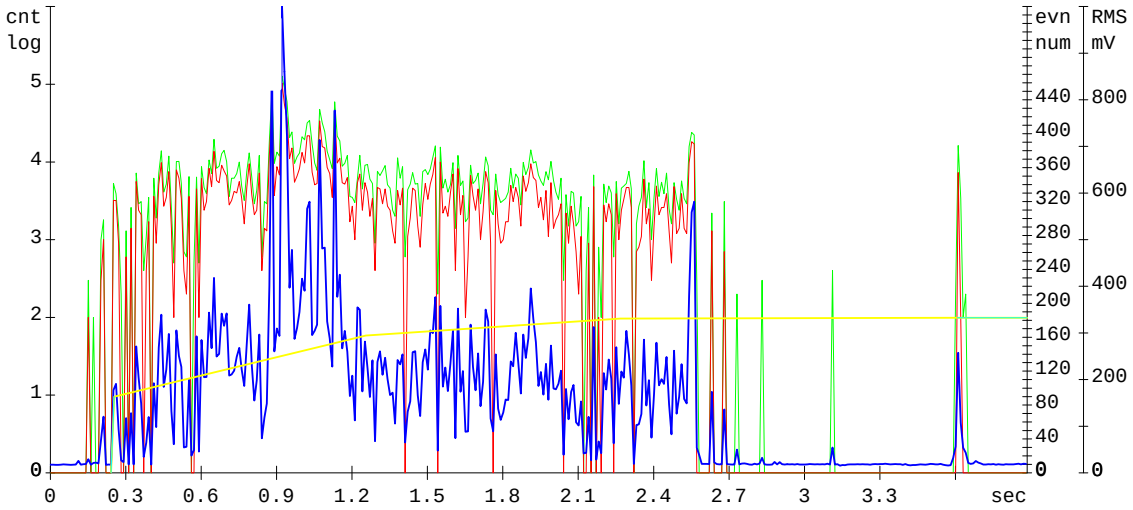


Figure 5 AE record of specimen 10/572 bending test

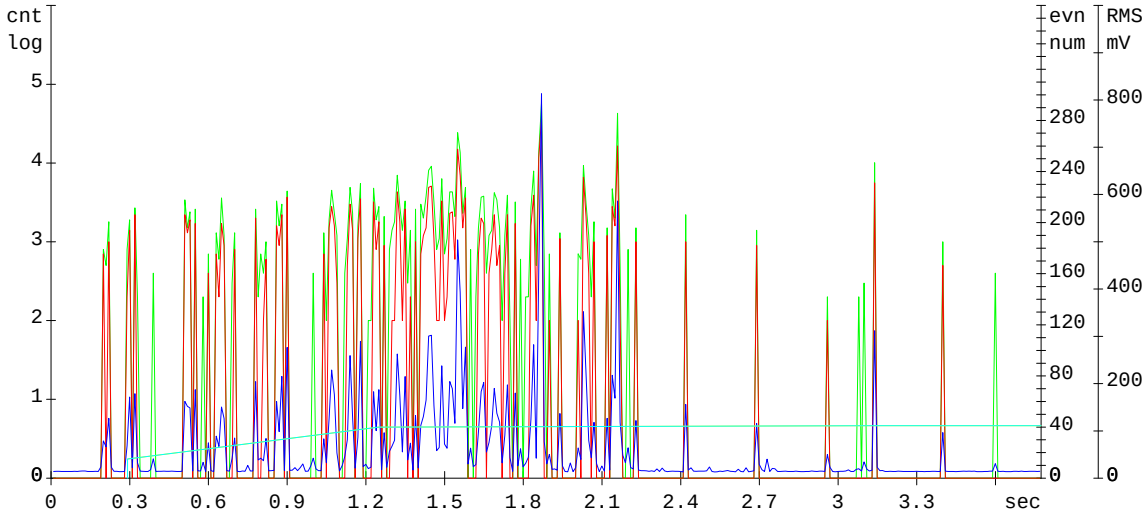


Figure 6 AE record of specimen 10/596 bending test

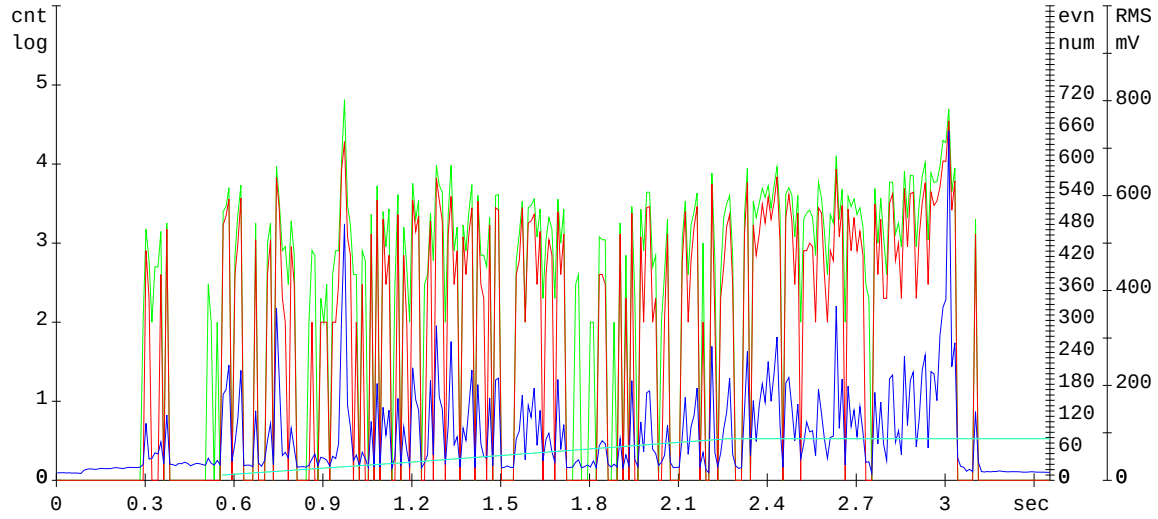


Figure 7 AE record of specimen 11/595 bending test

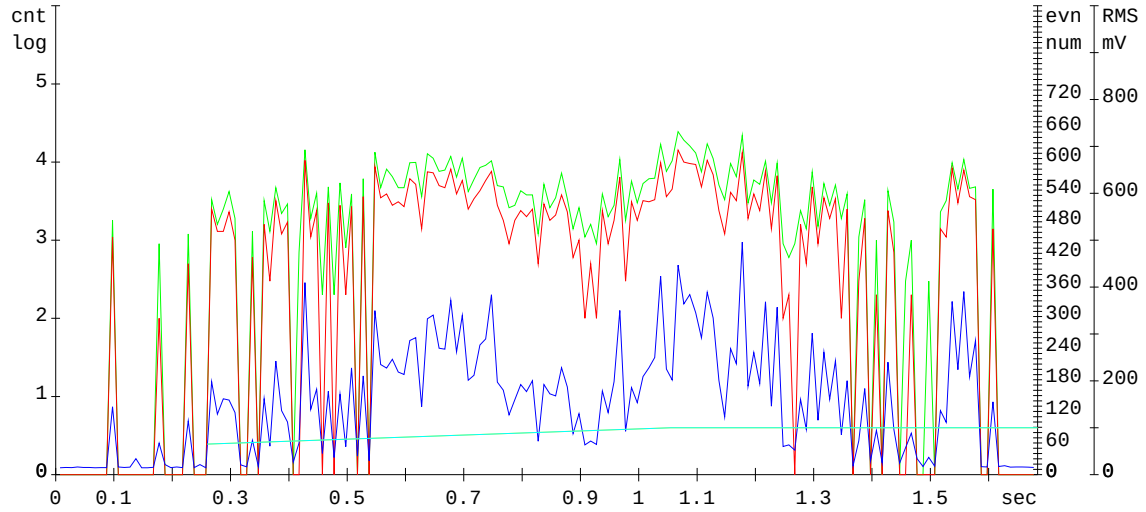


Figure 8 AE record of specimen 12/511 bending test

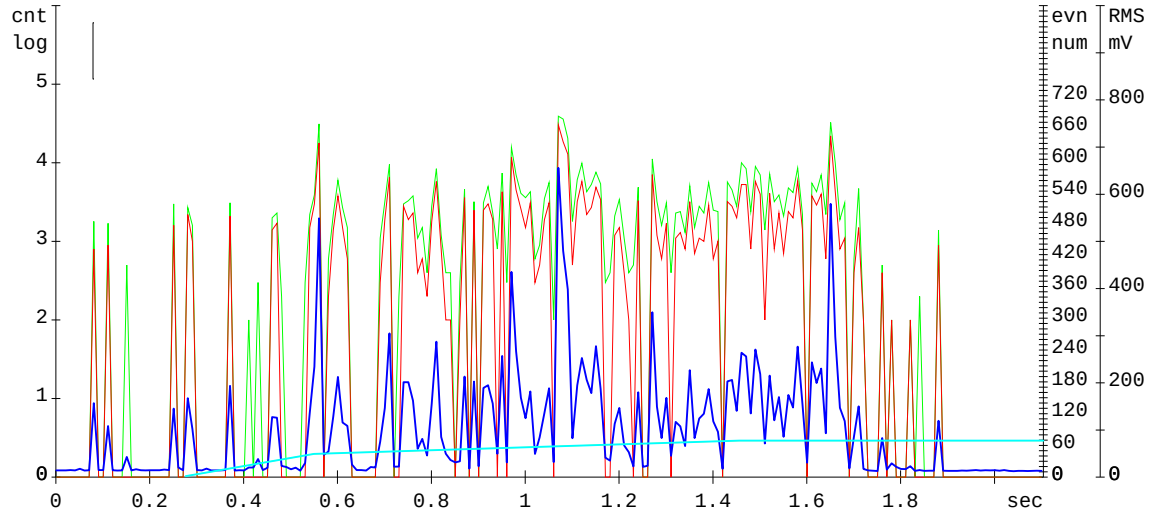
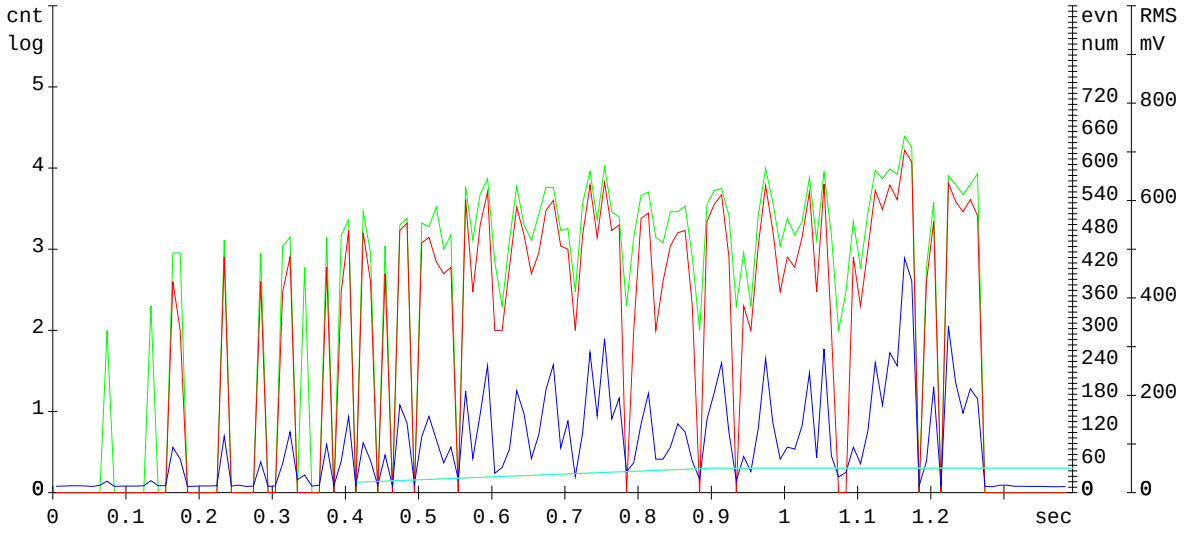


Figure 9 AE record of specimen 13/510 bending test



CONCLUSION

The pilot experiment demonstrated without a doubt that proposed method doesn't show significant shortcomings and it is fully usable in subsequent research including larger pool of stressed samples. AE sensor was fixed in simple and reliable manner. Configuration of measurement chain was calibrated thoroughly considering the predicted AE signal characteristics. Possible extraneous sources of AE signal were eliminated (e.g. run noise of the testing machine, fixture of samples). In entire sample set the AE signal was reliably detected and responded to applied stress load.

In course of recording the samples reported emergence of emission packets related to increasing load, therefore it is possible to clearly interpret the measured data; further advantage is the possibility to determine the fracture occurrence in course of force loading. Damage of internal structure in material is a crucial aspect for AE detection and measurement. Poultry bone resists the pressure until its firmness limit with significant recording of structural changes in material. After reaching this limit many acoustic pulses emerging from sample are recorded.

As in many other AE method applications here also applies that non-destructive AE testing provides invaluable information on processes taking place in internal structure of material and simultaneously presents the proper measured data explication for careful judgement of sometimes not clearly interpretable indices.

Experiment showed the applicability of AE survey for analysis and represented the interesting view on behaviour of internal AE sources in the structure of material subjected to mechanical pressure stress testing. From this hypothesis we can conclude that applicability of AE represents a viable approach for further research. One of possible practical applications is determination of mineral nutrient influence on bone firmness to prevent injuries in large capacity holdings, where intensive chicken breeding puts the bone structure under high stress due to high muscular growth rate. With this simple testing method, we are able to precisely assess the effect of various levels of nutrition onto the desired skeletal parameters.

ACKNOWLEDGEMENT

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Section – Plant Biology

ANALYSIS OF ROOT SECRETED PROTEINS IN *NICOTIANA TABACUM*

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Abstract: Plant secretomics is a new and largely unexplored area of the plant proteomics. Evidence is emerging on the important role of secreted proteins in plant's interactions with its environment. Here, we tested a hydroponic-based cultivation of *Nicotiana tabacum* and analyzed root secreted proteins that accumulated within 48 h. Altogether, more than 350 proteins were detected in the hydroponic medium.

Key Words: secretome, roots, LC-MS, proteome

INTRODUCTION

In plant cells, many proteins undergo secretion to the extracellular space in order to maintain cell structure, regulate the external environment or as a part of signaling and defense mechanisms. Plant secretomes have been studied in natural conditions, during nutritional deficiency, biotic and abiotic stress or after hormone treatment (Alexandersson et al. 2013). Previous analyses of plant secretome have shown that hundreds of proteins are secreted into the apoplast. These proteins constitute the interface between the plant cell and its environment and present an excellent framework for identification of biomarkers for signal and stress cues and biotic interactions. To identify apoplastic proteins, a cell culture experiment or vacuum-assisted extraction from separated plant organs have been used. Here, to analyze proteins secreted by an intact healthy plant into its environment, we tried to extract proteins secreted by plant roots in a hydroponic culture.

MATERIAL AND METHODS

Plant material

Plants of *Nicotiana tabacum* (SR1) were cultivated in ½ Murashige-Skoog medium in a hydroponic culture (e.g. Skalák et al. 2016). After four weeks, plantlets were separated into 15 ml tubes with 10 ml of hydroponic medium. Root secretome was collected after 48 h.

Protein extraction

The collected secretome was precipitated by acetone/TCA, dissolved in urea and digested with 20 µl of an immobilized trypsin (Promega) (Černý et al. 2014, Novák et al. 2015). The resulting tryptic digests were desalted by C18 SPE (Černý et al. 2013).

LC-MS proteome analysis

Analyses were performed using a combination of an SDS polyacrylamide gel electrophoresis separation (PAGE) and a gel-free shotgun protocol based on nano-HPLC and MS/MS (Baldrianová et al. 2015). Briefly, tryptic digests were dissolved in 0.5% (v/v) formic acid in 5% (v/v) acetonitrile, and then analyzed by nanoflow C18 reverse-phase liquid chromatography using a 40 cm column (0.075 mm inner diameter; NanoSeparations) and a Dionex Ultimate 3000 RSLC nano-UPLC system (Thermo) directly coupled to a CaptiveSpray nanoESI source (Bruker) and an UHR maXis impact q-TOF mass spectrometer (Bruker). Peptides were eluted with up to a 120-min, 4% to 40% acetonitrile gradient. Spectra were acquired at 2 Hz (MS) and 10 to 20 Hz (MS/MS) using an intensity-dependent mode with a total cycle time of 7 s.

Protein identification

The measured spectra were extracted by Bruker's Data Analysis 4.1 and processed as described previously (e.g. Cerna et al. 2016). In brief, recalibrated MGF files were searched against *Nicotiana* protein database (*N. tabacum* TN-90, Solgenomics, 7/2016) by Sequest HT, MS Amanda and Mascot 2.4 with the following parameters: Enzyme - trypsin, max two missed cleavage sites; Mass tolerance - 35 ppm (MS) and 0.1 Da (MS/MS); Modifications - up to three dynamic modifications including Met oxidation, Asn/Gln deamidation, Lys methylation, N-terminal acetylation, Ser/Thr/Tyr phosphorylation.

RESULTS AND DISCUSSION

Identification of proteins secreted by tobacco roots

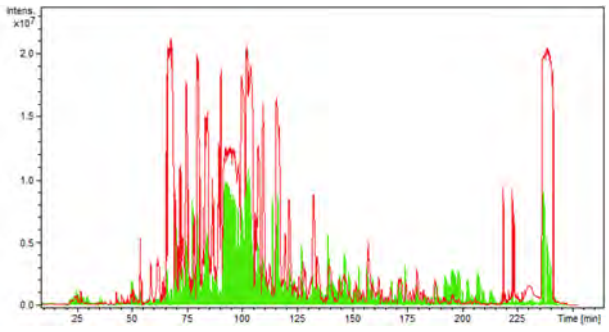
Proteins were precipitated and extracted as described in Materials and Methods. Two independent experimental replicates were analyzed, each consisting of extracts from at least three plants. Proteins were digested and analyzed by an LC-MS (Figure 1). To increase the proteome coverage, one replicate was prefractionated by one-dimensional PAGE. Altogether, 361 protein groups were identified (FDR < 1%). However, we note that even though the number of assigned peptide spectral matches (PSMs) is high (median >15), all of these identifications are based only on a single proteotypic peptide match per a protein group.

Function of proteins identified in root secretome

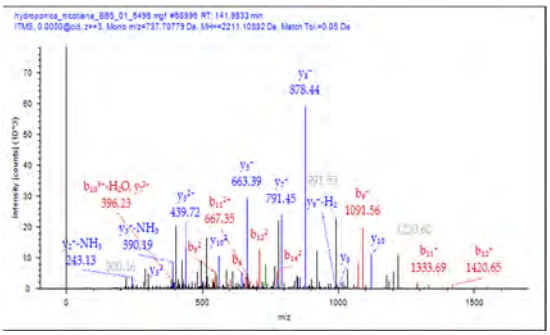
Annotations for tobacco genes/proteins are not comparable to those of other model plants. To elucidate functional relevance of identified secreted proteins, we employed annotations for homologous genes in *Arabidopsis thaliana* (TAIR 10 database) and analyzed the resulting dataset by STRING (http://string-db.org). String analysis highlighted several categories that are well in line with previously identified proteins in plants' secretome, including proteases (e.g. DegP2 protease), enzymes of carbohydrate metabolism (e.g. arabinose kinase 1), cell wall maintenance (e.g. cellulose synthase) or redox homeostasis (e.g. glutathione transferase) (Figure 2).

Figure 1 LC-MS analysis of root exudate tryptic digest

A) Representative base peak chromatogram (red) and MS/MS total ion chromatogram (green)



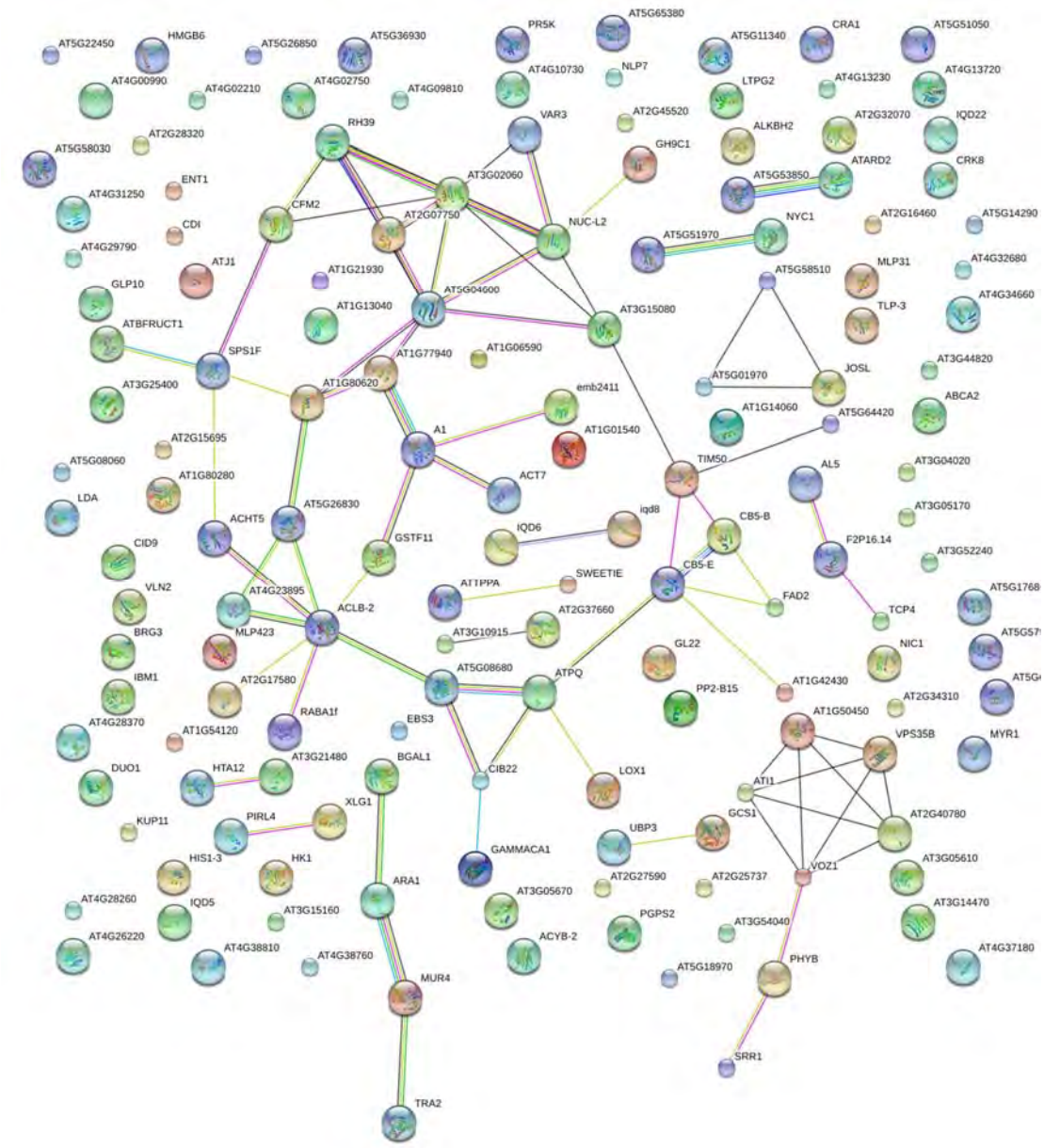
B) Representative MS/MS spectrum



Predicted localization of identified proteins

SUBA3 *Arabidopsis* database was used to determine localization of identified proteins. The experimental evidence is lacking for most of the detected proteins (less than 10%) and thus the analysis is based only on available predictions. In total, only six exclusively extracellular proteins were found. However, 74 of 203 identified *Arabidopsis* homologues are predicted to localize (among others) into extracellular space.

Figure 2 Protein–protein interaction network constructed using STRING



CONCLUSION

Plant secretomics are an important tool to analyze plant's interactions with its environment. Here, we employed a simple and fast technique to obtain proteins secreted by tobacco roots. Methods employed in secretome analyses often result in experimental artifacts, e.g. by a cell lesion that contaminates the secretome with content of damaged cells. Here, the plants were cultivated in the same environment and thus any contamination would most likely be a result of natural process. Indeed, functional analysis and predicted localizations indicate that most of 361 identified proteins truly belong to plant secretome.

ACKNOWLEDGEMENTS

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TRIPLOID VARIETIES OF *PETUNIA HYBRIDA* – PERSPECTIVE BREEDING POSSIBILITY

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Abstract: Petunia hybrida is an important annual plant. Current assortment of Petunias consists mainly of generatively propagated F1 varieties. Vegetatively propagated varieties are nowadays increasingly gaining popularity. In this experiment was successfully verified the possibility to create tetraploid varieties that would be propagated vegetatively. Large flowered hybrids with genotype Ggg were obtained. As a maternal component tetraploid genotype gggg and as a paternal component diploid GG genotype was used. From the seeds received from pollinating 120 flowers were obtained 10 triploid hybrids. Diameter of the flowers was 98 mm, which is 25% more when compared to parental component with big flowers. The growth characteristics as well as the health conditions were good. This is a new perspective possibility to create new varieties of *Petunia hybrida*.

Key Words: Petunia hybrida, triploid, G allele, variety, breeding

INTRODUCTION

Petunia hybrida belongs to the trio of the world's most important and popular annuals (Anderson 2007). It is subject of intensive breeding, mainly of American and Japanese companies (Murphy 2007). The core assortment consists of F1 varieties in the type multiflora and grandiflora (Ball 1991). A special group in assortment represent tetraploid varieties *Petunia h. superbissima*. This group was very popular in the early twentieth century with the launch of hybrid varieties to the market in the fifties, this type of Petunias nearly disappeared from the catalogues of seed companies (Gerats and Strommer 2009).

Superbissima type varieties are tetraploid (2 n = 28) (Matsuda 1933) and like most tetraploids are characterized by robustness of plants and flowers. Flower diameter is 160 mm (Maatsch and Nolting 1968, Reimann-Philipp 1969). Currently, F1 supperbissima hybrids don´t exist. Nor vegetative propagation - cuttings, which is used for multiplication of varieties of Petunias increasingly, is suitable for this type. Multiplying coefficient of tetraploids is very low. Yet completely unused option is the creation of large flowered triploid varieties, that would be then propagated vegetatively.

The flower size is in Petunia controlled by a dominant gene G (Plickert 1936) which is located on chromosome V (Cornu et al. 1980). Heterozygote Gg is a grandiflora, which is used in the creation of large flowered commercial F1 varieties. Homozygous paternal component with GG genotype is not very vivid. After crossing tetraploid and diploid plants mostly non germinating seeds are obtained. Only when a maternal component is tetraploid and paternal component is diploid, then in rare cases triploid hybrid could be found (Plickert 1936).

Triploid plant with genotype Ggg could combine big flower diameter, good growth characteristics and acceptable propagation coefficient needed for intensive vegetative propagation. Visual characteristics could be very similar to the attractive OP tetraploid varieties, but without their limitations and disadvantages (disparity, flowers deformations, bad plant habitus).

MATERIAL AND METHODS

Characterization of experimental design and methods

Plant growing and crossing were carried out in a greenhouse isolated against insects. Plants were grown in a commercial substrate manufactured by company Gramoflor. Once a week the plants were

fertilized. Plant protection was performed according to occurrence of diseases and pests. All plants were grown under the same conditions. Genetic materials used in the experiment were provided by company Černý-BioPro Ltd., Prague.

In 2013, a small pink flowered plant was selected from the group of plants of OP tetraploid variety Rosea. It was assumed to be homozygous recessive genotype gggg. The flowers were pollinated by their own pollen. In 2014, out of the seeds obtained after self-pollinating were cultivated and grown 100 plants. No large flowered plant was found. F1 generation only slightly differed in the flower colour from light to dark pink.

Four plants were used as maternal component (marked 2000 / 1–4). As a paternal component was used component KO1, which is used in production of current commercial varieties. It is a large flowered diploid with genotype GG, white colour, fimbriata type. Figure 1 represents the difference in size and colour of parental components and triploid hybrid. Maternal plants were emasculated. 30 flowers of every maternal plant were pollinated by pollen from KO1 plants. Seeds from this crossing, were sown in 2015. According to the size and shape of a flower, triploid plants were identified. Ploidy testing was carried out on the device Partec PA II with mercury UV lamp in flow cytometry in the Institute of Botany AS CR Pruhonice (Otto 1990, Doležel et al. 1994, Doležel et al. 2007).

Triploid plants were planted in pots with a diameter of 12 cm. In July 2015, the diameter of the flower (30 pieces) and plant height were measured. Plants were described using the descriptor used in the breeding station (Černý 1974). The best plants were transferred into *in vitro* gene bank (Šedivá 2009) to prevent accidental loss of genotypes or infection by viruses.

The aim of this experiment was to confirm the possibility of making Petunia triploid plants with genotype Ggg by crossing tetraploid and diploid components. The task was also to investigate the frequency of triploids in F1generation. The third task was to determine the expression of G allele in genotype of triploid Ggg. To do so, the flower size of parental plants and triploid hybrid was compared.

Table 1 Crossing in year 2014

Pair	Maternal component	Ploidy n	Paternal component	Ploidy n	Number of plants in F1 generation	Number of triploids
1	2000 / 1	4	KO1	2	42	3
2	2000 / 2	4	KO1	2	36	2
3	2000 / 3	4	KO1	2	51	3
4	2000 / 4	4	KO1	2	39	2
					168	10

RESULTS AND DISCUSSION

The aim of this experiment was to verify the possibility of making triploid varieties of *Petunia hybrida* and evaluate some of their features. These varieties would be reproduced vegetatively. F1 hybrid triploid varieties are used with success in some ornamental plants (Reimann-Philipp 1969). For example, in *Begonia semperflorens* this crossing scheme creates powerful and resistant hybrids. Another advantage is also so called self-cleaning ability. In seed pods of triploid plant, the seeds are not developed, therefore they fall down after several days. The plant is not burdened by the formation of the seeds and blooms continuously until the end of the growing season.

In *Petunia hybrida* is however the crossing between diploid and tetraploid lines not successful, so that this scheme can´t be used to produce hybrid varieties propagated by seed. As stated Seidel (Seidel 1962), only when crossed tetraploid maternal component and diploid paternal component, a small number of triploids could be obtained. This corresponds with results of our experiment, as shown in Table 1. Out of the 120 pollinated flowers 10 triploid plants were received. The total number of plants in the F1 generation was 168, but the 158 cases were not regular types, which happened by transferring pollen from other plants. Such plants were very similar to maternal plants, therefore, they varied in shades of pink and had small flower. So on average one triploid plant is received out of 12 pollinated flowers. Normally, in one seed pod of tetraploid OP variety are several hundred seeds (Anderson 2007).

Low number of triploids obtained is not a problem, when consider that received triploid plants will be reproduced asexually.

Specialized companies supplying horticultural firms with young plants are able to produce rooted cuttings in sufficient amount. The advantage of vegetatively propagated varieties is rapid development and change of assortment (Dole and Gibson 2006). Triploid hybrids fit this concept well. Our goal was to create a hybrid genotype Ggg. We assumed that one dominant G allele ensures big flowers of the hybrids. This was examined by measuring the diameter of flowers of various acquired triploids. The average size of the flower was 98 mm, which is almost 25% more than the paternal large-flowered component KO1. The flowers looked like OP tetraploid varieties – the size and shape were similar. The edge of the flower was fimbriata type, which increases its attractiveness. According to Table 2 there is a statistical significance (p = 0.05) that the flower diameter of all 10 triploid plants is statistically bigger then parental components 2000 and KO1.

Table 2 Comparison of flower size (mm) among triploid plants and parental components

	Mean Group 1	Mean Group 2	t-stat.	sv	p	Units per group 1	Units per group 2	Stand. deviation 1	Stand. deviation 2	F-stat.	p Variances
KO1 vs. triplo1	78.20	96.60	-65.486	38	0.00	20	20	1.105	0.598	3.412	0.010
KO1 vs. triplo2	78.20	97.20	-61.390	38	0.00	20	20	1.105	0.834	1.758	0.228
KO1 vs. triplo3	78.20	97.55	-64.547	38	0.00	20	20	1.105	0.759	2.119	0.110
KO1 vs. triplo4	78.20	97.50	-64.333	38	0.00	20	20	1.105	0.761	2.109	0.112
KO1 vs. triplo5	78.20	97.75	-64.464	38	0.00	20	20	1.105	0.786	1.974	0.147
KO1 vs. triplo6	78.20	97.65	-65.264	38	0.00	20	20	1.105	0.745	2.199	0.094
KO1 vs. triplo7	78.20	97.70	-65.774	38	0.00	20	20	1.105	0.733	2.275	0.081
KO1 vs. triplo8	78.20	97.75	-66.391	38	0.00	20	20	1.105	0.716	2.379	0.066
KO1 vs. triplo9	78.20	97.75	-70.830	38	0.00	20	20	1.105	0.550	4.035	0.004
KO1 vs. triplo10	78.20	97.60	-71.468	38	0.00	20	20	1.105	0.503	4.833	0.001
2000 vs. triplo1	59.55	96.60	-117.550	38	0.00	20	20	1.276	0.598	4.551	0.002
2000 vs. triplo2	59.55	97.20	-110.457	38	0.00	20	20	1.276	0.834	2.345	0.071
2000 vs. triplo3	59.55	97.55	-114.438	38	0.00	20	20	1.276	0.759	2.826	0.029
2000 vs. triplo4	59.55	97.50	-114.219	38	0.00	20	20	1.276	0.7609	2.814	0.029
2000 vs. triplo5	59.55	97.75	-113.957	38	0.00	20	20	1.276	0.786	2.634	0.041
2000 vs. triplo6	59.55	97.65	-115.290	38	0.00	20	20	1.276	0.745	2.934	0.024
2000 vs. triplo7	59.55	97.70	-115.931	38	0.00	20	20	1.276	0.733	3.034	0.020
2000 vs. triplo8	59.55	97.75	-116.723	38	0.00	20	20	1.276	0.716	3.174	0.015
2000 vs. triplo9	59.55	97.75	-122.920	38	0.00	20	20	1.276	0.550	5.383	0.001
2000 vs. triplo10	59.55	97.60	-124.053	38	0.00	20	20	1.276	0.503	6.448	0.000

Note: T-test for independent samples; p statistics p = 0.05; measures in mm; computed in STATISTICS 12.

Some plants of tetraploid OP varieties have a flower diameter up to 160 mm. The problem with these varieties is that they usually very differ in flower size. Some plants also have deformed flowers. Plant habit also does not fulfil the requirements of the growers, as the plants are high and don´t spread. These OP varieties are suitable for flower lovers and are sold in pictorial packages in the hobby market, but not for professional producers of flowers (Sink 1984). Based on the results we can say that triploid Petunia varieties with genotype Ggg is a good way of breading and producing new commercial varieties.

Figure 1 Flowers of parental plants and triploid hybrid

A) Maternal plant 2000 / 1 *B) Paternal plant KO1* *C) Triploid plant TRIPLO1*

CONCLUSION

Flower lovers and growers anxiously wait for new attractive varieties of ornamental plants. Petunia assortment is very broad and coming up with something new is difficult. Producing triploid varieties is still untapped opportunity to enrich the product range. In our work, we have demonstrated that triploid hybrids with genotype Ggg can successfully accomplish this task. The breeding cycle of such varieties is relatively short, and financially and technically not demanding. Triploids obtained in this experiment have attractive flowers and good growth characteristics.

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COMPARISON OF THE SPECIES COMPOSITION OF VEGETATION ON SELECTED SECTIONS OF RAILWAY

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Abstract: This paper compares the differences in species composition on different parts of the railway with the results of previous research. It evaluates the presence of individual species according to their harmfulness and links to the ecosystem. For this research were chosen the sections between the cities Chrudim and Úhřetice. The species composition of weeds was evaluated according to phytocoenology relevé in four periods: July and August 2013, July and August 2015. 100 weed species were found on the railway. The obtained data were statistically evaluated by DCA (Detrended Correspondence Analysis) and CCA (Canonical Correspondence Analysis) analysis. The highest coverage had species as: *Potentilla reptans, Urtica dioica, Equisetum arvense, Arrhenatherum elatius, Rubus caesius, Clematis vitalba, Galium mollugo*.

Key Words: weeds, railway, coverage, biodiversity, phytocoenology relevé

INTRODUCTION

Traveling by train is considered as safe and comfortable but not all passengers are aware that safety and convenience are highly dependent on both, railway line and railway bed. The latter is traditionally composed of coarse gravel, which ensures a solid surface of the track (Schweinsberg et al. 1999).

According to Jehlík (1998), habitat conditions of railway area are very specific. An anthropogenic railway land is characterized by the participation of coal, cinder, fly ash and SO_2. The chemistry of the railway soils affects mostly a brown coal, which often has a fertilising effect. A railway bed can be distinguished according to the mechanical and chemical composition. There are three main types of soils. Cinder soils composed from almost pure cinder, are soils with predominance of sand, and soils of the railway slopes and notches with a predominance of clay. Soils are affected by total herbicides used for weed control.

Weeds growing on the railway are considered as a negative element and consistent weed control is really important. Plants located on the railroad and its immediate vicinity, we can classify as so-called ruderal vegetation. This type of vegetation is often considered as hard to manage. High vegetation impairs visibility during a transport. Weeds located at tracks prevent visual inspection of the condition of the rails, sleepers and fasteners (Dvořák and Smutný 2003).

According to Ulrich et al. (2011) it is just up to operators of the railway infrastructure to maintain the patency of the railway lines, to remove growing trees over a path of trains and to prevent a possible danger of falling trees or self-seeding species growing in the protection zone of the railway.

Removal of weeds on the railroad is also important for a suppression of diseases and pests transmitting. Almost every of our cultivated crop has between weeds a related species, what significantly strenghtens a harmfulness of weeds on the railroad (Klingman et al. 1982).

Currently the most common way how to control weeds is application of herbicides. Choosing a right herbicide according to the plant species is very important (Torstensson 2001). Some of the weeds become resistant to a certain active herbicide compounds. Weeds on railways can be controlled by firing, high temperatures from special burners or heat lamps are destructive for weeds. Some of appropriate control uses a superheated steam (Dvořák and Smutný 2003).

The aim of this work was to evaluate the composition of the vegetation on the selected sections of railway, to compare the differences between habitats and weed composition between the utilized and unused sections of the railroad.

MATERIAL AND METHODS

Characteristics of the area

Section Chrudim-Úhřetice on the line Chrudim-Borohrádek was selected for mapping. The length of the monitored section is approximately 7 km. Major part of the monitoring was conducted in Chrudim city. Chrudim city is situated on the interface of the Iron mountain and the Elbe lowlands, 110 km east from Prague and 10 km south from the regional city Pardubice. The climate of monitored area is within the Czech Republic possible characterized as exceptionally warm with average total precipitation. The average temperature in Chrudim is 7 °C. July is the warmest month with an average temperature 17.5 °C. An altitude of railway section is 243–300 meters asl.

Part of the selected section is still utilized and the evaluation of vegetation took place directly in the track and embankment. Another part of the railway is unused and the evaluation was carried out in the track and embankment as well.

Methodology of evaluation and data processing

The observation was conducted in four terms. The first one was in July 2013, the second in August of the same year, the third in July of 2015 and the last one in August 2015. On the monitored section, four different stands were chosen, where three phytocoenology relevé were made. Weeds were evaluated by using the phytocoenology relevé with the size of 12 m^2. Species composition of weeds and their coverages were evaluated. Coverage was determined by Braun-Blanquet scale (Moravec 1994). Scientific names of weeds were used according to Kubát et al. (2002).

The obtained data were processed by multivariate analyses of ecological data, detrended correspondence analysis (DCA; detrended by segments) and canonical correspondence analysis (CCA). A total number of 999 permutations were calculated in Monte-Carlo test. Tested environmental factors were individual stands: embankment of utilizes section (Naps_pro), embankment of unused section (Nasp_nep), utilizes track of railway (Kol_prov), unused track of railway (Kol_nepo). Monitored years were used as covariates (*covariables*) in analysis. Collected data were processed in Canoco 4.0 (Ter Braak 1998).

RESULTS AND DISCUSS ION

85 plant species were found on the selected section of the railway in 2013. 83 species were identified in 2015. A total of 100 species was observed within all monitored period in both years together.

The obtained data about evaluation of weed infestation were initially processed by the DCA analysis which determined the length of the gradient, and it was 5.174. Based on this calculation, CCA was selected as most suitable for further data processing. CCA defines the spatial arrangement of plant species and selected environmental factors. This is subsequently graphically expressed by the ordination diagram. Weed species and monitored factors are shown by points of different shape and colour.

Table 1 The mean cover of species found on different sections of the railway (% - average coverage)

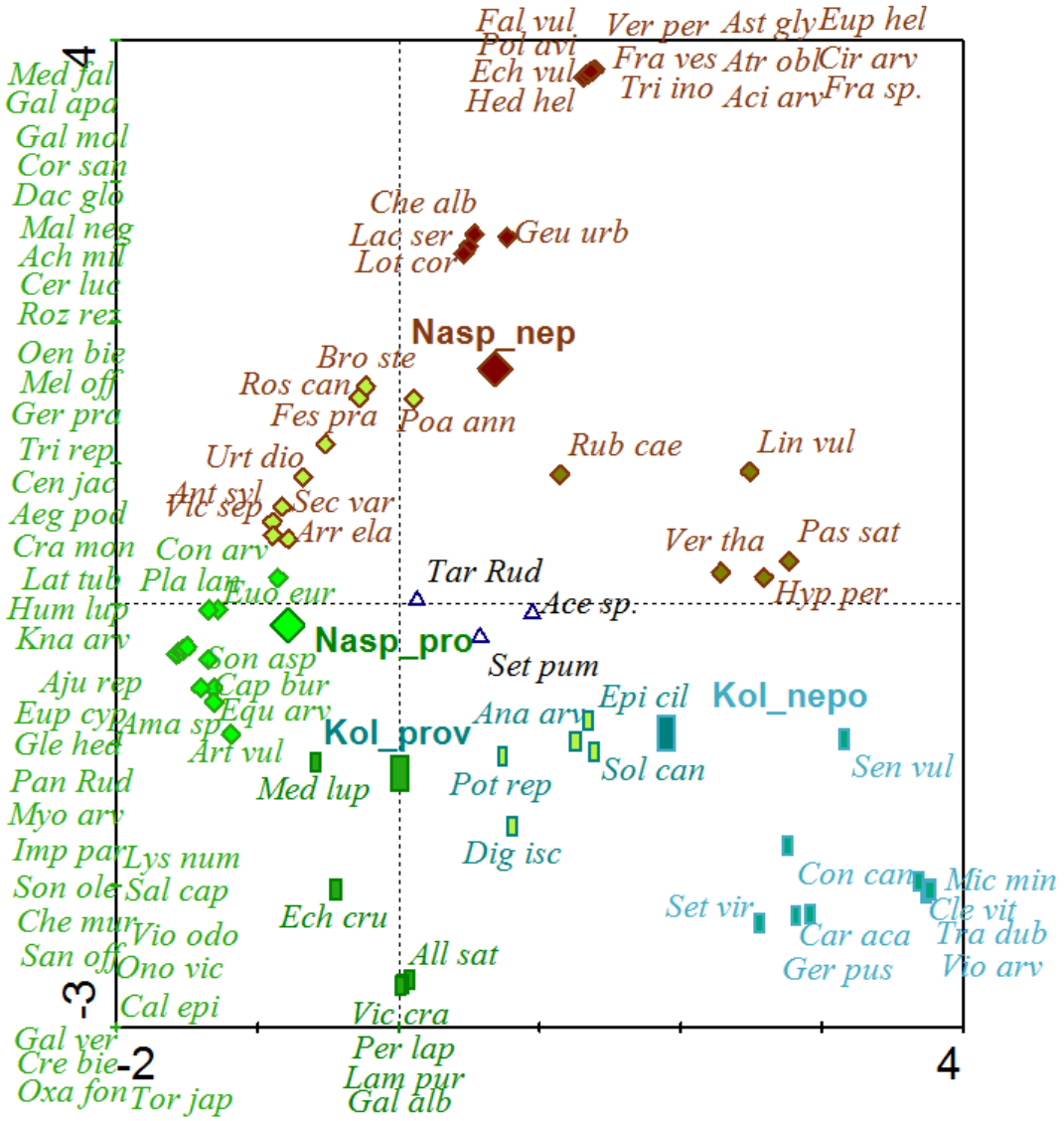
Species	*Abbrev.*	Utilized railroad		Unused railroad	
		Track	Embankment	Track	Embankment
Aegopodium podagraria	*Aeg pod*		2.40		
Amaranthus sp.	*Ama* sp.	0.01	0.05		
Acer sp.	*Ace* sp.	0.03	0.18	0.63	0.38
Acinos arvensis	*Aci arv*				0.06
Achillea millefolium	*Ach mil*		0.18		
Ajuga reptans	*Aju rep*		0.01		

Allium sativum	*All sat*	0.19			
Anagallis arvensis	*Ana arv*	0.06		0.03	0.03
Anthriscus sylvestris	*Ant syl*		0.09		0.06
Arrhenatherum elatius	*Arr ela*	0.31	7.21	0.06	4.13
Artemisia vulgaris	*Art vul*	0.03	0.04		
Astragalus glycyphyllos	*Ast gly*				0.38
Atriplex oblongifolia	*Atr obl*				0.06
Bromus sterilis	*Bro ste*		0.28		0.63
Calamagrostis epigejos	*Cal epi*		2.23		
Capsella bursa-pastoris	*Cap bur*	0.08	1.75		
Carduus acanthoides	*Car aca*	0.03		0.10	
Centaurea jacea	*Cen jac*		0.03		
Cerastium lucorum	*Cer luc*		0.05		
Cirsium arvense	*Cir arv*				0.69
Clematis vitalba	*Cle vit*			12.50	0.03
Convolvulus arvensis	*Con arv*	0.20	1.80		0.38
Conyza canadensis	*Con can*		0.03	0.39	
Cornus sanguinea	*Cor san*		0.26		
Crataegus monogyna	*Cra mon*		0.02		
Crepis biennis	*Cre bie*		0.18		
Dactylis glomerata	*Dac glo*		1.08		
Digitaria ischaemum	*Dig isc*	2.25	1.15	4.08	
Echinochloa crus-galli	*Ech cru*	2.94	0.28		
Echium vulgare	*Ech vul*				0.45
Epilobium ciliatum	*Epi cil*	0.13	0.20	0.65	0.15
Equisetum arvense	*Equ arv*	1.00	9.86		
Euonymus europaea	*Euo eur*	0.03	2.67		0.13
Euphorbia cyparissias	*Eup cyp*		0.18		
Euphorbia helioscopia	*Eup hel*				0.01
Falcaria vulgaris	*Fal vul*				0.75
Festuca pratensis	*Fes pra*		0.90		0.63
Fragaria vesca	*Fra ves*		0.00		0.13
Fraxinus sp.	*Fra sp.*		0.00		0.75
Galium album	*Gal alb*	0.15	0.00		
Galium aparine	*Gal apa*		0.01		
Galium mollugo	*Gal mol*		4.75		
Galium verum	*Gal ver*		0.04		
Geranium pratense	*Ger pra*		0.10		
Geranium pusillum	*Ger pus*	0.08		0.35	
Geum urbanum	*Geu urb*	0.13	0.10		4.38
Glechoma hederacea	*Gle hed*		0.01		
Hedera helix	*Hed hel*				6.25
Humulus lupulus	*Hum lup*		0.90		0.00
Hypericum perforatum	*Hyp per*	0.01	0.01	0.50	0.38
Chenopodium album	*Che alb*		0.01		0.08
Chenopodium murale	*Che mur*		0.01		
Impatiens parviflora	*Imp par*		0.06		
Knautia arvensis	*Kna arv*		0.90		
Lactuca serriola	*Lac ser*		0.05		0.40
Lamium purpureum	*Lam pur*	0.01			
Lathyrus tuberosus	*Lat tub*		0.06		
Linaria vulgaris	*Lin vul*			1.00	2.25

Lotus corniculatus	*Lot cor*		0.13		0.69
Lysimachia nummularia	*Lys num*		0.06		
Malva neglecta	*Mal neg*		0.03		
Medicago falcata	*Med fal*		0.04		
Medicago lupulina	*Med lup*	0.50	0.36	0.08	0.03
Melilotus officinalis	*Mel off*		0.01		
Microrrhinum minus	*Mic min*			0.01	
Myosotis arvensis	*Myo arv*		0.05		
Oenothera biennis	*Oen bie*		0.25		
Onobrychis viciifolia	*Ono vic*		0.08		
Oxalis fontana	*Oxa fon*		0.03		
Panicum miliaceum subsp. *ruderale*	*Pan Rud*		0.15		
Pastinaca sativa	*Pas sat*			0.09	0.06
Persicaria lapathifolia	*Per lap*	0.40			
Plantago lanceolata	*Pla lan*		0.19		0.03
Poa annua	*Poa ann*	0.04	0.06		0.19
Polygonum aviculare	*Pol avi*				0.33
Potentilla reptans	*Pot rep*		5.13	18.13	
Rosa canina	*Ros can*		0.05		0.10
Rubus caesius	*Rub cae*	0.44	0.54	3.95	10.14
Salix caprea	*Sal cap*		0.08		
Sanguisorba officinalis	*San off*		0.25		
Securigera varia	*Sec var*		0.09		0.08
Senecio vulgaris	*Sen vul*	0.01	0.01	2.56	0.33
Setaria pumila	*Set pum*		0.15	0.15	0.08
Setaria viridis	*Set vir*	0.20		0.69	
Solidago canadensis	*Sol can*	0.09	0.42	2.61	0.13
Sonchus asper	*Son asp*		0.16	0.01	
Sonchus oleraceus	*Son ole*		0.01		
Taraxacum sect. Ruderalia	*Tar Rud*	0.08	0.45	0.30	0.43
Torilis japonica	*Tor jap*		0.05		
Tragopogon dubius	*Tra dub*			0.15	
Trifolium repens	*Tri rep*		0.90		
Tripleurospermum inodorum	*Tri ino*				0.03
Urtica dioica	*Urt dio*		8.25		5.94
Verbascum thapsus	*Ver tha*		0.03	0.28	0.19
Veronica chamaedrys	*Roz rez*		0.03		
Veronica persica	*Ver per*				0.03
Vicia cracca	*Vic cra*	0.08			
Vicia sepium	*Vic sep*	0.01	0.20		0.14
Viola arvensis	*Vio arv*			0.08	
Viola odorata	*Vio odo*		0.10		

The results of the CCA analysis evaluating the effect of habitat on the occurrence of weeds is significant at the level of $\alpha = 0.001$ for all of the canonical axes. According to the ordination diagram (see Figure 1) plant species can be divided into five groups.

Figure 1 Ordination diagram expressing the relation between species and different habitats on the railway (CCA, Trace = 1.282, F-ratio = 3.118, P-value = 0.001)



Legend: Nasp_pro – embankment of utilized section; Nasp_nep – embankment of unused section; Kol_prov – utilized track of railway; Kol_nepo – unused track of railway

The first group of weed species occurred mainly on embankment of utilized section of railway: *Allium sativum, Artemisia vulgaris, Calamagrostis epigejos, Capsella bursa-pastoris, Convolvulus arvensis, Echinochloa crus-galli, Equisetum arvense, Euonymus europaea, Lysimachia nummularia, Medicago lupulina, Onobrychis viciifolia, Plantago lanceolata, Salix caprea, Sonchus asper, Vicia cracca, Viola odorata.*

The second group of weed species present mainly on embankment of unused section consists from: *Acinos arvensis, Astragalus glycyphyllos, Atriplex oblongifolia, Cirsium arvense, Echium vulgare, Euphorbia helioscopia, Falcaria vulgaris, Fragaria vesca, Fraxinus sp., Hedera helix, Polygonum aviculare, Tripleurospermum inodorum, Veronica persica.*

The third group of weed species occurred mainly on utilized track of railway: *Anagalis arvensis, Digitaria ischaemum, Epilobium ciliatum, Potentilla reptans, Solidago canadensis.*

The fourth group of weed species was present mainly on unused track: *Carduus acanthoides, Clematis vitalba, Conyza canadensis, Epilobium ciliatum, Geranium pussilum, Microrrhinum minus, Senecio vulgaris, Setaria viridis, Solidago canadensis, Tragopogon dubius, Viola arvensis.*

The last group of weed species was more influenced by other factors: *Acer* sp., *Setaria pumila*, *Taraxacum* sect. *Ruderalia.*

The differences between utilized and unused sections of railway are evident. The species with higher coverage, as *Clematis vitalba, Linaria vulgaris, Senecio vulgaris*, were more frequently observed on unused places. This fact can be link with an absence of chemical weed control. Some differences were observable also among the species composition. Species as *Falcaria vulgaris, Microrrhinum minus* or *Acinos arvensis* were found on utilized tracks. The greatest risk of further weed infestation threatens on unused sections. There is no regulation and a gradual expansion is expected.

CONCLUSION

Differences in vegetation between utilized and unused sections of railway are noticeable.

The selected section of the railway was interesting from the point of view of the high number of found species. Some species with negative effects on the ecosystem were observed on the selected section of the railway. These are invasive species, resistant to a herbicide substance, deep-rooted species, which affect the characteristics of the gravel bed, and some species cause pollen allergy. On the other hand, some of occurred species may affect the surrounding ecosystem in a positive way. These are entomophilous and medicinal herbs.

The species growing on unused section of railway will probably continue in spontaneous expansion due to the absence of chemical control. According to these findings it could be useful to continue in further monitoring.

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IMAGEJ SOFTWARE AS A TOOL FOR DETERMINING MORPHOMETRIC PARAMETERS

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Abstract: Most striking changes induced by treating biological samples are changes on the level of organism morphology. These changes are result of internal and external factors, but obviously the changes in body shape or topology affect the further development on the lower level by downward causation. Here, the use of image analysis software ImageJ is described. It is shown that ImageJ is not difficult to use and can easily produce valuable data which is demonstrated on the example of measuring rosette areas and length of leaf blades of plantlets treated by abiotic stress.

Key Words: ImageJ, image analysis, morphology, stress

INTRODUCTION

The word Morphology is from the Ancient Greek *μορφή* (morphé) meaning "Form" and *λόγος* (lógos) meaning "Word, Study". Morphology is the branch of biology which deals with the form of living organisms and with relationships between their structures. This includes external morphology, i.e. aspects of the outward appearance, as well as the internal morphology and shapes of organs and cells. The proper morphology is a key determinant essential for the survival and reproduction of any organism. In contrast to animals, plants are sessile organisms and are very limited in their movement. Changes of the shape, organ and organelle orientation and regulated growth are important responses of plants which cope with environmental changes and reduce their potential negative effects. Plant growth is regulated by external and internal stimuli which are integrated and result in controlled growth. Key environmental factors affecting plant growth and thus morphology are light conditions (Novák et al. 2015) and ambient temperature (Černý et al. 2014, Skalák et al. 2016). On the other hand plant hormones are important internal factors orchestrating and regulating plant growth (Baldriánová et al. 2015, Černý et al. 2016, Novák et al. 2013). Here we demonstrate the use of an open source software ImageJ which we found to be very useful in morphometric analyses.

MATERIAL AND METHODS

Plant material

Seeds (*Arabidopsis thaliana*, Columbia-0) were surface sterilized by 70% ethanol and sown on soil in pots. Seeds with pots were cultivated 2 days in dark and 4 °C for stratification. After stratification seedlings were cultivated under condition of long day (16 h light/8 h dark) at 20 °C under PPFD of 20 μmol/m^2/s. Seedlings were treated by abiotic stress for next 7 days. Consequently, plantlets have been cut and spread on Petri dishes on medium (1% agar). Photos of plantlets were taken by Canon camera.

Image analysis - calibration

Image analysis was performed in ImageJ downloaded from https://imagej.nih.gov/ij/ (ImageJ 1.45s, Java 1.6.0_20). For analysis of lengths, prior to measurement software was calibrated by using photo of calibrated ruler taken from the same distance as photos of samples. Software was calibrated by using icon 'straight line' and by extending a straight line over the length that defines a known distance of 2 cm. Then in panel 'Analyze/Set Scale…' distance in pixels is supplemented with a known distance from the ruler. Before 'confirmation', calibration was set for all images by check mark for the option –

'Global'. This sets the calibration for measuring lengths and areas for all images, including newly opened ones. The calibration will last till the restart of the software.

Image analysis - measurements

Lengths can be measured by straight or segmented lines depending on the shape of measured morphological parameter**.** For measuring plant areas, photos of samples are first adjusted in panel 'Image/Adjust/Color Threshold'. By adjusting settings of 'Hue', 'Saturation' and 'Brightness' plants' background can be filtered out. If plantlet rosettes are not properly spread in plane and leaves touch each other, then the border between leaves must be adjusted manually by using 'pencil tool' icon. Finally, the area is measured by using 'Wand (tracing)' tool.

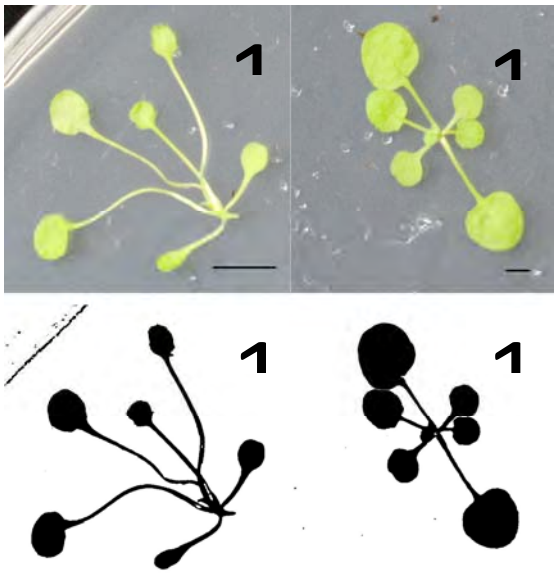
Statistics

In every experiment 15 independent plantlets were used and analyzed for each treatment. Data are presented by arithmetic means with standard deviations. Statistical evaluation was performed in Excel using Student unpaired two-sample *t*-test. Experiment was performed in three independent biological replicas.

RESULTS AND DISCUSSION

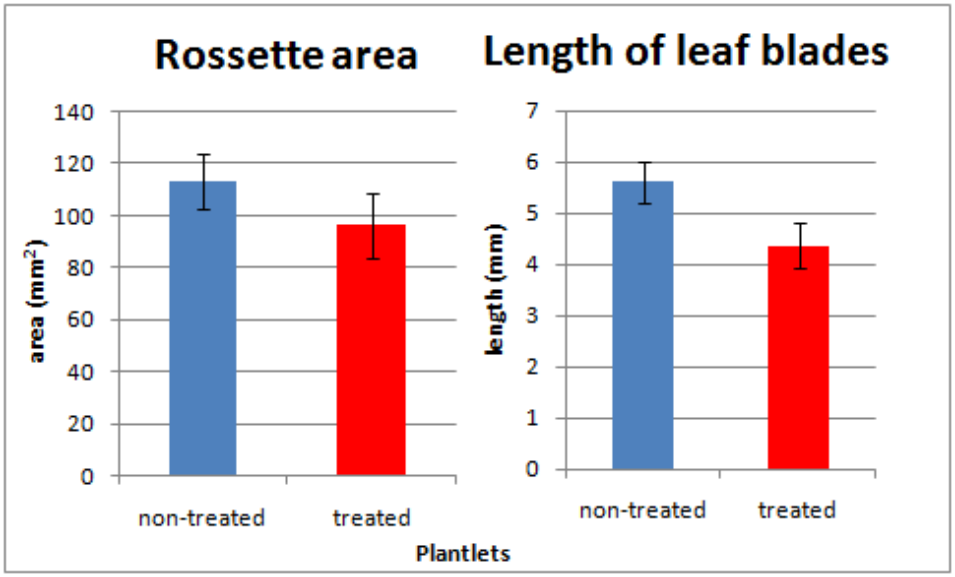
ImageJ is Java-based image processing program developed at the National Institutes of Health by Wayne Rasband. It was designed with an open architecture that provides extensibility by plugins and macros. Here, to evaluate its performance in a high-throughput morphometric screening, we analyzed morphological changes on series of plantlets treated with abiotic stress. Experimental samples were two weeks old *Arabidopsis* plantlets cultivated under standard conditions or treated with abiotic stress inducing changes in morphometric parameters. After the cultivation period, plantlets were spread on agar medium in plane to minimize measurement error from a depth of field discrepancy. Plantlets were digitized by standard HD camera (Canon), images were saved in JPEG file format and analyzed in ImageJ software. Plants cultivated under standard conditions showed compact plant rosette with short petioles and large leaf blades (Figure 1A). In comparison, plants treated with abiotic stress showed longer petioles with smaller leaf blades (Figure 1B). Moreover plantlets treated with abiotic stress were less green than controls, but level of chlorophyll haven´t been determined. First measured parameter was length of leaf blade (L 1/2). Analysis of leaf blade did not require any image adjustment. Lengths were determined directly from camera images. Analysis showed almost 25% reduction in length of leaf blades in plantlets cultivated under abiotic stress (Figure 2).

Figure 1 Planlets phenotype. Phenotype of 2 weeks old Arabidopsis plantlets cultivated under abiotic stress (1A, length of bar is 4 mm) or under standard conditions (1B). Prior rosette leaf area measurement images were adjusted to simplify analysis (1C – treated plantlets, 1D – non-treated).



For analysis of plant rosette area, images were first adjusted on the basis of light intensity and color on the image. Resulting adjusted images corresponding to non-adjusted images are presented in figure 1C and 1D. Plantlets treated with abiotic stress have smaller rosette area (Figure 2). Results showed that abiotic stress caused morphometric changes that are easily traceable by ImageJ software.

Figure 2 Effect of stress of plantlets morphology. Changes in length of leaf blade and rosette area in plantlets cultivated under standard conditions or abiotic stress treatment. Data are presented as means with standard deviations. Asterisk indicate statistically significant difference between treated and non-treated plantlets (P <0.05).



CONCLUSION

In this work a supervised automatic processing by an open source software ImageJ was used to determine two different morphometric parameters of two differently treated sets of plantlets. It can be concluded that ImageJ is easy to use and useful software which significantly boost the speed of morphometric analysis. Further, there are additional accessible plugins and new plugins in development that provide new functionalities and possibilities in image analyses.

ACKNOWLEDGEMENTS

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EFFECTS OF DIFFERENT MORPHOREGULATORS ON GROWTH AND DEVELOPMENT OF *CANNABIS SATIVA* L.

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Abstract: The aim of this study was to investigate the effects of concentration of different growth regulators (auxins and cytokinins) on morphological characteristics of hemp variety Bialobrzeskie. The plants were sprayed with 1-naphthaleneacetic acid (*NAA*) and 6-benzylaminopurine (*BAP*) and the resulting influence on the total height and lateral branching was observed. Expected results were obtained for variants treated with BAP, whereas the variants treated with auxin showed an increase in lateral branching.

Key Words: cytokinin, auxin, axillary branches, height, hemp

INTRODUCTION

Phytohormones are endogenous molecules occurring naturally in plants at very low concentrations. They do not have any nutritional function, but act as signalling compounds that regulate plant development and physiology (Sauer et al. 2013). Auxin and cytokinins are hormones influencing a wide range of plant development processes. Cytokinins favour the development of axillary buds whereas auxin supresses the development.

Via regulation of meristem activity, with opposite role in root and shoot morphogenesis (Werner et al. 2001), cytokinins determine plant shape, which allows plant to adjust to site conditions in order to take advantage of the environment. Both auxin and cytokinin have been known for a long time to act either synergistically or antagonistically to control several significant developmental processes, such as the formation and maintenance of meristem (Su et al. 2011). Shoot branching is a major determinant of plant architecture and is highly regulated by endogenous and environmental cues. Both classes of hormones, auxin and cytokinin, have long been known to have an important involvement in controlling shoot branching (Umehara et al. 2008)

Cannabis (*Cannabis sativa* L.) has been grown worldwide for thousands of years for its valuable properties, like fiber and oil content, and for medicinal purposes and as an intoxicant (Small and Cronquist 1976, Kojoma et al. 2005). Cannabis, or hemp, can provide high biomass quantities in a short time (Weiblen et al. 2015). The stem of this fiber crop supplies both cellulosic and woody fibres: the core is indeed lignified, while the cortex harbours long cellulose-rich fibres, known as bast fibers (Guerriero et al. 2013). The current climatic and economic scenario pushes towards the use of sustainable resources and hemp can be a source of fibres, oil, and hemp biomass. Exogenous phytohormones can be used to influence the shoot branching and can cause greater vegetative biomass, fruit and seed production. The effect of phytohormones on the shoot architecture of *Cannabis sativa* L. has never been studied before. The aim of the study was to document the changes in morphological characteristics of Bialobrzeskie, a hemp variety cultivated for fibres.

MATERIAL AND METHODS

The experiment on effects of plant hormones on the morphological characteristics of hemp variety Bialobrzeskie was carried out in the greenhouse of Mendel University in Brno. The seedlings were transferred to the greenhouse after one week after sowing. After acclimatization for three weeks the first measurements of the plants were carried out. The plants were measured for their total height and length

of axillary branches in individual nodes. In total there were seventy plants divided in seven different groups so there were 10 plants placed in each group. Three groups were treated with 1-naphthaleneacetic acid (1-*NAA*) in the concentrations of 5, 10 and 20 mg/l. The other three groups were treated with 6-benzylaminopurine (*BAP*) in the concentrations of 10, 25 and 50 mg/l. The phytohormone solutions were prepared from stock solutions of 100 mg/ml in dimethyl sulfoxide and 200 mg/ml in dimethyl sulfoxide for 1-NAA and BAP respectively. 1.25 ml of Tween was added as a surfactant and the volume of the mixture was adjusted to 250 ml using distilled water.

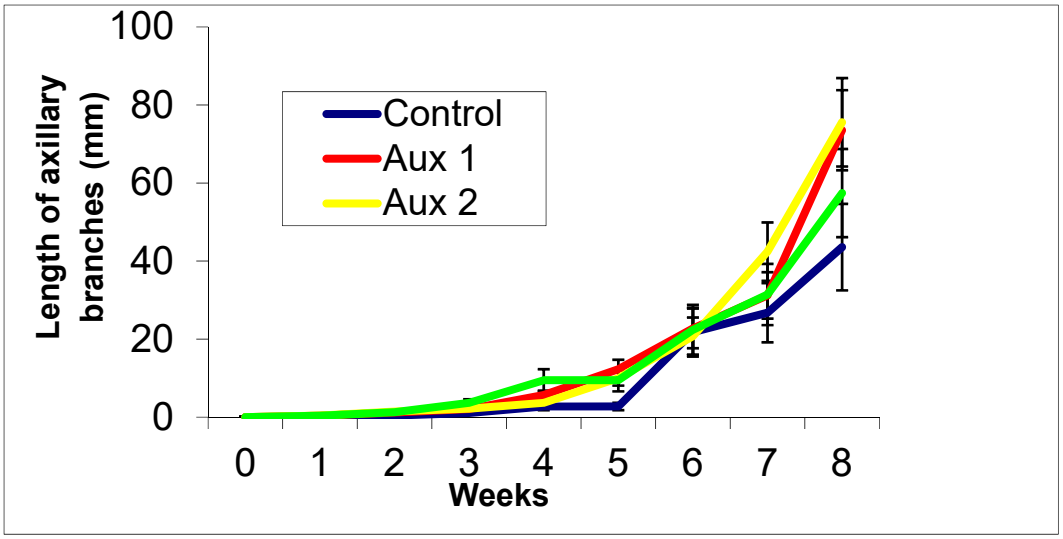
Both 1-NAA and BAP were applied by spraying on the leaves of the plants. The control group were treated with none of the growth hormones. The plants were sprayed with hormones every fortnight and the measurements were done every week on a fixed day. The measurements were noted for every plant in each group for 8 weeks. The mean values, standard deviation and standard error of total plant height and the length of axillary branches for all the variants were calculated.

RESULTS AND DISCUSSION

When comparing the growth of lateral branches in control group vs. NAA treated variants, we can see that the results are contradictory to what was expected (Figure 1). Auxins should inhibit the growth of lateral branches and elongate the stem, but significant increase in axillary branches length in some auxin treated variants in some intervals of measurement was observed. Plants treated with 1-NAA (auxin) variant should have shown an increase in stem length when compared with the control group, but this holds true only for one variant treated with 10 mg/ml of auxin (Figure 2). In many plant species the inhibition of shoot branching caused by exogenous auxin treatment (lanolin paste containing auxin) has been proven (Thimann and Skoog 1934, Morris 1977, Cline 1996) but also a few exceptions have been described (Cline 1996). This result could be caused by decreased sensitivity of hemp buds to inhibitory effect of auxin in apical dominance. Significantly increased length of lateral branches might be consequence of well-known stimulatory effect of auxin on stem elongation of already formed shoots which was described in some species (Yang et al. 1993, Haga and Lino 1997).

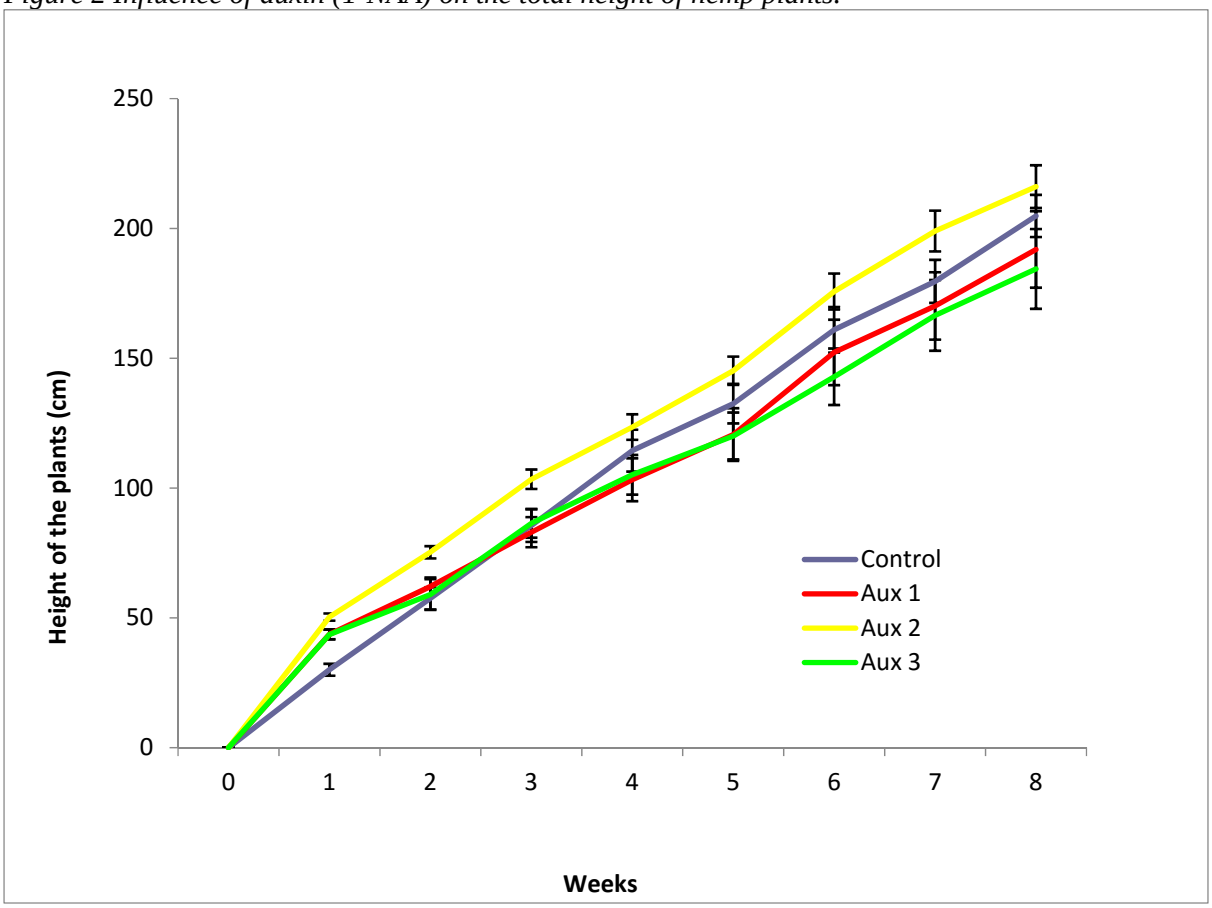
For the 6-BAP (cytokinin) treated group, the results were more in accordance with previous studies (Figure 3). Concentration dependant significant increase in axillary branches length in cytokinin treated variants was observed as known from literature (Sachs and Thimann 1967, Pillay and Railton 1983, Li and Bangerth 2003). The differences were the most significant in the highest dosing. Surprisingly, we can see some disproportion relation to concentration of BAP. Lowest dose promoted the growth of branches more than middle dose. However, the trend was normal before, and the reversal occurred during the sixth week of measurement. BAP did not affect the total height of the plants as they grew similarly with the control group (Figure 4). Exogenous application of cytokinin was not effective in modifying any evaluated plant growth variables also in soybean (Leite et al. 2003).

Figure 1 Influence of auxin (1-NAA) on the length of axillary branches.



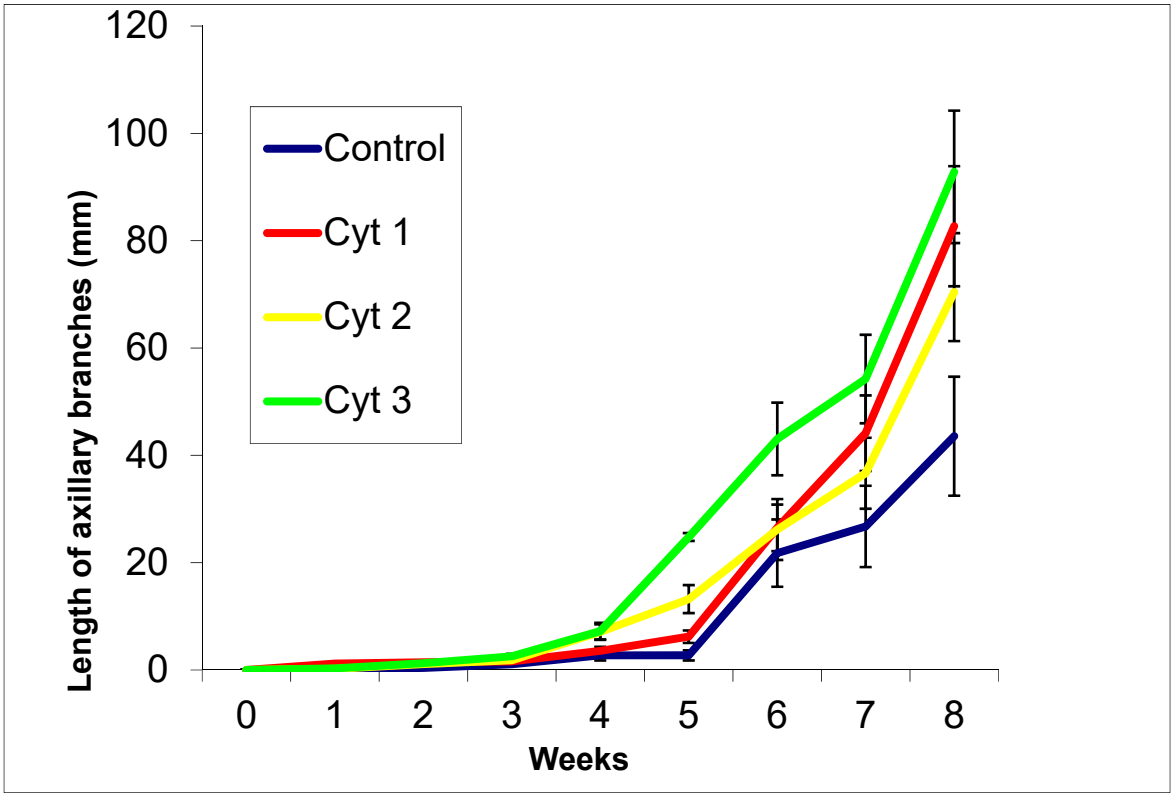
Legend: Aux 1 = 5 mg/ml, Aux 2 = 10 mg/ml, Aux 3 = 20 mg/ml of 1-NAA

Figure 2 Influence of auxin (1-NAA) on the total height of hemp plants.



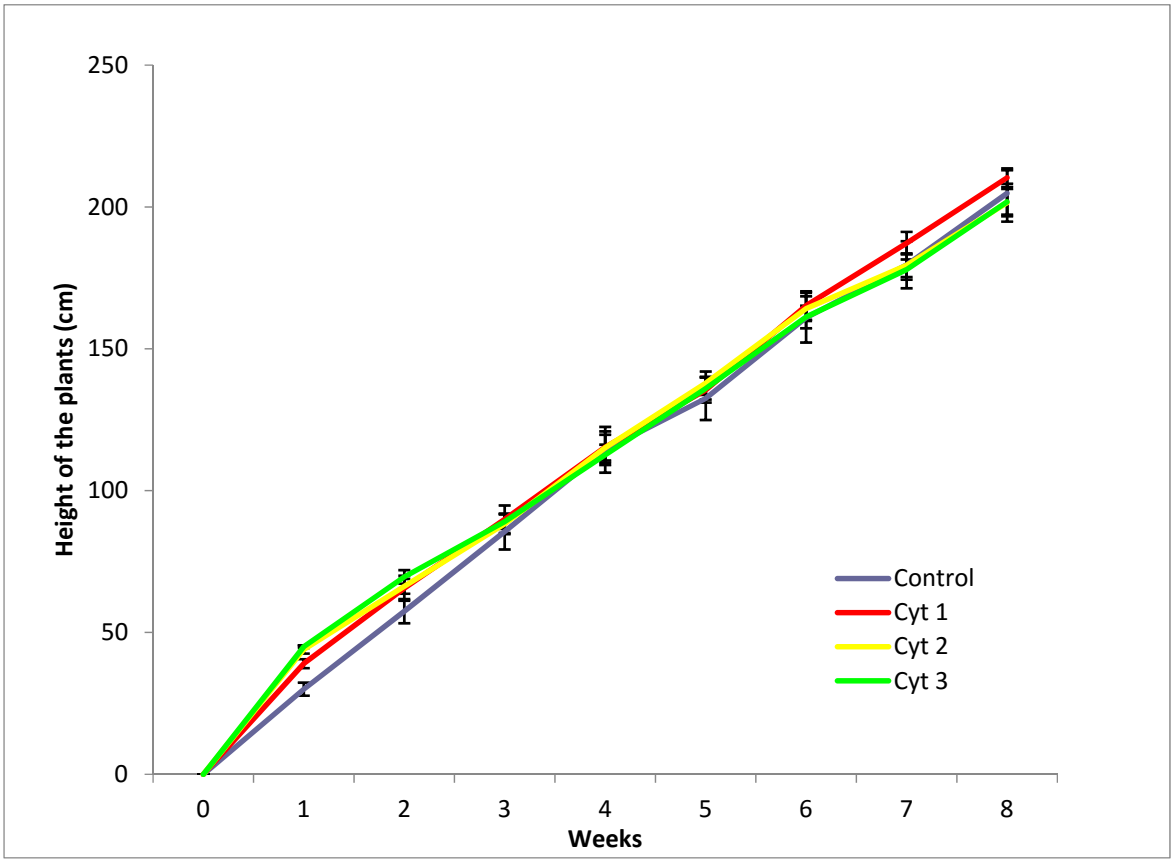
Legend: Aux 1 = 5 mg/ml, Aux 2 = 10 mg/ml, Aux 3 = 20 mg/ml of 1-NAA

Figure 3 Influence of cytokinin (BAP) on the length of axillary branches.



Legend: Cyt 1 = 10 mg/ml, Cyt 2 = 25 mg/ml, Cyt 3 = 50 mg/ml of BAP

Figure 4 Influence of cytokinin (BAP) on the total height of hemp plants.



Legend: Cyt 1= 10 mg/ml, Cyt 2 = 25 mg/ml, Cyt 3= 50 mg/ml of BAP

CONCLUSION

Hemp seems to have a standard response to cytokinins, even when applied externally via leaves. But this study suggests, that it may be less responsive to auxins, when applied exogenously. Another possible explanation is that individual differences between auxin metabolism in hemp and other species contribute to different behaviour when coping with NAA. In future experiments it will be necessary to verify response of hemp plants to auxin using another form of exogenous auxin application e.g. lanolin paste. The influence of increased shoot branching caused by morphoregulators treatment on crop characteristics will also be estimated.

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PROGRESS IN EARLY SEX DETERMINATION OF CANNABIS PLANT BY DNA MARKERS

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Abstract: The cannabis plant is a tall annual crop of economic importance. It is mostly dioecious, but fiber hemp varieties have been bred to be monoecious. Separating male and female plants at early developmental stage is useful due to the influence of gender at agriculturally significant traits. Several experiments have been focused on developing a reliable molecular marker for sex determination in cannabis plants. Our study compares three DNA markers for the detection of male genotype in totally twelve samples of industrial hemp and medicinal cannabis plants. Genotype scoring of SCAR119 marker appeared to be the most reliable, followed by MADC2 and SCAR323, when compared to the observed phenotype of plants. The results confirmed the insights given in previous studies. Research and *Polymerase Chain Reaction* (PCR) analysis should continue in order to find more advanced DNA markers for sex determination of cannabis plants.

Key Words: cannabis, sex determination, DNA markers

INTRODUCTION

Cannabis (*Cannabis sativa* L.) is a tall upright annual herb. It is generally dioecious i.e. producing separate male and female plants but fiber hemp varieties have been specifically bred to be monoecious (hermaphrodite) (Debruyne et al. 1994, Srivastava and Yadav 2013). The sex of most dioecious plants can only (reliably) be determined at the time of flowering. The significance of separating male and female plants at seedling stage lies in the fact that in many dioecious plants gender influences the economic value, breeding schemes and opportunities for commercial use of genetically modified materials (Parker and Clark 1991).

The discussed demand for tools supporting sex determination in plants gave rise to a series of molecular studies investigating DNA markers that could be used for that purpose. A molecular marker (DNA marker) is a DNA sequence observed in at least two versions that are easy to distinguish (Brown 2002), which reveals individual polymorphisms. The preferred marker should demonstrate the widest possible range of variation in the analyzed trait, and it should not be affected by environmental factors. An effective marker should guarantee reproducibility, and it should be easy to detect. Molecular markers facilitate analyzes of variations between individuals, regardless of their development stage (Sztuba-Solińska 2005), which is particularly useful in sex determination studies of plants. Some researchers have suggested that effective markers for plants should be relatively short to support sex determinations in herbarium specimens with damaged DNA. Shorter sequences increase the probability of successful amplification (Korpelainen et al. 2008).

This study is focused on testing the reliability and reproducibility of three developed molecular markers linked to male sex in the cannabis plant (*Cannabis sativa* L.). Standard technical hemp varieties, as well as medicinal ones were included.

MATERIAL AND METHODS

DNA isolation and samples used

Four well known varieties of industrial hemp and six experimental varieties of medicinal cannabis were included in this experiment (Table 1). The medicinal cannabis samples were all provided by Bedrocan International in form of DNA. For the variety Bialobrezskie, three plants from an experiment, where plants were treated with phytohormones were selected: a NAA (naphtylacetic acid) – auxin analogue variant, a BAP (benzylaminopurine) – cytokinin analogue variant and an untreated control group. Chemicals were applied every two weeks by spraying it on the leaves in six different doses, each for different group: 5, 10 and 20 mg/l for NAA and 10, 25 and 50 mg/l for BAP. After 11 weeks of application, one plant from the most concentrated auxin group as well as one from highest cytokinin dosing, showed male flowering structures. DNA was isolated from these two plants as well as from one plant of the untreated control group. These three plants were included in the sex determination experiment.

Total genomic DNA was isolated from 0.1 g of fresh leaves homogenized by mortar and pestle and liquid nitrogen. A DNeasy Plant Mini Kit (Quiagen) was used for the isolation process. Concentration and purity of isolated DNA was measured using Picopet 1.0 spectrophotometer (Picodrop).

Table 1 Overview of used samples

Sample marking	Variety/ genotype	Material	Source/ provider
CAR	Carmagnola	seeds	Hempoint Ltd.
KHT	Kompolti Hybrid TC	leaves	Hempoint Ltd.
UNI	Unikó	leaves	Hempoint Ltd.
B-C	Bialobrezskie	leaves	Hempoint Ltd./ untreated control
B-M1	Bialobrezskie	leaves	male plant treated with 20 mg/l NAA
B-M2	Bialobrezskie	leaves	male plant treated with 50 mg/l BAP
BK1	medicinal cannabis *(unspecified)*	DNA	Bedrocan International
BK4	medicinal cannabis *(unspecified)*	DNA	Bedrocan International
BK5	medicinal cannabis *(unspecified)*	DNA	Bedrocan International
BK7	medicinal cannabis *(unspecified)*	DNA	Bedrocan International
BK8	medicinal cannabis *(unspecified)*	DNA	Bedrocan International
BK9	medicinal cannabis *(unspecified)*	DNA	Bedrocan International

PCR conditions

Three different primer pairs for sex determination were tested (Table 2). The first two markers (SCAR – sequence-characterized amplified region) were developed from RAPD primers by Törjék et al. (2002), the other marker (MADC – male-associated DNA from *Cannabis sativa*) is based on the research of Mandolino et al. (1999).

PCR was performed in a total volume of 25 µl consisting of 0.5 U *Taq* polymerase (Promega), 1× aliquot buffer, 0.1 mM of each dNTP(Promega), 0.3 M of each primer and 20 ng of template DNA in a T3 thermocycler (Biometra) for the SCAR markers and gradient thermal cycler QB-96 (Quanta

Biotech) for the MADC marker. We used the same PCR protocols as the aforementioned authors, without any further optimization for the SCAR (Törjék et al. 2002) and the MADC (Mandolino et al. 1999) markers.

Electrophoresis was performed in a 1.5 % agarose gel on a Blue Marine 200 apparatus (Serva), with Tris-acetate-EDTA buffer (TAE) and ethidium-bromide used for for staining. The presence and size of PCR products was visualized by using a UV transluminator and VisionCapt software (Vilber Lourmat).

Table 2 Primers used for sex determination

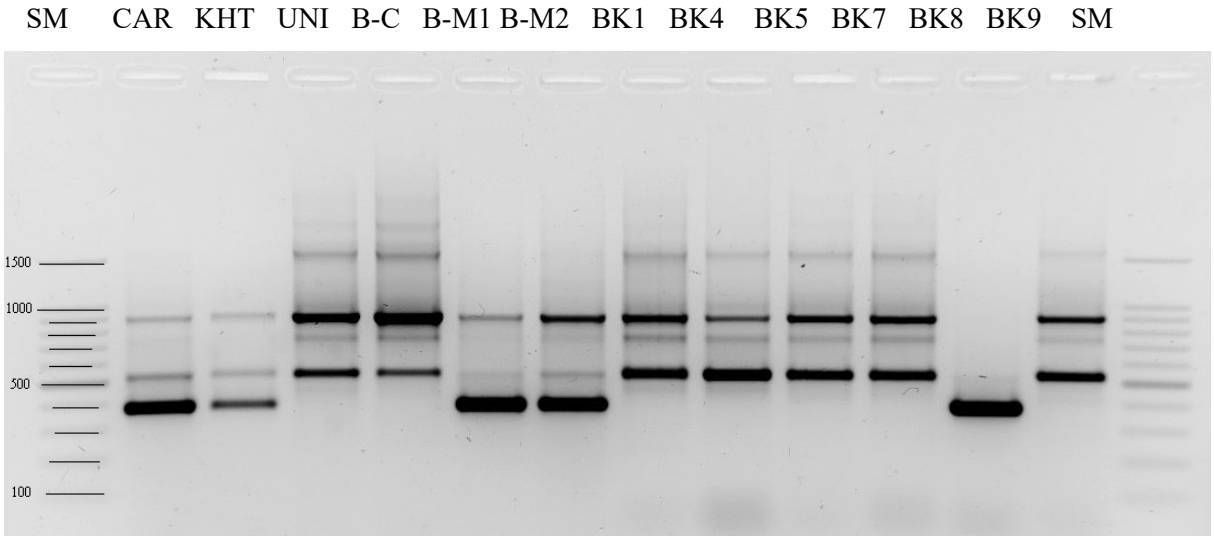
Marker name	Amplification product	Primer sequence 5'-3'
SCAR119_F	male sex linked DNA	TCAAACAACAACAAACCG
SCAR119_R	(119 bp)	GAGGCCGATAATTGACTG
SCAR323_F	male sex linked DNA	GAGCGGACATCATTGCCT
SCAR323_R	(323 bp)	ATCACCCCACCGTTTAGG
MADC2_F	male sex linked DNA	GTGACGTAGGTAGAGTTGAA
MADC2_R	(390 bp)	GTGACGTAGGCTATGAGAG

RESULTS AND DISCUSSION

MADC marker amplification products

Total genomic DNA of all twelve cannabis plant samples was amplified by specific primers. A single DNA band of size about 390 bp was expected for all putative male plants, while all female and monoecious plants were expected to have two products of about 560 and 870 bp. This appeared to be the case with ten of the samples, two deviations – in the case of Carmagnola and Kompolti Hybrid TC have possible explanations (Figure 1).

Figure 1 Visualisation of PCR products for sex determination of cannabis varieties (MADC2 marker)



Legend: SM – 100 bp size marker, CAR – Carmagnola, KHT – Kompolti Hybrid TC, UNI – Unikó, B-C – Bialobrezskie monoecious control, B-M1 – Bialobrezskie male plant treated with NAA, B-M2 – Bialobrezskie male plant treated with BAP, BK1, BK4, BK5, BK7, BK8, BK9 – unknown genotypes of medicinal cannabis (Phyto Engineering Department, Bedrocan International)

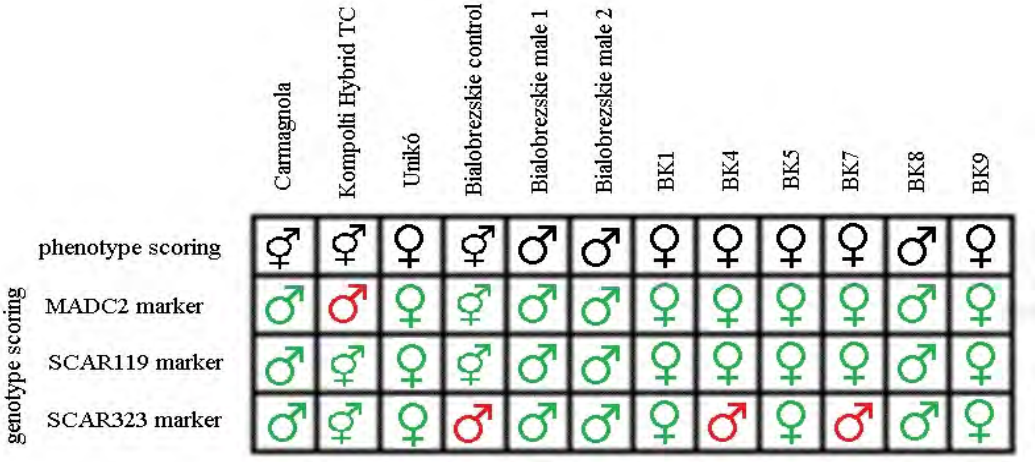
As mentioned by Mandolino et al. (1999), MADC2 is probably a non-coding genome region and it is not confirmed whether the sequence is a part of the genes for sex determination. Most likely, it is not located solely on the male chromosome, as in previous studies MADC2 failed to discriminate sex phenotype in some cases (Sakamoto et al. 1995). Our results support this hypothesis, as the first two samples should be phenotypically female/ monoecious, but show products supposedly corresponding to the male genotype. In addition, DNA of the first variety (Carmagnola) was isolated from seeds. Sex

determination in cannabis is complex and can even be reversed or modified by chemical treatment and environmental factors (Chailakhyan 1979, Mohan Ram and Sett 1979). At the same time, male flowers are able to develop on female plants under extreme conditions (Clarke 1997). BK8, although being a medicinal cannabis plant, was observed to form male flowering structures and was, based on the examination of the plant morphology, classified as a male and possibly dioecious plant. This theory can help us explain the fact, that during our experiment with phytohormones two unexpected staminate plants with male phenotypes appeared (they produced a lot of pollen), while the used variety Bialobrezskie is normally monoecious, as stated in a list (2014) by the Czech Central Institute for Supervising and Testing in Agriculture. Two slightly visible bands of higher molecular weight can be seen on the gel (Figure 1) in case of the second male plant. This suggests some gradual development from a previously monoecious plant. Another hypothesis is genetic recombination between the sex locus and the marker, on which both authors (Mandolino et al. 1999, Törjék et al. 2002) - speculated. The research group of Techen et al. (2010) was dealing with sex determination of cannabis and certain types of MADC and SCAR markers as well, being able to reliably identify female individuals in all cases. However, the experiment was carried out in very early seedling stage and it is not exactly known whether they included monoecious plants, in which stage of onthogenesis the development of male phenotype really begins and what mechanisms are underlying it.

Comparison with SCAR markers

The same set and order of DNA samples (Table 1) was used to test the SCAR markers. In case of SCAR119, our sex determination results seem to be generally in accordance with MADC2 and with the information from Törjék et al. (2002) – that male plants are presented with single band of 119 or 323 bp (for SCAR323). And while some female plants may show the same product as well, it is much less intensive. This, however, appeared to be in contrast with our results for SCAR323 – the high intensity for male might be corresponding in case of Carmagnola seeds, previously mentioned BK8 and two phenotypic males (B-M1, B-M2), but there was still quite a robust product of 323 bp in the case of the monoecious Bialobrezskie plant (B-C) and some of the medicinal genotypes (BK4 and BK7). An overall evaluation of phenotype versus genotype scoring of all varieties and DNA markers used in this study is shown in Figure 2.

Figure 2 Final comparison of DNA markers results for all varieties



Legend: ♀ - plant with female phenotype, ♂ - plant with male phenotype, ⚥ - monoecious plant, GREEN colour – sample with genotype scoring in accordance to phenotype, RED colour – samples with incorrect genotype indication

CONCLUSION

Three molecular markers were tested for their ability to detect male plants in cannabis. DNA material of various origin was used, twelve different varieties of cannabis plants in total.

The SCAR119 marker appeared to be the most reliable, with genotype scoring results corresponding to phenotype in case of all twelve samples. The MADC2 marker showed only one

contradictive sample result. The SCAR323 marker appeared to be the most controversial, with three deviant results.

This study confirmed that sex determination of the cannabis plant is a complex process, for which further research is needed to develop a reliable molecular tool to distinguish male and female plants at an early developmental stage.

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ANTIBACTERIAL EFFECT OF SELECTED NANOPARTICLES AS REVEALED BY DOUBLING TIME OF TREATED *XANTHOMONAS CAMESTRIS* PV. *CAMPESTRIS* CULTURES

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Abstract: Besides many possibilities of applications of nanoparticles in the field of medicine, diagnostics, molecular biology, bioorganic chemistry or remediation of environment, there is also a potential of employment of nanoparticles as a tool for elimination and control of bacteria invading plant tissue. In this experiment an antibacterial activity of selected nanoparticles based on silver, gold and bimetallic silver/copper was tested on bacteria *Xanthomonas campestris* pv. *campestris* (Xcc*)* (strain 1279a). The strongest inhibitory effect represented by doubling time of treated cultures was measured in the presence of the smallest silver nanoparticles (9 nm) at the highest concentration (5 ppm).

Key Words: nanoparticles, *Xanthomonas campestris,* doubling time, antibacterial effect

INTRODUCTION

Borm et al. (2006) consider nanoparticles (NPs) as particles with size from 1 to 100 nm. Properties of NPs depend on the used material, size, shape and their surface area. Recently, the use of NPs in industry and in manufacture of commercial products recorded large increase. Understanding of interaction mechanisms between NPs and biological systems on the molecular level is still insufficient (Barren et al. 2009). Simultaneously, new ways of NPs applications are found but many of that are still in the phase of testing (Ngomsik et al. 2005, Uheida el al. 2006). One of the most important properties of metal nanoparticles is their antibacterial activity. Nanoparticles slow down growth of bacteria or eliminate bacteria by disrupting the function of the cell wall (Ahmed et al. 2016). The antibacterial properties of silver NPs are widely employed in healthcare industry and they can also be used for the control of bacterial and fungal plant diseases (Yo et al. 2009). Similar effects have nanoparticles based on copper (Cu NPs) or titanium dioxide (Giannousi et al. 2013, Paret et al. 2013). On the other hand, certain concentrations of zinc oxide NPs proved an inhibition of seed germination and root elongation (Hrdinová 2011). Study focused on the application of gold nanoparticles on aquatic plant *Ceartophyllum demersum* showed an inhibitory effect as well (Ostroumov et al. 2014).

Xanthomonas campestris is a gram-negative quarantine bacterium belonging to strain of proteobacteria. This bacterium has been genetically divided into more than 140 pathovars and each of them has a different range of hosts. *Xanthomonas campestris* pv. *campestris* (Xcc) usually infects system of species from Brassicaceae family and exhibits characteristic symptoms as black rot caused by blackening of vascular bundles and V-shaped necrosis proceeding from the edges to central vessel (Park et al. 2004). Xcc belongs to seed-borne pathogens and it enters plants through hydatodes, stomata, roots or injuries. Xcc infects a wide scale of Brassicaceae family species including cabbage, cauliflower, broccoli, radish, or *Arabidopsis*. Therefore, this bacterium is an economically important pathogen (Williams 1980). The epidemics of Xcc are repeated worldwide and regularly while areas with high temperature and humidity are the most affected. Thus, significant losses of agricultural products are

caused by Xcc epidemics (Qian et al. 2005). Despite increasing knowledge, there are no effective approaches for elimination of Xcc from seeds and crops.

MATERIAL AND METHODS

The nanoparticles were purchased from NPIC s.c. (G. Celichowski J. Grobelny, Poland). Silver nanoparticles were tested in 9, 19, 35 and 61 nm sizes, gold NPs in 9, 19 and 35 nm sizes and silver-copper NPs in sizes of 29 and 69 nm. Particular emphasis in the choice of supplier was placed on ability to get nanoparticles in solutions without any biological toxicity for biologically active systems such as plants or bacteria. Dissolving medium was used as a control in this experiment.

Measurement of inhibitory effect of NPs against Xcc

For testing, the strain 1279a of *Xanthomonas campestris* pv. *campestris* was chosen. The strain was obtained from the collection of The University of Warwick HRI (UK). Antibacterial activity of NPs was tested by spectrometric measurement of growing activity under influence of different types of nanoparticles. For this measurement, the spectrophotometer SpectraMax PLUS384 from Molecular Devices (Sunnyvale, California) and 96-well plates were used.

Firstly, Xcc were cultivated overnight in liquid LB medium (20 ml of medium, 30 °C, 160 rpm). Obtained substance was used to inoculate experimental cultures. Cultivation of bacteria with added nanoparticles was performed in 96-well plates. The final volume of cultures was 200 µl. Cultures were inoculated into a new LB medium (initial OD_{600} nm=0.08). The final concentration of NPs (5; 3.75; 2.5; 1.25; and 0.5 ppm) was added into the cultures. As a control, a reference solution (NPIN s.c.) was used. Each type and each concentration of NPs was prepared in triplicate. Cultures were incubated at 30 °C with continual mixing (100 rpm) for 6 hours. During the cultivation, the optical density (600 nm of wavelength) of cultures was measured. Sterile LB medium was used as a blank. Interval of measurement was determined on every 10^{th} minute. Values of obtained ODs were used for calculation of bacteria doubling time (T min.). Software SoftMax Pro v4.6 (Molecular Devices, Sunnyvale, California), Statistica and Microsoft Excel were employed for data acquisition and evaluation.

RESULTS AND DISCUSSION

Au NPs

The highest increase of Xcc doubling time (31 minutes) with gold nanoparticles was measured at the size of 19 nm with a concentration of 5 ppm (see Figure 1). The doubling time was also increased at the 35 nm Au NPs but the effect was not as strong as at 19 nm Au NPs (less than 15 minutes at the concentration of 5 ppm). In most of the concentrations of 9 nm Au NPs the doubling times were below the control. The explanation of this phenomenon requires additional experiments but is out of our scope to search for growth inhibition.

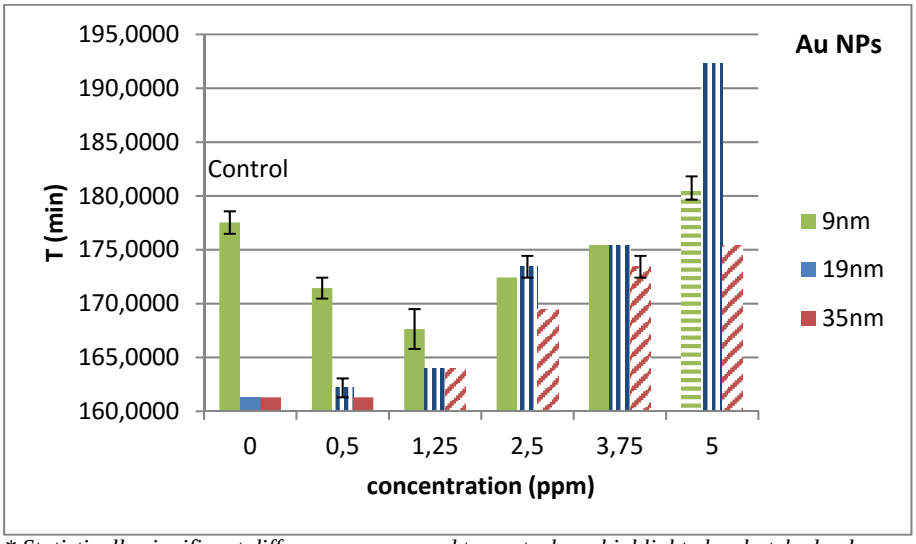
Ag/Cu NPs

Description of Ag/Cu-based NPs antibacterial activity is possible to find in literature (Singh et al. 2014, Yasir et al. 2016). The aim of including this type of NPs was to verify the possibility of synergistic effect of silver-copper composited nanoparticles. The doubling time was affected at the variant with size of 29 nm in a concentration of 5 ppm (16 minutes). Variants with lower concentrations as well as 69 nm sizes didn´t have any significant effect on the doubling time (see Figure 2).

Ag NPs

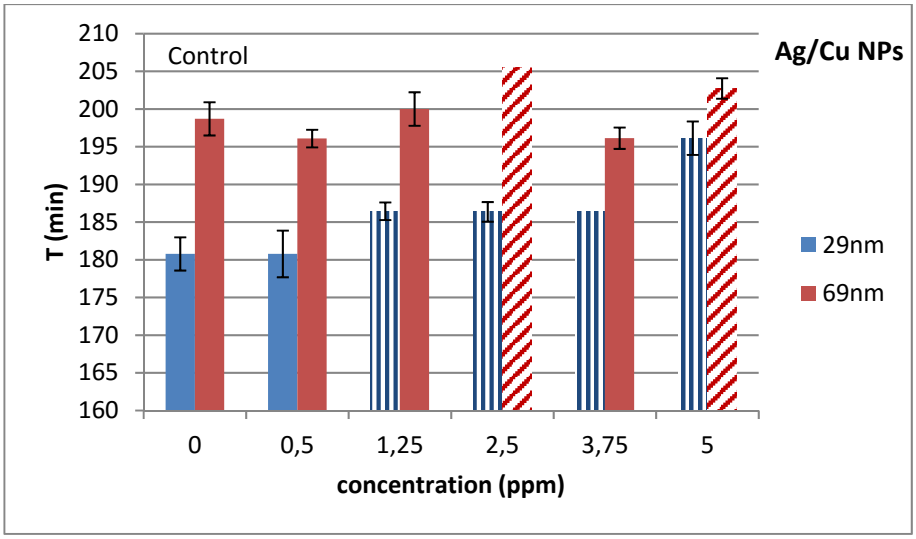
Four kinds of Ag NPs with diameter of 9, 19, 35 and 61 nm were tested. Variant of 35 nm diameter showed statistically significant effect with concentrations of 1.25 ppm and higher. Other sizes described within Fig 3 (19 nm, 61 nm) showed significant effect from the concentration of 2.5 ppm and higher. An influence of concentration is noticeable, when in the case of variants with the highest concentration (5 ppm) the difference of doubling time compared to control was from 18 to 21 minutes (see Figure 3).

Figure 1 Doubling time of Xcc 1279a in the presence of 9, 19, and 35 nm Au NPs



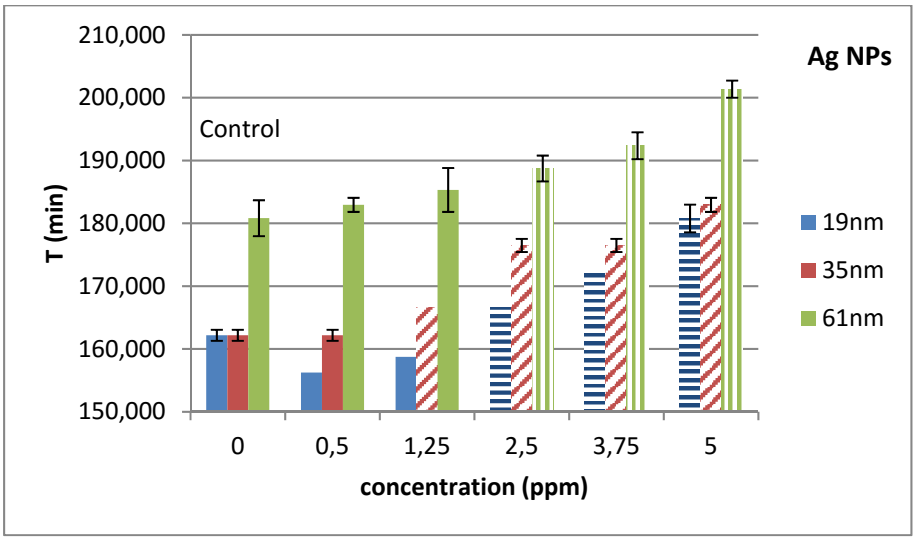
** Statistically significant differences compared to control are highlighted as hatched columns*

Figure 2 Doubling time of Xcc 1279a in the presence of 29 and 69 nm Ag/Cu NPs



** Statistically significant differences compared to control are highlighted as hatched columns*

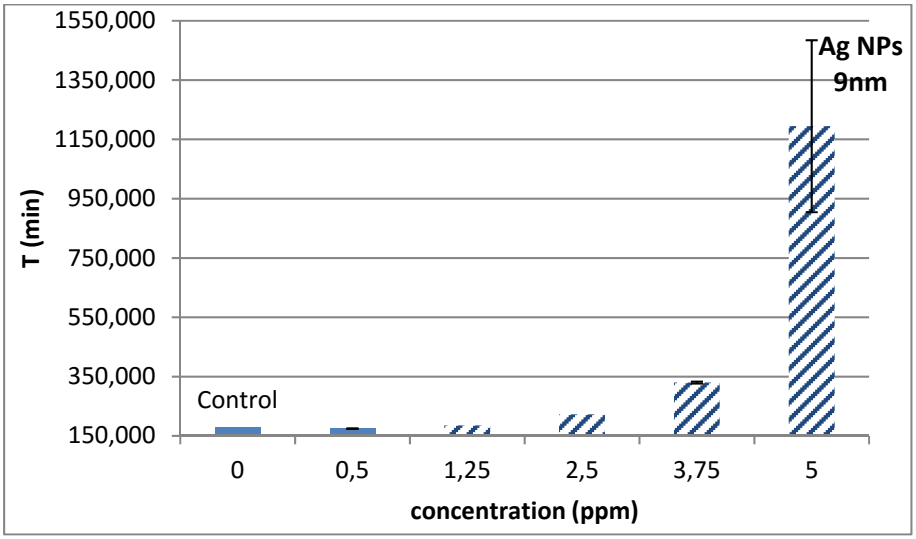
Figure 3 Doubling time of Xcc 1279a in the presence of 19, 35 and 61 nm Ag NPs



** Statistically significant differences compared to control are highlighted as hatched columns*

A unique position in the context of this study was showed by the variant of 9 nm diameter Ag NPs. Doubling time of 5 ppm concentration was increased by 1016 minutes compared to the control. This is many times more than other tested variants. Significantly above the average of the experiment, it was also the variant with the concentration of 3.75 ppm (doubling time increased by 151 minutes) (see Figure 4).

Figure 4 Doubling time of Xcc 1279a in the presence of 9 nm Ag NPs



** Statistically significant differences compared to control are highlighted as hatched columns*

There are many studies focused on the antibacterial activity of nanoparticles. Nanoparticles have an influence on the growth and development of bacteria and development of bacterial biofilm. Shristavata et al. (2007) reported 60% slowdown of the gram-negative bacterium *Escherichia coli* grown in LB medium in the presence of silver nanoparticles at a concentration of 5 mg/ml. Concentration of 10 mg/ml showed 90% growth retardation and concentration of 25 mg/ml showed complete inhibition. On the other hand, gram-negative bacteria *Staphylococcus aureus* showed no slowdown at a concentration of 25 mg/ml of Ag NPs and concentration of 100 mg/ml showed only partial slowdown. Thus there are indications that the antibacterial effect of individual nanoparticles is specific for each species and before targeted application it is necessary to verify this effect on the specific bacterium. Based on our preliminary study, use of Ag NPs against Xcc seems to be promising.

Gold nanoparticles used in this study didn´t prove so high inhibitory effect as for silver NPs. But interestingly, Mu et al. (2016) published a study which showed significantly higher antimicrobial properties of chitosan-streptomycin conjugates bound to the gold nanoparticles. These substances had higher ability to eliminate biofilms of gram-positive and gram-negative bacteria and proved to be more effective than chitosan conjugates, or streptomycin alone. However, limited availability of this type of nanoparticles didn´t let us to verify these effects against Xcc, we received information only about the unmodified Au NPs.

Ag/Cu NPs showed the smallest effect on the doubling time of Xcc cultures from all three types of NPs used in this experiment. On the other hand, Giannousi et al. (2013) published study describing the positive antibacterial effect of copper-based nanoparticles. *Bacillus cereus*, *Bacillus subtilis*, *E. coli*, *X. campestris* and *S. aureus* were exposed to Cu and Cu_2O NPs. The half-maximal inhibitory concentration (IC_{50}) and 100% inhibitory concentration (IC_{100}) were determined and were various depending on the size, type and concentration of nanoparticles. However, the results of our study didn´t confirm potential synergistic effect of these composite nanoparticles. Effect of Ag/Cu NPs was smaller than Ag NPs.

CONCLUSION

The results of this experiment showed as the most effective silver-based nanoparticles. Ag NPs 9 nm size variant at a concentration of 5 ppm showed an increase of Xcc doubling time by more than 570% compared to control. Same size of these nanoparticles at concentration of 3.75 ppm also increased

the doubling time of Xcc by more than 84%. The best result for the gold nanoparticles was showen in the case of 19 nm size at the concentration of 5 ppm (doubling time extended by 19%). Bimetallic Ag/Cu NPs showed the slightest antibacterial effect of all tested nanoparticles. Although the effects of nanoparticles on biological systems are intensively studied, the exact mechanism of their action is not completely understood. There is no possibility to exactly determine which property of the nanoparticles is the most important in its antimicrobial effect. Acquired results represent solid basis for further experiments.

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NEW REAL-TIME RT-PCR ASSAYS FOR DETECTION OF TYMV (TURNIP YELLOW MOSAIC VIRUS) AND EVALUATION OF REACTION OF CABBAGES TO TYMV INFECTION

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Abstract: Samples of Turnip yellow mosaic virus obtained from two Czech viral collections were used for design and optimization of real-time PCR detection system for its presence. Samples were sequenced and the phylogenetic analysis was done. The new real-time PCR primer pair and probe were designed and the systems based on TaqMan probe and SYBR Green were tested. The detection system for TYMV using SYBR Green was optimized and evaluated on 6 different cultivars of *Brassica* species artificially inoculated by TYMV.

Key Words: Turnip yellow mosaic virus, real-time PCR, molecular detection, infection

INTRODUCTION

Turnip yellow mosaic virus (TYMV) is a plant pathogenic virus that belongs to the *Tymovirus* genus. It causes mosaic disease on species of *Brassicaceae* family and two closely related families, the *Capparidaceae* and *Resedaceae* (Alfaro-Fernández et al. 2016). The virus is transmitted by insects as leaf flea beetles (*Phyllotreta* genus) and also by seeds (Lee and Rho 2015). Contemporary phylogenetic analyses have identified two distinct groups of isolates based on the studies of coat protein (CP) gene sequence of different isolates. The TYMV-1 group includes the European and Australian subgroups, the TYMV-2 different UK isolates. Moreover, further studies identified other subgroup of Japanese isolates which is closely related to subgroups of TYMV-1 (Alfaro-Fernández et al. 2016).

The possibility of spreading by seed makes TYMV potential economically important pathogen that should be monitored and tested in seed production. The real-time RT-PCR systems provide many advantages compared to standard RT-PCR, especially the quickness, accuracy and high sensitivity of detection. On the basis of these facts, the objective of this study was to design real-time PCR system for end-point detection of TYMV in plant material and its evaluation on potential breeding material of cabbages artificially inoculated by TYMV.

MATERIAL AND METHODS

Viral isolates, design of real-time PCR reaction

Positive isolates of TYMV were obtained from collections of Crop Research Institute (CRI, Prague-Ruzyně, Czech Republic) and Central Institute for Supervising and Testing in Agriculture (CISTA, Olomouc, Czech Republic). Both collections stored isolates on dried leaves of headed Chinese cabbage (*Brassica rapa* var. *pekinensis* L.), cultivars 'Bristol' (CRI) and 'Hilton' (CISTA). Part of each sample was used for direct isolation of RNA, second part for mechanical re-inoculation with phosphate buffer; isolate TYMV to plants of Chinese cabbage 'Bristol' and 'Nozaki', isolate TYS to *Chenopodium quinoa* L. For optimization of real-time PCR systems, 10 samples were used (Table 1).

Table 1 Overview of samples used for real-time RT-PCR optimization

Sample	Host	Origin	Processing	Sample	Host	Origin	Processing
TYMV	*B. rapa* var. *pekinensis* L. 'Bristol'	CRI	Direct isolation of RNA	MY1	*Chenopodium quinoa* L.	CISTA	Inoculated by TYS
TYV1	*B. rapa* var. *pekinensis* L. 'Bristol'	CRI	Inoculated by TYMV	MY2	*Chenopodium quinoa* L.	CISTA	Inoculated by TYS
TYV2	*B. rapa* var. *pekinensis* L. 'Bristol'	CRI	Inoculated by TYMV	MY3	*Chenopodium quinoa* L.	CISTA	Inoculated by TYS
Y	*B. rapa* var. *pekinensis* L. 'Nozaki'	CRI	Inoculated by TYMV	MY4	*Chenopodium quinoa* L.	CISTA	Inoculated by TYS
TYS	*B. rapa* var. *pekinensis* L. 'Hilton'	CISTA	Direct isolation of RNA	MY5	*Chenopodium quinoa* L.	CISTA	Inoculated by TYS

The total RNAs of samples were isolated by Spectrum Plant Total RNA Kit (Sigma Aldrich, St. Louis, MO, USA) and reverse-transcribed by RevertAid reverse transcriptase (ThermoFisher Scientific, Waltham, MA, USA). As an internal positive control the detection of Malate dehydrogenase gene was used. To verify presence of studied virus, the standard PCR with primer pair TY 109N0-109M9 (Týcová 2008) was done. Moreover, the PCR fragments of representative samples (TYMV, TYV1, TYS, Y and MY1) were sequenced. The sequencing was done on genetic analyser ABI PRISM 310 as described by Eichmeier et al. (2010) using the TY 109N0 primer. Obtained nucleotide sequences were analysed using CLC Main Workbench 5.0 (CLC bio, Aarhus, Denmark). Cladogram was constructed with MEGA 7.0.18 (Kumar et al. 2016) using Muscle alignment (Edgar 2004) with UPGMB clustering method.

Two frequently used approaches for real-time PCR - the TaqMan probes and SYBR Green were tested. Primers and probe were designed to target the nucleotide sequence of TYMV coat protein gene (Table 2) on the basis of TYMV genome sequences cited in the GenBank database. For TaqMan and SYBR Green approach, the same set of primers and temperatures were used.

Table 2 Primers and probe used in the study

Primer/Probe	5'-3' sequence	Size of product	Reference
TY 109N0	TGGTCGGGAAAGCTGGGGC	192 bp	Týcová (2008)
TY 109M9	CCGGCCCATCACCTCTCACC		Týcová (2008)
ETYMV1	CAATTCCCCAGTCACTCC	125 bp	This study
ETYMV2	CGGGGGTTCATCATTTCA		This study
ETYS	CAACACCCTCTCACCTCTCA	-	This study

The reaction mix with probe consisted of 12.1 µl of water (HPLC purity), 2.5 µl of 10× Taq Buffer (MCLAB, San Francisco, CA, USA), 0.5 µl of dNTPs (10mM, MCLAB, San Francisco, CA, USA), 1 µl of each primer (10µM), 0.4 µl of TaqMan probe (10µM), 0.5 µl of HoTaq DNA Polymerase (200 U/µl, MCLAB, San Francisco, CA, USA) and 2 µl of cDNA template.

For the real-time RT-PCR approach with the SYBR Green, the commercial 2× HotSybr qPCR kit (MCLAB, San Francisco, CA, USA) was used. The reaction mix consisted of 10 µl of kit, 6.4 µl of water (HPLC purity) and 0.8 µl of each primer (10µM). 2 µl of cDNA were added.

The reactions were carried out on Rotor-Gene 3000 (Corbett Research, Venlo, Netherlands) on following program: denaturation at 95 °C for 3 min, 40 repeats of 95 °C for 40 s, 52 °C for 40 s, 72 °C for 40 s and final extension at 72 °C for 7 min. The result of reaction using probe was also checked on 1.2% agarose gel by electrophoresis.

Inoculation and testing of plants

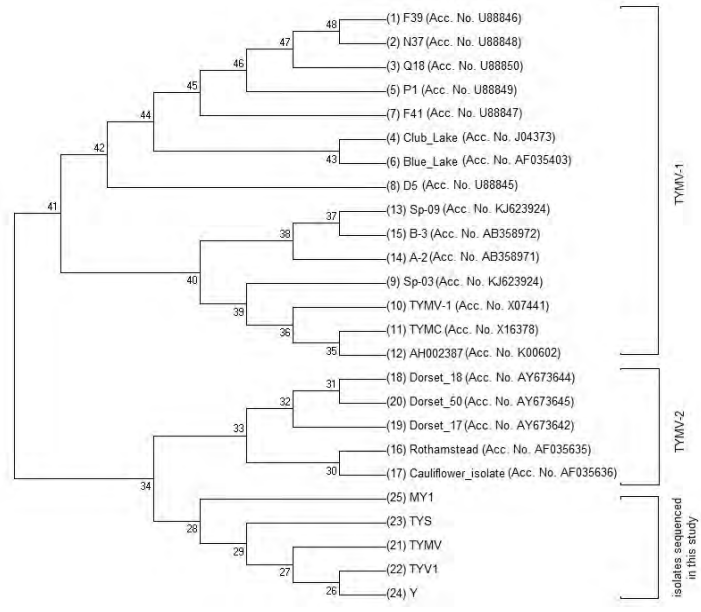
Designed detection system was used for comparison of reaction of six different varieties from the genus *Brassica* to an artificial inoculation by studied virus. They were tested two varieties of three species of *Brassica* genus; varieties 'Orange Heart' and 'Beijing Spring Yellow' of *B. rapa* var. *pekinensis* L., the 'Dwarf Milk' and 'Zi Guan No. 1' of *B. chinensis* L. and 'Avak' and 'Albatros' of *B. olearacea* var. *capitata* L. The experiment was carried out in 3 repetitions and control variant without inoculation. The inoculation was performed at the beginning of October 2015 by the suspension of isolate TYMV1 and phosphate buffer. Leaves of plants (one month old) were dusted with carborundum powder and inoculated by rubbing two leaves of each plant with viral suspension. Plants were cultivated in isolated conditions, in temperature of 18.8 ± 0.9 °C, under Valoya AP673L LED Light (Valoya, Helsinki, Finland). As an indicator plant, *Nicotiana tabacum* L. was used. The sampling was done one month after inoculation; the mixed sample for each variety and repetition was collected and tested by RT-PCR and real-time PCR used SYBR Green method. Obtained data were statistically analysed by Statistica.CZ software (StatSoft, Prague, Czech Republic).

RESULTS AND DISCUSSION

Viral isolates, design of real-time PCR reaction

The sequencing of fragments obtained by standard RT-PCR proved differences between tested isolates. Sequence variability in CP gene may cause also important changes in amino acid variation of capsid protein in different isolates. Moreover, it could bring subsequent differences in the pathology and ecology of isolates (Mitchell and Bond 2005). That could be reason for observed weak symptom expression of MY isolates. The phylogenetic tree (Figure 1) showed division of isolates in two main clusters (TYMV-1 and TYMV-2) as stated in Kirino et al. (2008) or Alfaro-Fernández et al. (2016) and three groups. The third group is represented by isolates sequenced in this study. These isolates showed higher similarity to TYMV-2 group and referred the possible origin of isolates in UK.

Figure 1 Phylogenetic analysis of CP nucleotide sequences of TYMV by Maximum likelihood method based on Tamura-Nei model (Tamura and Nei 1993). All nodes supporting a threshold of 26 are indicated.

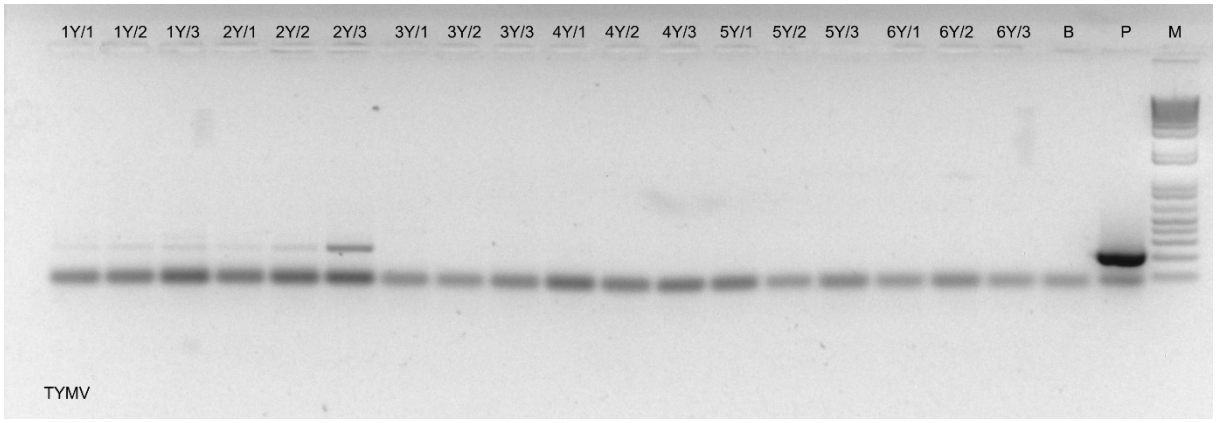


The system using TaqMan probe also confirmed the variability of isolates found by sequencing. Designed probe has detected only isolate TYS and MY1-5 where all MY isolates originated from the TYS. Samples after real-time RT-PCR run were separated on agarose gel. This confirmed positive detection of all tested isolates to TYMV presence. Unfortunately, the probe was not functional for the detection of second group of TYMV isolates probably because of the lack of complementarity of the sequence. The SYBR Green based real-time RT-PCR option detected all available TYMV isolates. Therefore it was selected for the detection of TYMV presence in tissues of tested plants.

Inoculation and testing of plants

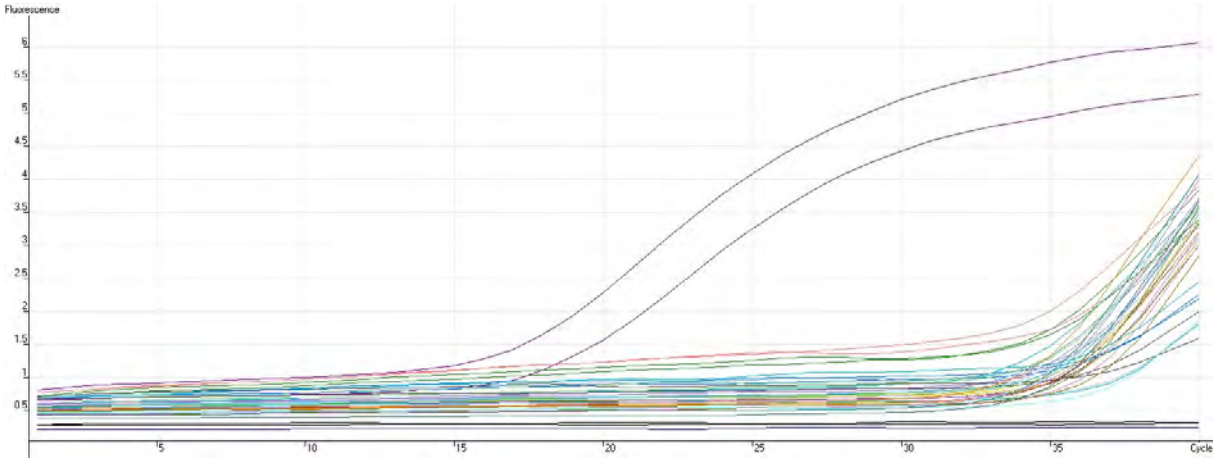
Results of RT-PCR showed positive reaction only for cultivars 'Orange Heart' and 'Beijing Spring Yellow' (Figure 2) whereas the real-time RT-PCR detected TYMV in all inoculated plants (Figure 3). The results of real-time PCR were also confirmed by gel separation of half of reaction volume removed before Melt analysis. Additionally, all control variants were negative for TYMV presence.

Figure 2 Results of RT-PCR for TYMV infection



Legend:1Y – Orange Heart, 2Y – Beijing Spring Yellow, 3Y – Dwarf Milk, 4Y – Zi Guan No. 1, 5Y – Avak, 6Y – Albatros, B - template control, P – positive control, M – 1 Kb Plus DNA Ladder (Invitrogen)

Figure 3 Amplification curve of real-time PCR for inoculated varieties of Brassica plants (SYBR Green, raw data)



Values of measured Ct (threshold cycle, Table 3) were statistically analysed by Tukey HSD test ($P < 0.05$) but no significant differences were found (Table 4). Inoculated plants showed only mild symptoms of virus infection that correspond to values of Ct in real-time RT-PCR.

Table 3 Ct values for real-time PCR detection of TYMV in different Brassica species (SYBR Green)

Sample	Ct	Sample	Ct	Sample	Ct
1Y/1	34.11	3Y/1	31.38	5Y/1	32.17
1Y/1	33.59	3Y/1	31.86	5Y/1	31.40
1Y/2	33.13	3Y/2	32.49	5Y/2	31.56
1Y/2	31.39	3Y/2	32.02	5Y/2	30.17
1Y/3	33.12	3Y/3	33.69	5Y/3	32.92
1Y/3	31.73	3Y/3	35.06	5Y/3	31.69
2Y/1	34.06	4Y/1	30.84	6Y/1	31.09
2Y/1	30.76	4Y/1	35.01	6Y/1	32.00
2Y/2	32.12	4Y/2	32.16	6Y/2	30.96
2Y/2	31.38	4Y/2	31.60	6Y/2	33.61
2Y/3	33.15	4Y/3	31.67	6Y/3	30.59
2Y/3	30.70	4Y/3	34.00	6Y/3	30.88

Legend:1Y – Orange Heart, 2Y – Beijing Spring Yellow, 3Y – Dwarf Milk, 4Y – Zi Guan No. 1, 5Y – Avak, 6Y – Albatros

Table 4 Tukey HSD test; var.: ct. Significant differences on the level of P < 0.05 were not found

Variety	{1} M=32.845	{2} M=32.028	{3} M=32.750	{4} M=32.547	{5} M=31.652	{6} M=31.522
Orange Heart {1}		0.868897	0.999995	0.998422	0.581211	0.471068
Beijing Spring Yellow {2}	0.868897		0.917430	0.979122	0.995144	0.981113
Dwarf Milk {3}	0.999995	0.917430		0.999763	0.662315	0.551177
Zi Guan No.1 {4}	0.998422	0.979122	0.999763		0.819618	0.722734
Avak {5}	0.581211	0.995144	0.662315	0.819618		0.999974
Albatros {6}	0.471068	0.981113	0.551177	0.722734	0.999974	

CONCLUSION

Phylogenetic analysis of CP sequences of TYMV showed that obtained viral isolates are closely related to TYMV-2 group. The real-time PCR primers developed for this study provide in described condition reliable and sensible end-point detection of TYMV in the tissues of horticultural crops which was proved on cabbage plants. The SYBR Green real-time RT-PCR method showed high efficiency in the detection of TYMV in artificially inoculated plants of cabbages and proved even higher sensitivity of reaction than the standard RT-PCR. Neither symptoms of plants inoculated by TYMV suspension nor measured Ct showed significant differences. Therefore additional tests for inclusion or rejection of these varieties for breeding to TYMV resistance were recommended.

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DETECTION OF LMW GLUTENIN ALLELIC COMPOSITION IN *GLU-A3* LOCI OF WHEAT (*TRITICUM AESTIVUM* L.) WITH NON-STANDARD COLOR OF CARYOPSIS

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Abstract: Using 7 specific STS markers, allelic composition of *Glu-A3* locus was examined among set of 36 winter wheat genotypes containing blue, purple, yellow and red caryopses. Allele *Glu-A3d* occurred in all analyzed samples. Together with the *Glu-A3d* allele were detected *Glu-A3f* (53%) and *Glu-A3c* (8%) alleles. Two heterozygous gene forms were detected in wheat collection (*Glu-A3d/f* and *Glu-A3c/d*). Other *Glu-A3a*, *Glu-A3b*, *Glu-A3e* and *Glu-A3g* alleles were not detected in any sample. Selected yield and quality characteristics are also presented. Lines with non-standard caryopsis color had lower yields in comparison with standard ones. Group of lines with purple pericarp had high falling number which is connected with low activity of the hydrolytic enzymes and good resistance to sprouting. The non-standard color of the caryopsis is determined by anthocyanins and carotenoids which are important antioxidants.

Key Words: bread wheat, caryopsis color, LMW-GS, glutenins, wheat, Glu-A3, quality

INTRODUCTION

Common bread wheat (*Triticum aestivum* L.) is one of the most important crops in food industry in the world. Standard color wheat caryopsis is red, which is determined by 13 dominant *R* alleles located on the long arms of the third homologous groups of chromosomes (Himi and Noda 2005) and white is determined by a triplet of recessive alleles. Plant pigments are found in the whole plant, but significant amounts with practical importance are stored in caryopses (Khlestkina 2013). In wheat also exists non-standard caryopsis color: the yellow endosperm, purple pericarp, and blue aleurone (Zeven 1991). The carotenoids and anthocyanins with antioxidative and photoprotective effects are responsible for these types of pigmentation (Kong et al. 2003). Caryopsis is formed by seed coat, endosperm, and embryo (Zimolka 2005). In the endosperm mainlystarch (70%) and proteins (10–15%) can be found as storage compounds. Proteins are primarily located in the aleurone layer. Storage proteins consist of monomer gliadins and the polymer glutenins which are divided to high-molecular weight (HMW-GS) and low-molecular weight (LMW-GS) by mobility in a polyacrylamide gel. LMW-GS and HMW-GS play an important role for baking quality (Branlard et al. 2003). Wheat baking quality is composed of many environmental factors, but also by genotype (Svec and Hruskova 2009). One of the most important components of the wheat endosperm are storage proteins, which are divided into gliadins and glutenins, forming gluten together. Low molecular weight glutenin subunits (LMW-GS) are encoded by genes located on *Glu-3* locus on the short arms of chromosomes 1A, 1B and 1D. Allelic combination of glutenin subunits is directly linked with bread-making quality. The aim of this work was to detect allelic variability in the locus *Glu-A3* for low molecular weight glutenins and thereby contribute to the characterization of individual groups of wheat genotypes differing in caryopsis color.

MATERIAL AND METHODS

The analyzed breeding materials and control varieties were grown in 2016 in the company Agrotest Fyto, Ltd. in central Moravia region (Table 1).

Table 1 Yield, quality parameters, and allelic composition in Glu-A3 locus of the lines with different caryopsis color

Breeding line/ Variety	Caryopsis color	Yield (t/ha)***	Thousand kernel weight (g)	Volume weight (kg/hl)	Protein content (N×5.7) in DM (%)	Falling number (s)	Zeleny test (ml)	Gluten index (-)	Resulting allele of the *Glu-A3* locus
V1-104-15	Ba	8.45 e-k	40.3	74.3	11.1	258	21	40	*Glu-A3d*
V1-106-15	Ba	9.72 b-i	47.9	78.7	12.0	92	36	46	*Glu-A3d*
V1-107-15	Ba	9.92 a-h	46.3	76.6	11.2	164	31	13	*Glu-A3d*
V1-103-15	Ba	7.89 h-k	40.8	73.6	12.3	276	28	51	*Glu-A3d*
V1-114-15	Ba	8.73 d-k	50.5	73.9	11.3	340	39	82	*Glu-A3d*
V1-116-15	Ba	8.21 f-k	48.2	74.5	10.5	287	33	94	*Glu-A3d*
V1-117-15	Ba	7.62 i-k	46.5	71.0	11.6	400	39	76	*Glu-A3d/f*
V2-88-15	Ba	9.89 a-h	49.4	76.9	11.4	344	32	88	*Glu-A3d*
KM 53-14*)	Ba	10.93 a-c	48.9	75.9	11.3	390	47	93	*Glu-A3d*
V1-126-15	Ba	7.97 g-k	48.0	72.9	11.6	194	38	91	*Glu-A3d*
V1-127-15	Ba	8.76 c-k	48.7	73.0	11.0	282	41	94	*Glu-A3d*
V1-129-15	Ba	7.50 jk	51.8	75.0	11.3	288	36	92	*Glu-A3d/f*
Skorpion	Ba	9.99 a-h	49.7	73.7	10.0	82	33	91	*Glu-A3d/f*
V1-141-15	Ba	7.21 k	34.1	74.3	12.8	276	38	49	*Glu-A3d/f*
mean		***8.77***	***46.5***	***74.6***	***11.4***	***262***	***35***	***71***	
V1-180-15	Ye	10.31 a-f	39.8	77.3	11.7	349	46	96	*Glu-A3d/f*
Bona Vita	Ye	9.27 c-k	38.1	78.3	15.4	274	70	60	*Glu-A3d*
Citrus	Ye	9.66 b-j	43.1	77.4	10.8	219	21	96	*Glu-A3d/f*
mean		***9.75***	***37.3***	***77.6***	***12.0***	***281***	***46***	***84***	
V1-142-15	Pp	9.02 c-k	36.8.	78.2	12.5	392	30	77	*Glu-A3d/f*
V1-143-15	Pp	9.49 b-j	33.8	77.6	12.7	405	35	77	*Glu-A3d/f*
V2-60-15	Pp	10.14 a-g	36.3	78.2	12.4	337	32	66	*Glu-A3d/f*
V2-62-15	Pp	9.84 a-h	35.1	77.3	13.6	338	34	66	*Glu-A3d/f*
KM 178-14*)	Pp	11.49 ab	37.4	80.4	10.3	310	22	96	*Glu-A3d/f*
V3-5ab-15	Pp	10.05 a-h	37.2	77.8	12.4	348	32	68	*Glu-A3d/f*
V1-170-15	Pp	10.51 a-e	39.3	79.2	10.5	340	31	70	*Glu-A3d/f*
V1-169-15	Pp	9.83 b-h	37.6	77.6	10.7	322	33	83	*Glu-A3d/f*
V1-168-15	Pp	9.89 a-h	36.8	77.9	12.0	394	37	68	*Glu-A3d/f*
V1-167-15	Pp	9.70 b-i	38.0	77.7	12.6	377	45	58	*Glu-A3d/f*
PS Karkulka	Pp	9.75 b-i	42.7	76.9	11.9	380	28	81	*Glu-A3d*
V2-98-15	Pp	10.21 a-f	36.3	78.5	12.4	382	26	81	*Glu-A3d/f*
V2-95-15	Pp	10.74 a-d	35.7	80.5	12.4	400	26	89	*Glu-A3d/f*
V2-68-15	Pp	9.60 b-j	38.0	74.2	11.1	349	35	94	*GluA3c/d*
V1-176-15	Pp	8.56 e-k	36.4	74.3	13.1	392	33	48	*GluA3c/d*
V1-175-15	Pp	9.27 c-k	34.2	77.2	12.5	426	30	68	*Glu-A3d/f*
mean		***9.88***	***37.0***	***77.7***	***12.1***	***368***	***32***	***74***	
Bohemia**)	R	10.87 a-d	49.2	77.5	13.7	376	66	52	*GluA3c/d*
Vanessa**)	R	11.53 ab	40.0	73.9	11.8	293	24	35	*Glu-A3d*
Matchball**)	R	12.01 a	41.9	75.5	12.3	349	37	38	*Glu-A3d*
mean		***11.47***	***43.7***	***75.6***	***12.6***	***339***	***42***	***42***	

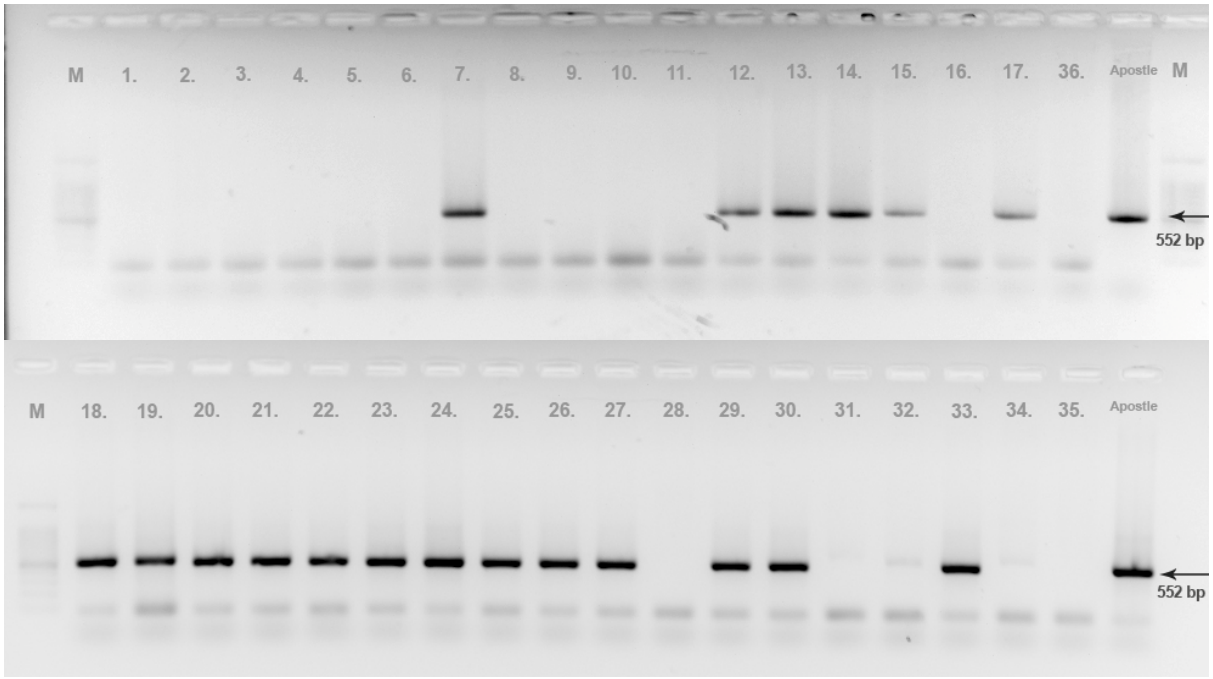
*Legend: DM – dry matter, Ba – blue aleurone, Ye – yellow endosperm, Pp – purple pericarp, R – red caryopsis, *) line tested in the Official State Tests, **) commercial variety with standard caryopsis colors a check, ***) the values indicated in the column with the same letters are not statistically different (LSD, P<0.05)*

The conventional cultivation technology and 10 m^2 plots with four replications were used. The presented yields are results from the harvest 2016, the results of the quality of grain and the analysis

of glutenin alleles were used from the harvest of 2015. 36 samples of bread wheat with nonstandard colored caryopsis were analyzed. The results characterizing the individual groups of genotypes with individual caryopsis colors are in Table 1.

Varieties Chinese Spring (*Glu-A3a*), Gabo *(Glu-A3b*), Gawain (*Glu-A3c*), Abbodanza (*Glu-A3d*), Liocorno (*Glu-A3e*), Apostle (*Glu-A3f*), Glenlea (*Glu-A3g*) from Crop Research Institute in Praha – Ruzyne were used as positive controls for each allele in *Glu-A3* locus. Genomic DNA was isolated from 5–7 days old wheat seedlings planted in laboratory conditions. Specific STS markers, developed by Wang et al. (2010) were used for identification of alleles in *Glu-A3* locus. PCR reaction was performed with the following conditions: 5 min 94 °C, 38 cycles: 94 °C 35 s, 60 °C 35 s, and 72 °C 90 s, final extension 72 °C 8 min. PCR products were electrophoretically separated on a 1% agarose gel according to the size of the individual fragments and visualized under UV light. Individual alleles were detected together with the positive control sample. Control variety Chinese Spring specified the product of the size 529 bp for *Glu-A3a* or *Glu-A3c* allele. Other control varieties: Gabo (*Glu-A3b*) with product size 892 bp, Abbodanza (*Glu-A3d*) - 967 bp, Liocorno (*Glu-A3e*) - 158 bp, Apostle (*Glu-A3f*) - 552 bp and Glenlea (*Glu-A3g*) amplified a product of 1345 bp. Allele *Glu-A3c* was detected using the common marker for *Glu-A3a* and *Glu-A3c*, distinguishable by checking Gawain with product size 573 bp. Electrophoreograms of 36 analyzed samples are on Figure 1.

Figure 1 The PCR products obtained using the marker for Glu-A3f



Legend:1 – V1-104-15, 2 – V1-106-15, 3 – V1-107-15, 4 – V1-103-15, 5 – V1-114-15, 6 – V1-116-15, 7 – V1-117-15, 8 – V2-88-15, 9 – KM 53-14, 10 – V1-126-15, 11 – V1-127-15, 12 – V1-129-15, 13 – Skorpion, 14 – V1-141-15, 15 – V1-180-15, 16 – Bona Vita, 17 – Citrus, 18 – V1-141-15, 19 – 1-143-15, 20 – V2-60-15, 21 – V2-62-15, 22 – KM 178-14, 23 – V3-5ab-15, 24 – V1-170-15, 25 – V1-169-15, 26 – V1-168-15, 27 – V1-167-15, 28 – PS Karkulka, 29 – V2-98-15, 30 – V2-95-15, 31 – V2-68-15, 32 – V1-176-15, 33 – V1-175-15, 34 – Bohemia, 35 – Vanessa, 36 – Matchball

RESULTS AND DISCUSSION

Evaluated wheat genotypes were grouped according to the color of the caryopses. Individual groups differed significantly among themselves by yield parameters and some parameters of grain quality (Table 1). Well-known commercial varieties (Bohemia, Vanessa, and Matchball) with red (standard) color of caryopsis used here as a controls had predictably the highest average yield (11.67 t/ha). They were followed by wheats with purple pericarp (9.87 ± 0.69 t/ha), yellow endosperm (9.77 t/ha), and blue aleurone (8.77 ± 1.14 t/ha). Wheat breeding for different caryopsis color is only marginal in comparison with the main flow of commercial breeding. They have lower yields (Garg et al. 2016) and this is compensated by higher cost of the cereal products. We believe that repeated backcrossing with major high yielding varieties will gradually increase the yield of colored wheat. It is

interesting that the group of wheat with a blue aleurone were characterized by a relatively high 1000 kernel weight (TKW) (46.5 ± 4.8 g) while wheats with yellow endosperm and purple pericarp had TKW much lower (37.3 ± 2.9 g and 37.0 ± 2.1 g, respectively). Wheats with a blue aleurone had a relatively lower mean content of proteins (11.4 ± 0.7%) and falling number (262 ± 90 s). Purple wheats markedly differed by a high falling number (368±34 s). The described genes for different caryopsis color are located on other chromosomes than genes for HMW and LMW glutenins and gliadins. We believe that the technological properties of the grain and color of caryopsis are inherited independently. Technological quality of grain is influenced by many genes. Significant role have the genes affecting the structure of storage proteins. Genes for the LMW glutenins are only a small part of genes that affect the final baking quality. The most frequent *Glu-A3d* allele occurred in all analyzed samples. Together with the *Glu-A3d* allele were detected *Glu-A3f* (53%) and *Glu-A3c* (8%) alleles. The presence of two alleles in one genotype could be explained by impurities in the sample or the fact that the samples are heterozygous. Other *Glu-A3a*, *Glu-A3b*, *Glu-A3e* and *Glu-A3g* alleles were not detected in any sample. Zhang et al. (2012) reported that *Glu-A3a*, *c*, *d* and *f* alleles have a positive impact on the resistance and extensibility of the dough. It is assumed that the assessed set of genotypes will be tested in the following years and detailed data will be gradually collected to find relationships among the evaluated characteristics.

CONCLUSION

36 genotypes of bread wheat (*Triticum aestivum* L.) with blue, yellow and purple colored caryopsis were tested with specific STS markers. Three different alleles *Glu-A3c*, *d* and *f* which positively effects bread quality, were detected. In some cases a heterozygote constitution of analyzed alleles was detected. These analyzes could be helpful in breeding programs in the selection of varieties for baking purposes. Lines with non-standard caryopsis color had lower yields in comparison with important standard varieties. Lines with purple pericarp had high falling number and smaller caryopsis. Wheats with purple pericarp had lower falling number and larger caryopsis.

ACKNOWLEDGEMNTS

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STUDY OF EXTENSIN GENE EXPRESSION AS A CANDIDATE RESPONSIBLE FOR PEA POD DEHISCENCE

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Abstract: The aim of this work is the study of one of the key genetic principles in domestication traits in pea – pod dehiscence by comparative analysis of wild and domesticated pea genotypes. Pod dehiscence is one of various mechanisms of seed spreading into their surroundings for the purposes of species preservation. Wild pea after maturing pods suddenly opens and fruits are scattered far and wide. This undesirable characteristic is for humans problematic because collection pea pod when touched if bursts is difficult and may cause yield loss in legumes before or during harvest. Pea is an example of crops that have undergone a process of domestication. Non-pod dehiscence, non-dormant seeds or bigger seeds are domestication traits that differentiate cultural kinds from wild species peas. There was evaluated a group of several RILs formed by crossbreeding of contrast parent genotypes of wild JI 64 *Pisum sativum* ssp. *elatius* L. and cultural pea JI92 *Pisum sativum* ssp. *sativum* L. This work was preceded by the method called MACE (Massive Analysis of cDNA Ends) to identify differences in gene expression during pod maturation. The expression of a selected gene was determined using qRT-PCR for pod dehiscence.

Key Words: pea, pod dehiscence, qRT-PCR

INTRODUCTION

Domestication is a phenomenon which in wild-type plants causes genetic changes through selection controlled by man. It is an evolutionary process in which a result of the selection are plants changed genetic, morphological and physiological characteristics. The result of the domestication is a plant adapted to survive in culture conditions, and having characteristics that prefer producers and consumers. Domesticated plants are different from their wild ancestor in several morphophysiological characters, most of which are associated with seed retention and germination, growth habit, size and coloration (Ladizinsky 1998, Sakuma 2011).

Pea is one of the oldest legumes on the world. The legume seeds are contained in the pod, which is composed of a single seed-bearing carpel. Pea pod have a seam that runs along both sides that can split open. When matures, splits open along two seams, a process called pod dehiscence. Purpose is to propel seed from pot away from the plant (Gao and Zhu 2013, Ambrose et al. 2008).

The genetics of the dehiscence was first reported by Marx (Marx 1971) where he described pods as being tough and leathery and prone to dehiscence (Ambrose et al. 2008). Genetic nature of the loss of pod dehiscence during domestication of a legume was illustrated for example in soybeans (Dong et al. 2014). In this case SHAT1-5 gene was identified as domain NAC transcription factor. It is believed that this gene affects the process of the secondary cell wall thickening. For pea it is probably a similar mechanism of loss of pod dehiscence but with a different genetic nature. Wild pea exhibits full pod dehiscence upon maturity, while cultivated peas have indehiscent pods that allow all the seeds to be retained at maturity. Despite its long history as a genetic model system, dating to Mendel's famous early studies, surprisingly little is known about pea domestication genes. Previously, it was a character of pod dehiscence located on LGIII in *Dpo1* (Weeden 2007, Bordat et al. 2011), but the gene has not yet been published.

MATERIAL AND METHODS

Characterization of plant material and DNA extraction

Two lines of wild and cultivated pea and their 134 RILs were used for this research. Evaluated plants of pea (*Pisum* L.*)* were grown in the greenhouse conditions in Mendel University in Brno. As a parental lines were used lines obtained from the John Innes Centre, Norwich Research Park, Norwich, United Kingdom: JI64 (*Pisum sativum* ssp. *elatius* L.) wild pea with dehiscent pods and dormant seeds collected in Turkey and JI92 (*Pisum sativum* ssp. *sativum* L.), with indehiscent pods and non-dormant seeds and which we rank among the cultural pea. It is a landrace from Afghanistan. Recombinant inbred lines (RILs) were obtained by reciprocal crosses of both parental lines (North et al. 1989). The evaluation of pod dehiscence phenotype (indehiscent / dehiscent) is not clearly visible and is thus difficult.

For our study RNA of both parents as well as several selected RILs (indehiscent / dehiscent) of F6 were isolated from tissues directly related to the pod dehiscence – dorsal and ventral pod suture.

The RNA was isolated from pod sutures of three developmental stages: 10, 15 and 20 days after flowering using NucleoSpin RNA Plant kit (Machery-Nagel, Düren, Germany). RNA isolated from dehiscent/indehiscent RILs and their parents was used for Massive analysis of cDNA Ends (MACE) to identify differences in gene expression during pod maturation. The cDNA for qRT-PCR was obtained from isolated RNA using Promega (Madison, USA) chemicals.

PCR amplification

Expression of candidate gene for pod dehiscence was assigned using Real-Time qRT-PCR by using of LC 480 SYBR Green I Master kit (Roche, Basel, Switzerland). The forward primer sequences used: 5'- CACCGTCATCTACTACCACA -3'; reverse: 5'- ACCGACCAAAATTGTAATGGA -3' and as a reference published specific primers for β-tubulin were used (Die et al. 2010). Based on qRT-PCR results the ΔCt values were calculated.

For detailed study of the candidate gene also genomic DNA of parents and RILs were used. Genomic DNA was isolated from young leaves by Invisorb Spin Plant Mini Kit (STRATEC Biomedical AG, Birkenfeld, Germany). Differences in candidate gene DNA sequence were tested by sequencing (Macrogen, Amsterdam, The Netherlands) of parent lines by PCR (MyTaq DNA Polymerase kit, Bioline, Taunton, Massachusetts, USA) with primers designed based on GenBank (National Center for Biotechnology Information) sequence JI917454 using Primer-BLAST tool.

RESULTS AND DISCUSSION

This work was focused on the evaluation of one candidate gene for pod dehiscence of pea. This candidate gene was predicted by the method MACE (Massive Analysis of cDNA Ends) which was used to identify differences in gene expression during pod maturation between parental lines and bulks of indehiscent / dehiscent RILs. Based on MACE results we identified 77 genes with different expression between indehiscent and dehiscent genotypes (minimal difference in expression fold log2>3). The gene with the highest difference in expression (our main candidate gene) was identified as an extensin-like protein (GenBank accession No.: JI917454.1). In parental lines we can see differences in the expression of the candidate gene, corresponding with contrasting phenotypes of pod dehiscence. Genotype with dehiscent pods (JI64) showed higher expression of the candidate gene and genotype with indehiscent pods (JI92) lower than the reference gene (Figure 1). However, it can be seen that differences in expression between the developmental stages are not significant as well as there is no significant difference between the expression of the dorsal and ventral seam. Therefore, RNA from RILs was isolated from the mixture of both seams.

Evaluated lines were divided based on phenotype into two groups (Table 1): indehiscent (Figure 2) and dehiscent (Figure 3).

Figure 1 Evaluation of qRT-PCR: expression of candidate gene for pod dehiscence in parental pea lines

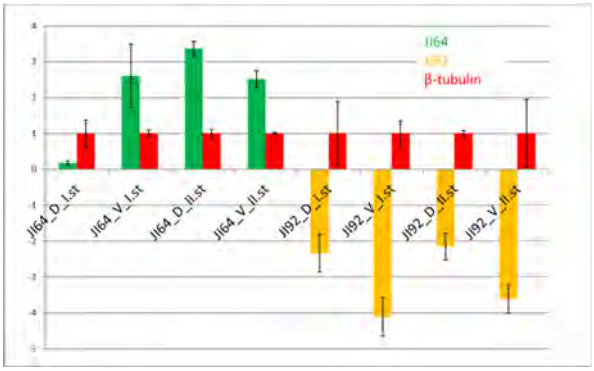
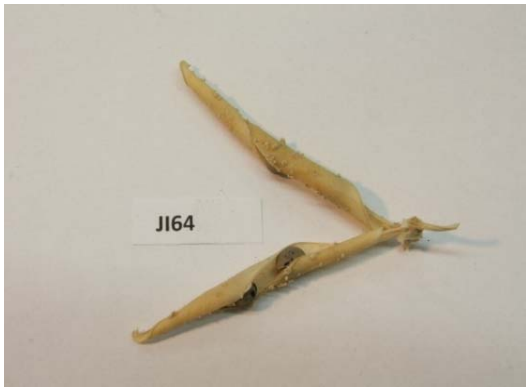


Table 1 RILs divided into groups according to phenotype

Indehiscent lines	Dehiscent lines
16 64x92	23 64x92
45 64x92	42a 64x92
61 64x92	60 64x92
63 64x92	93 64x92
	99 64x92

Figure 2 Indehiscent pea pod – JI92

Figure 3 Dehiscent pea pod – JI64

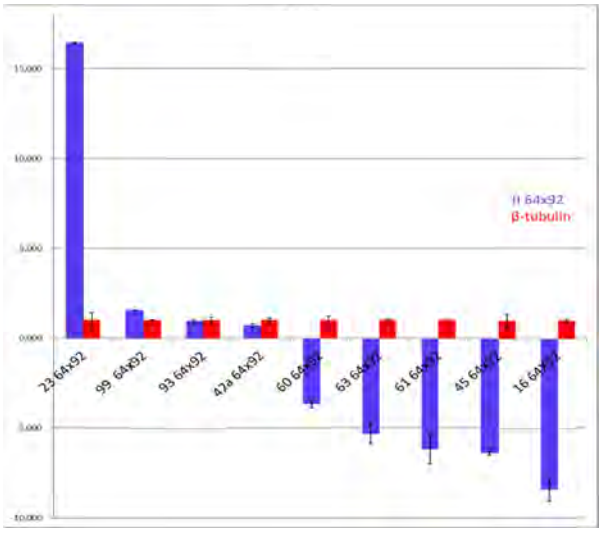


In Figure 4 expression of the candidate gene in evaluated RILs can be seen. It is obvious that indehiscent samples tested for expression of the candidate gene have lower expression then the reference gene. The opposite was observed in dehiscent samples where gene expression of the candidate gene was higher compared to the reference gene. Between the values of indehiscent and dehiscent test samples is thus a significant difference. Except for one line (60 64x92) all others correspond in phenotypic evaluation of pod dehiscence with results of gene expression (Table 1). The evaluation of electrophoresis of the line 60 64x92 revealed its heterozygous state. It may explain the differences in phenotypic and genotypic evaluation. In dehiscent RILs the expression is slightly higher than the reference gene, except of line 23 62x92, which has a very high value. All indehiscent lines have values lower than the reference gene.

Identification of new markers for domestication traits is in the interests of breeders for many years. The reason is the possible use of the potential of landraces, which contain in their genome a variety of resistance genes. But landrace crops often have traits not suitable for cultivation. In the case of legumes one of the most undesirable is dehiscence of matured pods causing a yield loss.

For example, in *Arabidopsis thaliana* has already been identified the gene *SHATTERPROOF (SHAT),* which controls the deposition of lignin during maturation of the fruit. Also another gene *INDEHISCENT (IDEH)* was identified, which belongs to regulators of formation of lignified layer of the fruit (Fourquin et al. 2013).

Figure 4 Evaluation of qRT-PCR: expression of the candidate gene for pod dehiscence in tested pea samples (ΔCt)



The genetic basis of pod dehiscence loss during domestication was recently explained in soybeans, where the identified gene *SHATTERING 1-5* (Dong et al. 2014) functionally activates creation of the secondary cell wall.

Funatsuki et al. (2014) identified a gene *Pdh1* controlling dehiscence of the soybean pod, which supports deposition of lignin to the cells of endocarp. These genes are the only identified genes associated with a domestication of legumes. In case of peas is in cultural forms probably similar mechanism of pod dehiscence loss but with a different genetic basis. In the studies of Weeden et al. (2009) and Weeden (2007) the authors claim that pod dehiscence of pea is controlled by two loci, semidominant and monogenic *Dpo1* locus (binding group LGIII) and by *Dpo2* locus (found only in some crossbreeds) (Bordat et al. 2011) but the specific gene is not yet published. Based on comparison of our candidate gene position in *Medicago truncatula* genome and SNPs map which was created by Tayeh et al. (2015) we recognized that our candidate gene is present on pea LGIII in the estimated region of *Dpo1* locus. This fact corresponds with Weeden (2007) and Bordat et al. (2011).

CONCLUSION

The expression of our candidate gene for dehiscent pea pod by qRT-PCR in JI64 (*Pisum sativum* ssp. *elatius* L.), JI92 (Afghan cultural pea *Pisum sativum* ssp. *sativum* L.) and their recombinant inbred lines (RILs) created from reciprocal crossing was evaluated. Gene expression in lines with dehiscent and indehiscent pea pods were detected. Lines with dehiscent pods showed expression higher than the reference gene and conversely, lines with indehiscent pods showed lower expression.

This candidate gene is present on pea LGIII in the estimated region of *Dpo1* locus and could therefore be responsible for pod dehiscence in peas.

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GENERATION OF ARABIDOPSIS LINES WITH ALTERED CYTOKININ LEVEL EXPRESSING GFP-FUSED CYTOSKELETAL PROTEINS

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Abstract: Cell division, expansion and differentiation require a sophisticated spatial arrangement of the cytoskeleton. The ever increasing progress in fluorescent microscope techniques has allowed to visualize rearrangements and dynamics of actin and microtubule arrays in various cell types and tissues in plants. Processes controlling proper cytoskeleton organisation and its response to various stimuli, mediated by actin and microtubule binding proteins as well as plant hormones, are being an area of active investigation. As the current research of cytoskeleton regulation focused mainly on hormone auxin, the involvement of cytokinin is still unclear. In this study, we describe generation of transgenic lines of *Arabidopsis thaliana* comprising inducible system for manipulation of endogenous cytokinin level and simultaneously GFP-labelled actin or microtubule proteins. These lines serve as a tool for the study of cytokinin mode of action in the course of cell/organ development. Based on our results, it is obvious that cytokinins play a considerable role in modulation of plant cytoskeleton. However, further research is necessary to elucidate precise cytokinin signalling pathways and cross-talk with other hormones, which participate in such complex processes of cell morphology.

Key Words: actin, microtubule, *Arabidopsis*, cytokinins, GFP

INTRODUCTION

Microfilaments and microtubules, two major types of cytoskeletal elements in plant cells, mediate together with so called motor proteins cytoplasmic streaming and direct organelle movement, processes that accompany plant growth and development and provide a rapid response system to both external abiotic and biotic stimuli. Whereas microfilaments create solid chains of actin protofilaments, microtubules are composed of α- and β-tubulin heterodimers resulting in polymeric cylinder structure. Actin microfilaments and microtubules undergo cycles of polymerization-depolymerisation causing an incessant remodelling and assembly or disassembly within seconds, which is essential for cell life. The dynamic changes in cytoskeleton arrangement are determined by accessory proteins such as actin-binding proteins or microtubule-associated proteins (MAPs) (Taiz et al. 2015). To analyse the behaviour of cortical microtubules and actin filaments *in vivo*, a number of transgenic lines has been created. For this purpose, genes encoding important cytoskeletal proteins or their binding domains were fused with green fluorescent protein (GFP). Stably transformed *Arabidopsis thaliana* lines expressing GFP-FABD2, an N-terminal fusion of GFP to the C-terminal half of *Arabidopsis* fimbrin 1, provided an opportunity to report the dynamic changes in the architecture of filamentous F-actin in all cell types (Voigt et al. 2005). For instance, visualization of GFP-FABD2 fusion protein in hypocotyl epidermal cells revealed that cell elongation in *Arabidopsis* is controlled by profilin binding proteins or that application of heat stress caused severe actin rearrangements in *Arabidopsis* (Cao et al. 2015, Fan et al. 2016). A microtubule reporter gene *GFP-MBD*, prepared by fusing the microtubule binding domain of the mammalian microtubule-associated protein 4 (MAP4) and GFP-TUA6, representing N-terminal fusion protein of GFP with *Arabidopsis* α-tubulin 6 (TUA6), were widely used for studies concerning microtubule arrays organization in response to diverse stimuli (Marc et al. 1998, Vineyard et al. 2013, Sambade et al. 2012, Shaw et al. 2003, Chan et al. 2007).

Cytokinins belong to the family of well-studied plant hormones, having a key role in many aspects of plant growth and development, including embryogenesis, maintenance of root and shoot meristems modulation of root elongation, apical dominance or response to environmental stimuli (Osugi and Sakakibara 2015, Skalák et al. 2016, Novák et al. 2015). The prominent role of cytokinins in plant cell expansion has been already suggested (Schaller et al. 2014) and several important mutants in cytokinin signalling pathway were shown to mediate the proper plant cell development (Li et al. 2013). Last years, researchers have been following a question, how plant hormones interact or regulate the cytoskeletal proteins and searched for signalling molecules/pathways, which could mediate these interactions. In 2012, Lanza et al. using GFP-FABD2 *Arabidopsis* marker lines, revealed that actin acts as a node of interaction of brassinosteroid and auxin signalling pathway. Recently, components of auxin signalling pathway, such as ROP6 GTPase and ROP-interactive protein RIC1, were shown to be required for auxin-mediated re-orientation of microtubule arrays. These findings on the regulation of microtubule organisation by ABP1-mediated auxin signalling pathway describe molecular mechanism by which can plant hormones employ their effects on plant cell growth (Chen et al. 2014).

In past few years, advances in confocal microscopy allowed researchers to study thoroughly the variety of ongoing processes in living cells. As mentioned above, growing evidence that phytohormones cause alteration in cytoskeleton organization have emerged. However, the involvement of cytokinins, and mainly their mode of action in cytoskeleton organization remains unclear, since there are no studies concerning this topic available to date. Thus, we decided to generate *Arabidopsis* lines with GFP-marker proteins for cytoskeleton imaging and with proteins of cytokinin metabolism, which will provide a first look at cytokinin action on the level of plant cell mobility.

Here, we describe a preparation of lines with inducible increase (using agrobacterial isopentenyl transferase gene, *ipt*) or decrease (using cytokinin dehydrogenase 2 gene from *Hordeum vulgare*, *HvCKX2*) of cytokinin levels by synthetic steroid dexamethasone (DEX) with GFP-labelled proteins or protein-binding domains of cytoskeletal components, namely TUA6, MBD and ABD. Successfully crossed lines were scanned using epifluorescent (segregation analysis) and confocal microscopy.

MATERIAL AND METHODS

Plant material and cultivation

Genetic crosses were performed using transgenic lines of *Arabidopsis thaliana*: CaMV35S>GR>ipt (pOpBK-ipt; Craft et al. 2005), CaMV35S>GR>HvCKX2 (pOpOn-HvCKX2, Černý et al., 2013); proCaMV35S::GFP-TUA6 (Shaw et al. 2003), proCaMV35S::GFP-MBD (Marc et al. 1998); proCaMV35S::GFP-ABD (Voigt et al. 2005). All transgenic lines were in the Columbia ecotype (Col-0) background. For segregation analysis plants were cultivated on half-strength Murashige and Skoog (MS) medium without sucrose, supplemented with appropriate selection agents. According to Harrison et al. 2006, after 2–4 days of stratification, seeds were illuminated for 6 h and then placed in the dark for 2 days. Transgenic seedlings were further cultivated for 1 or more days at long-day conditions (16 h light/8 h dark photoperiod, 100 µmol/m^2/s light intensity, 21 °C) until antibiotic resistant seedlings were easily distinguished from non-resistant seedlings (Figure 1–2).

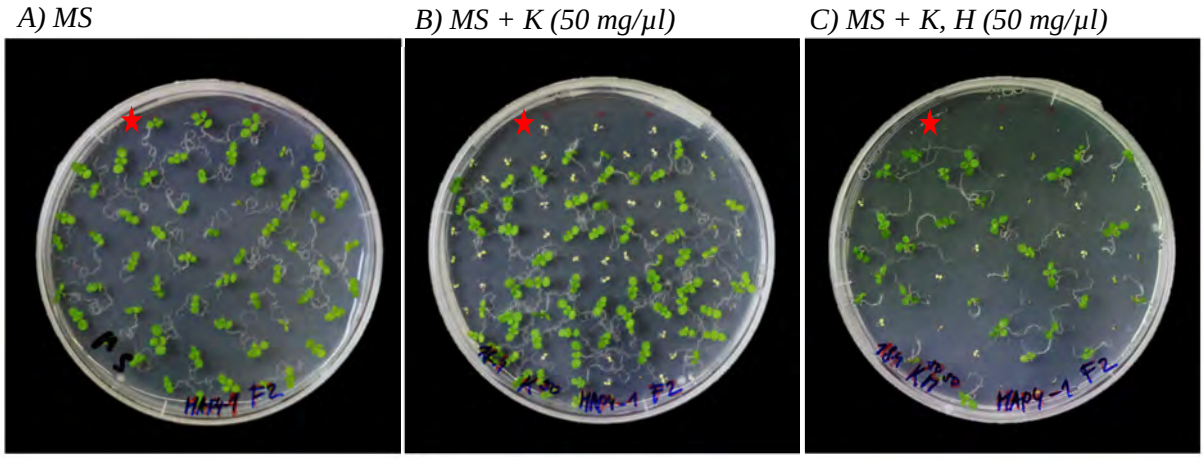
For confocal microscopy, seeds of homozygous lines were sawn on sterile nylon meshes placed on half-strength MS medium without sucrose and placed in the dark (4 °C) for 2–4 days and then cultivated under long-day conditions (16 h light/8 h dark photoperiod, 100 µmol/m^2/s light intensity, 21 °C). Three days after stratification, seedlings on nylon meshes were transferred to half-strength MS media supplemented with 10 µM DEX. 12-day old plants were scanned by confocal microscope Axio Imager Z.1 platform, equipped with the LSM 710 (Zeiss) using 63x oil objective. The light source included argon-neon laser with wavelength set up 488 nm for GFP fluorescence and 639 nm for chlorophyll auto-fluorescence, to avoid a crosstalk between the emission spectra of both fluorescent signals.

RESULTS AND DISCUSSION

Selection of homozygous lines

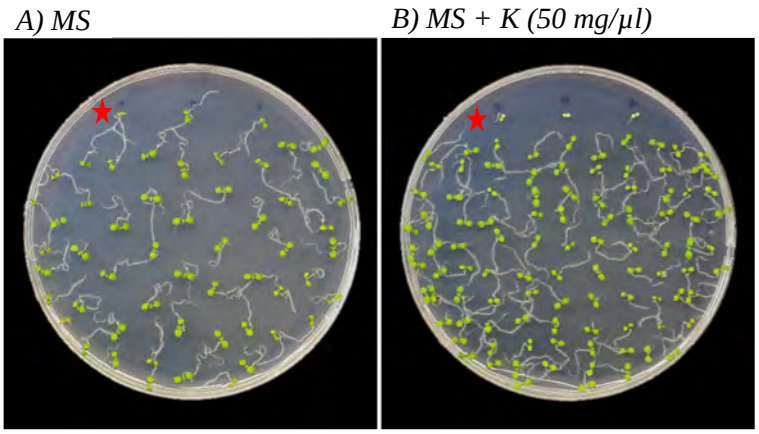
Arabidopsis lines CaMV35S>GR>HvCKX2 or CaMV35S>GR>ipt were crossed with individual GFP reporter lines proCaMV35S::GFP-TUA6, proCaMV35S::GFP-MBD and proCaMV35S::GFP-ABD. Progeny of F1 plants was analysed using kanamycin, hygromycin and L-phosphinothricin as selection antibiotics and positive plants were screened for the presence of GFP signal via epifluorescent microscope. At least six plants of each crossed line were cultivated to next generation. Heterozygous F2 plants (Figure 1) with 3 : 1 segregation ratios for both genes (HvCKX/ipt and GFP-labelled gene), representing a single copy of T-DNA inserts, were screened for GFP signal and three or more plants were cultivated to next generation. Finally, homozygous lines of F3 generation (Figure 2) HvCKX2 x GFP-ABD and HvCKX2 x GFP-TUA6, showing 100% resistance on selection media with no phenotypic changes were characterized and propagated to next generation. Genotyping of F2 and F3 generation for the presence of HvCKX2 gene was performed using T-DNA specific primers. However, due to the presence of two loci of activator and reporter genes in the line CaMV35S>GR>ipt, crosses with marker lines did not allow to generate homozygous lines, since the segregation of three genes would be time-consuming. Thus, successfully crossed F1 lines ipt x GFP-ABD, ipt x GFP-TUA6 and ipt x GFP-MBD were directly analysed using confocal microscope.

Figure 1 Segregation analysis of F2 generation of heterozygous HvCKX2 x GFP-MBD line



Legend: MS – Murashige and Skoog medium, K - kanamycin, H – hygromycin. Red asterisks show wild-type plants Col-0.

Figure 2 Homozygous HvCKX2 x GFP-FABD2 line, F3 generation



Legend: MS – Murashige and Skoog medium, K – kanamycin. Red asterisks show wild-type plants Col-0.

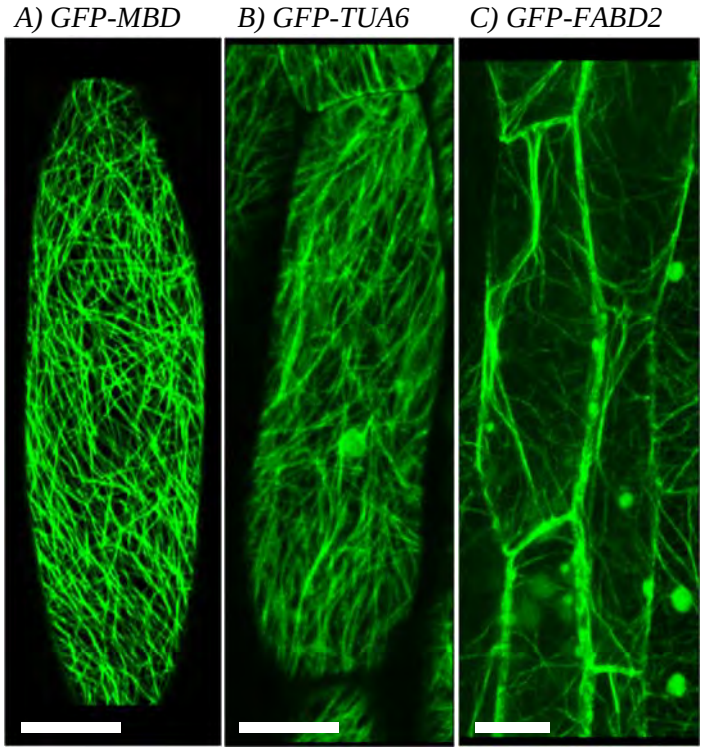
Typical cytokinin-deficient phenotype of homozygous crossed lines, caused by overexpression of *HvCKX2* (Werner et al. 2003, Černý et al. 2013), confirmed the proper function of DEX inducible system. Similarly, inducible expression of *ipt* led to opposite effect (Skalák et al. 2016). Cytokinins are known to act as regulators of plant cell morphogenesis. One of the important features of the plant cell is

regulation of its expansion to balance the growth towards the light (shoot) or gravity (root). Cytokinins were shown to modulate auxin transport to control the root cell elongation in response to gravity (Pernisova et al. 2016). However, the role of cytokinins in light-induced hypocotyl cell elongation with cytoskeletal modification remains rudimentary.

Cytokinins change the cytoskeletal organisation

First step was analysis of GFP signal of individual GFP marker lines in order to configure software settings of the confocal microscope (Figure 3). Afterwards, 12-day old homozygous transgenic plants with altered cytokinin level, were used to explore the microtubule and actin orientation in hypocotyl cells. All crossed lines exhibited strong GFP signal and uniform response to DEX (data not shown). The primary results obtained from confocal microscopy indicate that cytokinins modify the cytoskeletal structure during intracellular processes.

Figure 3 Visualization of cortical microtubules and actin microfilaments in Arabidopsis hypocotyl epidermal cells.



Legend: GFP – green fluorescent protein, MBD – MAP4 binding domain, TUA6 – α-tubulin 6, FABD2 – F-actin binding domain 2. Scale bars 20 µm.

CONCLUSION

We generated *Arabidopsis* reporter lines containing either *ipt* or *HvCKX2* genes of cytokinin metabolism and cytoskeletal marker proteins/protein-binding domains labelled with GFP. Inducible expression of *ipt* or *HvCKX2* genes in these lines resulted in significant reorganization of cortical microtubules and actin microfilaments. Thus, we suggest cytokinins act upstream of cytoskeletal compartments and through unknown transduction pathway, cytokinin signal is translated into plant growth responses. To statistically quantify the effect of cytokinins on specific cytoskeletal proteins, sophisticated software for analysis of microtubule fibres and actin microfilaments reorientation will be used in future experiments.

Our findings can contribute to uncover the hidden mechanism of cytokinin-regulated control of spatial arrangement of plant cells. Moreover, these reporter lines will serve as a tool for study of changes in plant proteome and extend the current knowledge about the role of plant hormones in abiotic stress response, as the changes on proteome level have become the main interest lately (Johnová et al. 2016, Skalák et al. 2016, Černý et al. 2016, Takáč et al. 2016).

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THE ABILITY TO DECOLORIZE DIFFERENT SYNTHETIC DYES DUE TO LACCASE PRODUCED BY *TRAMETES VERSICOLOR* AND *FOMES FOMENTARIUS*

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Abstract: The textile industry, by far the most frequent user of synthetic dyes, is in need of ecologically efficient solutions for its colored wastewaters. White-rot basidiomycetes are among the most potent organisms to biodegrade and detoxify a wide range of pollutants and synthetic dyes, enabled by ligninolytic enzymes, namely, laccases. Present study was undertaken to explore white-rot fungi *Trametes versicolor* and *Fomes fomentarius* for their laccase production and potential in dye decolorization. The laccase production was induced by 0.5 mM copper sulphate and their effectity in decolorization of five types of synthetic textile dyes (N-heterocyclic, azo, triphenylmethane and triarylmethane) was studied. The most effective laccase decolorization was observed for the dyes Malachite Green and Bromothymol Blue using 0.5 mM copper as inducer for both fungi.

Key Words: decolorization, copper inducer, laccase, *Trametes versicolor*, *Fomes fomentarius*

INTRODUCTION

Wastewater from textile industries is characterized by high concentration of chemicals suspended solids and intensively colored aromatic structure due to the extensive use of synthetic dyes and pigments. Based on the chemical structure of the chromophore group, these dyes are classified as azo, anthraquinone, triphenylmethane, heterocyclic and polymeric dyes (Yang et al. 2009).

Typically, these dyes are removed by chemical and physical methods like adsorption, coagulation-flocculation, oxidation, filtration and electrochemical treatments. All these methods have different color removal capabilities, capital costs and operating speed. However, these methods create huge amounts of sludge which become a pollutant on its own creating disposal problems and can cause the formation of toxic carcinogenic and mutagenic breakdown products. There is a great need to develop an economic and effective way of dealing with the textile dyeing waste at the level of the industry itself in the face of the ever increasing production activities.

Biological treatment using white-rot fungi have been demonstrated to be capable of transforming, mineralizing and removing of a wide range of organopollutants, such as polycyclic aromatic hydrocarbons, chlorophenols and polychlorinated biphenyls and various azo, heterocyclic and polymeric dyes due to containing rich ligninolytic enzymatic system. The most important enzyme is laccase (Lac, E.C. 1.10.3.2), which is a multicopper enzyme, which catalyses the oxidation of phenolic and non-phenolic compounds and belongs to the group of phenol oxidases.

However, ligninolytic enzymes from white-rot fungi are only secreted in small amounts, so their using in industrial applications has been limited due to low productivity and high economic cost (Rivera-Hoyos 2013).

Enzyme production can be affected by many factors. One of the most critical factors is type and concentration of the inducer agent (Majeau 2010). The inducer is a specific molecule that induces synthesis of the relevant inducible enzyme and is usually a substrate for a given enzyme. Collins and Dobson (1997) and Palmieri et al. (2000) found that laccase expression of basidiomycetes is induced by copper at a transcriptional level. It is known that the most important metal for white-rot fungi is copper, which is a cofactor in the catalytic center of laccase; thus, a minimum concentration (millimolar range) of copper ions was necessary for production of the active enzyme.

The possible mechanism for this phenomenon is that copper ions enhance the laccase genetic transcription level during the laccase synthesis. A higher enzyme activity guarantees a higher and faster transformation of the target substrate and improves the applicability and effectiveness of the enzyme catalyzed processes (Rao et al. 2014).

In this paper, the most effective laccase produced by *Trametes versicolor* and *Fomes fomentarius* was chosen based on previous study and induced by 0.5 mM copper sulphate and further investigated with respect to the effects on synthetic dye decolorization.

MATERIAL AND METHODS

Fungal strains and culture conditions

Locally isolated fungal strains *Trametes versicolor* and *Fomes fomentarius* obtained from the Culture Collection of the Faculty of Forestry and Wood Technology of the Mendel University in Brno (Czech Republic) were used in this study. Cultures were cultivated on Potato Dextrose Agar (PDA) for 10 days at 22 °C.

Dyes decolorization

The dye decolorization experiment was performed using five different synthetic dyes from different dye classes such as Malachite Green (MG), Crystal Violet (CV), Bromothymol Blue (BB), Methyl Red (MR) and Methylene Blue (MB). Table 1 shows the final concentration and the maximum wavelength of the dyes. The light absorption for each dye was monitored using a VISIONlite SCAN program on Helios Epsilon spectrophotometer.

Table 1 Concentration and wavelength of synthetic dyes

Dyes	Concentration [mg/l]	Wavelength [nm]
Malachite Green	7	615
Crystal Violet	20	590
Bromothymol Blue	50	605
Methyl Red	100	530
Methylene Blue	240	665

Decolorization of dyes by *Trametes versicolor* and *Fomes fomentarius* fungal culture

For decolorization experiments, three agar plugs (5 mm^2) of active mycelium from PDA plates were transferred aseptically into 50 ml Erlenmeyer flasks containing 30 ml of PDB (Potato Dextrose Broth) medium with different concentration of dyes (Table 1) and incubated at 28 °C in dark for 20 days. Decolorization experiment was prepared in different variants: sterilized medium with dyes; sterilized medium with dyes and 0.5 mM copper inducer and sterilized medium containing the dyes but not inoculated with the fungus. Culture samples were collected every 4 days, centrifuged at 10 000 g for 10 min at 4 °C and the supernatants obtained were used for decolorization assay by fungal culture.

Decolorization assay by fungal culture

Dye concentrations were selected in order to obtain approximately 1.5 absorbance units at the maximum wavelength in the visible spectrum. All the tested flasks were incubated at room temperature, without shaking and in dark. The residual dye concentration was measured spectrophotometrically at their maximum wavelength, as shown in Table 1, and calculated from measured absorbance according to the following expression:

$$\% = \frac{A_0 - A}{A_0} \times 100$$

,

where % is the decoloration percentage obtained, A_0 the initial absorbance and A is the final absorbance. A control test containing the same amount of a heat-denatured laccase was also performed in parallel.

RESULTS AND DISCUSSION

The decolorization of model synthetic dyes is a simple method to assess the bioremediation potential of ligninolytic enzymes, especially laccase. In general, the efficiency of decolorization depends on the structure of dye, fungi and enzymes used and experimental conditions as well as presence of inducer (Zhang et al. 2006). The ability of fungal mycelium obtained from *Trametes versicolor* and *Fomes fomentarius* to decolorize 5 synthetic dyes with different structure was examined. Acquired results were compared with experiment when the fungal laccase was induced by 0.5 mM copper sulphate.

Day decolorization by two different white-rot fungi

Many studies with *Trametes* strains have been extensively conducted. In our study *Trametes versicolor* and *Fomes fomentarius* were chosen as significant producers of laccase (Rodrigues et al. 2008). Higher effect of dye decolorization was observed using *Trametes*, which agree with observation of Selvam et al. (2002), who used *Fomes* and *Trametes* strains to decolorization of industrial dyes and maximal effect was observed using *Trametes* on the fourth day (Figure 1). The slow increase of decolorization by *Fomes* was observed during all the time of cultivation (Figure 2). Large variations exist among different white-rot species in the ability to produce the different isoenzymes, but also they can vary over time (Hatakka and Hammel 2010). However, Neifar et al. (2011) studied *Fomes fomentarius* and showed a promising future of applying laccase system of this fungus for industrial wastewater decolorization and bioremediation.

Dye decolorization with and without copper inducer

The positive effect of copper addition in the form of copper sulphate on laccase activity has been reported by many authors (Palmieri et al. 2000, Levin et al. 2002). In our study the copper was used as inducer to increase dye decolorization effect.

Addition of copper sulphate stimulated decolorization, the presence of copper increased around 3-4-fold the degradation capability by both fungi throughout the experiment in comparison with cultivation without laccase inducer.

The higher decolorization effect using copper suggested that induction of laccase significantly influenced decolorization process. It is known that the presence of laccase inducer (Cu^{2+}) in the process increased the range and rate of decolorization (Levin et al. 2002).

Our finding is in accordance with work of Zouari-Mechichi et al. (2006), who observed that the decolorization using *Trametes trogii* is possible in the presence of Cu^{2+} and it was faster when Cu^{2+} was present in the medium. Lorenzo et al. (2006) observed that addition of copper increased laccase activity and this factor played an important role in the decolorization of the textile dye.

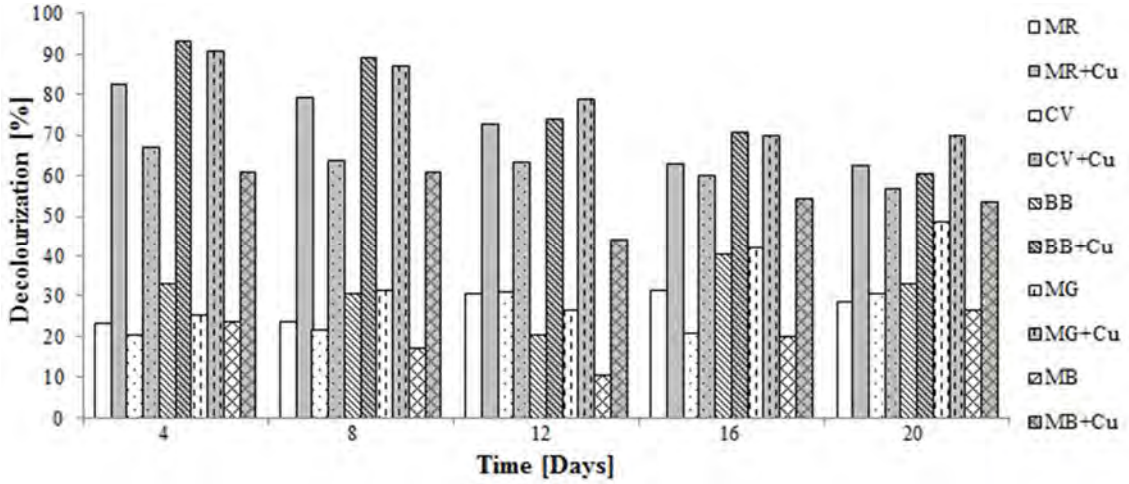
Decolorization effect of five types of synthetic dyes

The synthetic dye decolorization efficiency of the biomass from fungus *Trametes versicolor* was the highest for the triarylmethanic dyes Malachite Green and Bromothymol Blue with a maximum of 95% after 4 days of cultivation using 0.5 mM copper inducer. For *Fomes fomentarius* the ability to decolorize the triarylmethanic dyes around 70% during the period of cultivation was observed. Bromothymol Blue and Malachite Green share very close structure with three benzene rings and these dyes might fit well with the enzyme activity centers and can be degraded (Ling et al. 2015). It is in contrary with work of (Jayasinghe et al. 2008), who tested different fungal strains and *Fomes fomentarius* showed better mycelial growth and decolorization of Malachite Green than *Trametes versicolor*.

The decolorization ability for Methyl Red, which belongs to azo dyes, was around 80% for both fungal strains, which agree with work of Sharma et al. (2015), who tested ability of white-rot fungi to decolorize azo dyes. Wong and Yu (1999) reported that dye decolorization by fungi was dependent on dye structure. Different dyes have different molecular structures. So a fungus capable of decolorizing

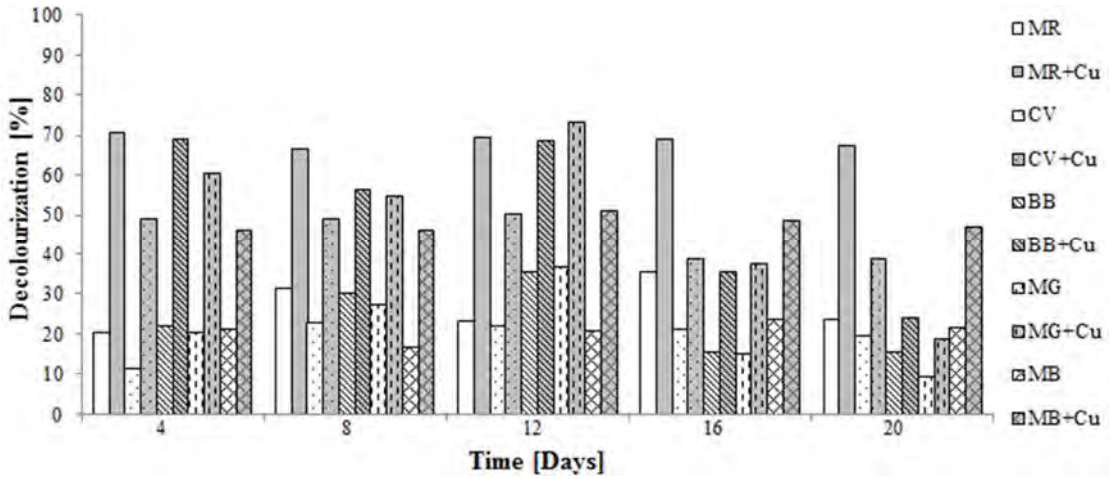
one dye may have different capacities for other dyes. One of the lowest decolorization was observed for Crystal Violet, which belongs to triphenylmethane dye and for heterocyclic dye Methylene Blue. It can be due to the fact that the triphenylmethane dyes are known to be resistant to enzymatic decolorization in comparison with azo dyes and hence more time for decolorization is required (Hughes and Poole 1991). It is in contrast with the study Yan et al. (2009), who suggested the efficient decolorization of Crystal Violet using fungus *Trametes trogii*.

Figure 1 The synthetic dye decolorization of fungal mycelium from Trametes versicolor with and without copper



Legend: MR – Methyl Red, MR+Cu – Methyl Red + copper, CV – Crystal Violet, CV+Cu – Crystal Violet + copper, BB – Bromothymol Blue, BB+Cu – Bromothymol Blue + copper, MG – Malachite Green, MG+Cu – Malachite Green + copper, MB – Methylene Blue, MB+Cu – Methylene Blue + copper, standard deviation is less than 0.5%

Figure 2 The synthetic dye decolorization of fungal mycelium from Fomes fomentarius with and without copper



Legend: Legend: MR – Methyl Red, MR+Cu – Methyl Red + copper, CV – Crystal Violet, CV+Cu – Crystal Violet + copper, BB – Bromothymol Blue, BB+Cu – Bromothymol Blue + copper, MG – Malachite Green, MG+Cu – Malachite Green + copper, MB – Methylene Blue, MB+Cu – Methylene Blue + copper, standard deviation is less than 0.5%

The mechanism for decolorization by white-rot fungi consist of a combination of biosorption by fungal mycelia and biodegradation by extracellular laccase. High decolorization effect by fungal mycelium is through adsorption of the dyes onto its cell surface. This type of decolorization has been reported to be the primary mechanism of decolorization and major to decolorization by enzymatic preparations (Selvam et al. 2002).

CONCLUSION

In this study white-rot fungi *Trametes versicolor* and *Fomes fomentarius* have been investigated as potential producers of laccase enzyme and copper sulphate was successfully used as potential inducer of laccase. Fungi were used to decolorize five synthetic dyes in the form of fungal mycelium. This effect is more pronounced, when copper inducer was present in cultivation medium. Therefore, laccase inducer showed great potential to be used in process of color removing from textile wastewaters.

Higher decolorization effect was observed using *Trametes* strain than *Fomes* and our study confirmed that each strain has different capability to decolorize synthetic dyes.

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Section – Animal Biology

THE IMPACT OF AMYGDALIN ON THE OXIDATIVE PROFILE OF RABBIT TESTICULAR TISSUE

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Abstract: Amygdalin (AMG) is a cyanogenic glucoside primarily found in the seeds of bitter almonds (*Prunus dulcis*). It is a biomolecule exhibiting antitumor activity which has also been used for the treatment of asthma, bronchitis, emphysema, leprosy and diabetes. This *in vivo* study was designed to reveal whether amygdalin is able to cause changes in the oxidative profile of rabbit testicular tissue. Twelve adult male rabbits were randomly divided into three groups: the Control group received no AMG and two experimental groups receiving daily intramuscular AMG injections at 0.6 and 3.0 mg/kg b.w. respectively over the period of 28 days. At the end of the experiment testicular tissue was collected from each animal and tissue lysates were prepared. Reactive oxygen species (ROS) production were assessed by chemiluminescence assay. Protein oxidation was evaluated using the traditional 2,4-dinitrophenylhydrazine method and lipid peroxidation (LPO) was assessed with the help of the TBARS assay. The resulting data reveal, that 0.6 mg/kg b.w. AMG administration led to an insignificant decrease of ROS production, protein and lipid oxidation. On the other hand, administration of 3.0 mg/kg b.w. AMG resulted in an insignificant increase of LPO while the ROS production and protein oxidation were significantly ($P < 0.01$) increased in comparison with the Control. Our results reveal that AMG administration may have a dose-dependent impact on the testicular tissue, acting as an antioxidant at low doses, while high doses may compromise the delicate oxidative balance in male reproductive structures.

Key Words: amygdalin, reactive oxygen species, oxidative profile, testicular tissue, rabbits

INTRODUCTION

Amygdalin ($C_{20}H_{27}NO$), commercially called „vitamin B17“, is found primarily in the seeds of apricots and bitter almonds. This bioactive substance is consisting of glucose, benzaldehyde exhibiting analgesic effects and hydrocyanic acid, which is an antineoplastic agent (Fukuda et al. 2003, Chang et al. 2006).

Several studies state that AMG is capable of preventing and/or treating numerous diseases including migraine, chronic inflammation, pain and fever (Fukuda et al. 2003, Zhou et al. 2012). In the 1970s, AMG was one of the most attractive ways to cure cancer (Moss 2005). Due to insufficient clinical evidence on its therapeutic effects, FDA disapproved AMG as a therapeutic agent (Hwang et al. 2008). Acute toxicity experiments proved much more considerable toxicity by oral administration than the intravenous route. It was reported that the mean lethal dose (LD50) of MAG in rats was 880 mg/kg body weight by oral administration (Adewusi and Oke 1985, Park et al. 2013).

Spermatogenesis is an exceptionally active process able to generate thousands of cells in a short period of time. These high rates of cell division inherent in this process imply correspondingly high rates of mitochondrial oxygen consumption by the germinal epithelium. Nevertheless, the poor testicular vascularization implies that oxygen tension in the testes is low (Free et al. 1976) and that competition for this vital element is highly intense. Since oxidative stress (OS) has been demonstrated to play a key role in male reproductive dysfunction, it is crucial to identify measures that would help predict, with

accuracy, if OS is a significant contributor of infertility in any given clinical or research setting (Sharma et al. 1999).

Resuming the very sparse data available on the *in vivo* effects of AMG on mammalian tissues and/or cells it may be hypothesized that low doses of AMG may exhibit protective effects, yet higher AMG concentrations may be toxic to the biological system. As such, the aim of this *in vivo* study was to reveal whether AMG is able to cause changes in the oxidative profile of rabbit testicular tissue.

MATERIAL AND METHODS

Rabbits are commonly used as experimental subjects for their numerous advantages in comparison to mice, rats or larger animals, including their docile and non-aggressive nature as well as short vital cycles. Rabbits are easy to handle and observe, widely bred and very economical (Wang et al. 1998). As such, meat line P91 Californian rabbit males (n = 12) from the experimental farm of the Animal Production Research Centre Nitra (Slovak Republic) were used in this study. The rabbits were 150 days old and weighing 4.00 ± 0.5 kg. The animals were randomly divided into the three groups, leading to 4 male rabbits in each group. Amygdalin (AMG) from apricot kernels (≥ 99% purity) was purchased from Sigma-Aldrich (St. Louis, MO, USA). AMG was freshly dissolved in sterile saline and 0.5 ml were applied intramuscularly (IM) to the *musculus biceps femoris* on a daily basis. The control (Ctrl) group received no AMG while the experimental groups Exp. 1 and Exp. 2 received a daily IM AMG injection at a dose of 0.6, which is considered to be a preventive dose as a dietary supplement, and 3.0 mg/kg b.w. as a therapeutic dose (Chandler et al. 1984) respectively during 28 days. Institutional and national guidelines for the care and use of animals were followed, and all experimental procedures were approved by the State Veterinary and Food Institute of Slovak Republic, no. 3398/11-221/3 and Ethic Committee (Tusimova et al. 2016).

At the end of the experiment the animals were paralyzed using electric stunning and sacrificed by cutting the carotid artery. Ten testicular tissue samples were collected from each rabbit. Tissue aliquots of approx. 50 mg were subjected to lysis by sonication at 28 kHz for 30 s on ice using RIPA buffer (Sigma-Aldrich) with protease inhibitor cocktail (Sigma-Aldrich). The samples were centrifuged at 11,828 × g, 4 °C for 15 min in order to purify the lysates from the residual cell debris. The resulting supernatants involving the intracellular content were kept on ice for further analyses.

ROS production in each sample was assessed by the chemiluminescence assay using luminol (5-amino-2, 3-dihydro-1,4-phthalazinedione; Sigma-Aldrich) as the probe. The test samples consisted of luminol (2.5 µL, 5 mM) and 100 µL of control or experimental sample. Negative controls were prepared by replacing the lysate with 100 µL of PBS (Dulbecco's Phosphate Buffer Saline; Sigma-Aldrich). Positive controls included 100 µL PBS, 2.5 µL luminol and 12.5 µL H_2O_2 (30%; 8.8 M; Sigma-Aldrich). Chemiluminescence was measured on 96-well plates in 15 cycles of 1 min using the Glomax Multi$^+$ Combined Spectro-Fluoro-Luminometer (Promega Corporation, Madison, WI, USA). The results are expressed as relative light units (RLU)/s/g protein (Tvrda et al. 2016).

Carbonyl group quantification was performed through the traditional 2,4-dinitrophenylhydrazine (DNPH) method. Briefly, 1 mL of the pretreated sample solution was added to 1 mL of DNPH (10 mM in 2 NHCl; Sigma-Aldrich), mixed, and incubated for 1 h in the dark at room temperature. After the addition of 1 mL of trichloroacetic acid (20% w/v; Sigma-Aldrich) the mixture was incubated at 4 °C for 10 min before centrifugation at 11,828 × g for 15 min. The supernatant was discarded without disturbing the pellet that was washed three times with 1 mL of ethanol/ethyl acetate (1/1; v/v) to remove free DNPH reagent. The sample pellet was resuspended in 1 mL of 6 M guanidine-HCl (Sigma-Aldrich) before absorbance measurement at 360 nm. The molar absorption coefficient of 22,000 1/M.cm was used to quantify the concentration of protein carbonyls groups. Protein carbonyls are expressed as nmol/mg protein (Weber et al. 2015).

Lipid peroxidation (LPO) expressed through malondialdehyde (MDA) production was assessed with the help of the TBARS assay, modified for a 96-well plate and ELISA reader. Each sample was treated with 5% sodium dodecyl sulfate (SDS; Sigma-Aldrich), and subjected to 0.53% thiobarbituric acid (TBA; Sigma-Aldrich) dissolved in 20% acetic acid, and subsequently boiled at 90–100 °C for 1 h. Following boiling, the samples were placed on ice for 10 min and centrifuged at 1,750 × g for 10 min. Supernatant was used to measure the end-product resulting from the reaction of MDA and TBA under

high temperature and acidic conditions at 530–540 nm with the help of the Multiskan FC microplate photometer (Thermo Fisher Scientific Inc.). MDA concentration is expressed as μmol/g protein (Tvrda et al. 2016).

Protein concentration was quantified using the DiaSys Total Protein (DiaSys, Holzheim, Germany) commercial kit and the semi-automated clinical chemistry photometric analyzer Microlab 300 (Merck, Darmstadt, Germany). The measurement is based on the Biuret method, according to which copper sulfate reacts with proteins to form a violet blue color complex in alkaline solution, and the intensity of the color is proportional to the protein concentration when measured at 540 nm.

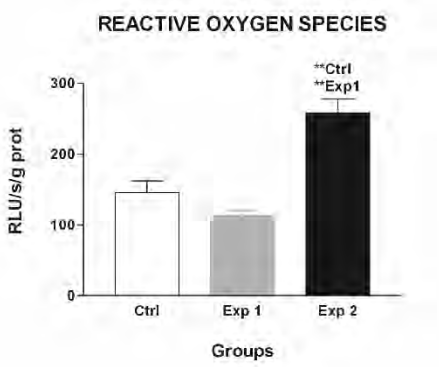
All data were subjected to statistical analysis using the GraphPad Prism program (version 3.02 for Windows, GraphPad Software incorporated, San Diego, California, USA, http://www.graphpad.com/). Results are quoted as arithmetic mean ± standard error of mean (SEM). Comparative analysis of selected parameters in the groups was carried out by one-way ANOVA with the Bonferroni multiple comparison test. The level of significance for the comparative as well as correlation analysis was set at * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

RESULTS AND DISCUSSION

Over the past years, notable attention has been driven towards the exploration of natural biomolecules and their impact on reproductive functions in animals as well as in humans (Tanyildizi and Bozkurt 2004, Kolesarova et al. 2011, Halenar et al. 2015). AMG has captured the interest of numerous researchers (Fukuda et al. 2003, Hwang et al. 2008, Halenar et al. 2015) principally because its potential pharmacological properties.

This study was therefore focused to provide more specific information on the *in vivo* effects of AMG on the male reproductive tissue.

Figure 1 Reactive oxygen species (ROS) generation assessed in the testicular tissue of the Control and Experimental groups.



Legend: Ctrl group: without amygdalin administration; Exp. 1 group: 0.6 mg/kg IM AMG; Exp. 2 group: 3.0 mg/kg IM AMG. Mean ± SEM. ** $P < 0.01$. Ctrl – vs. Control group; Exp1 – vs. Experimental 1 group.

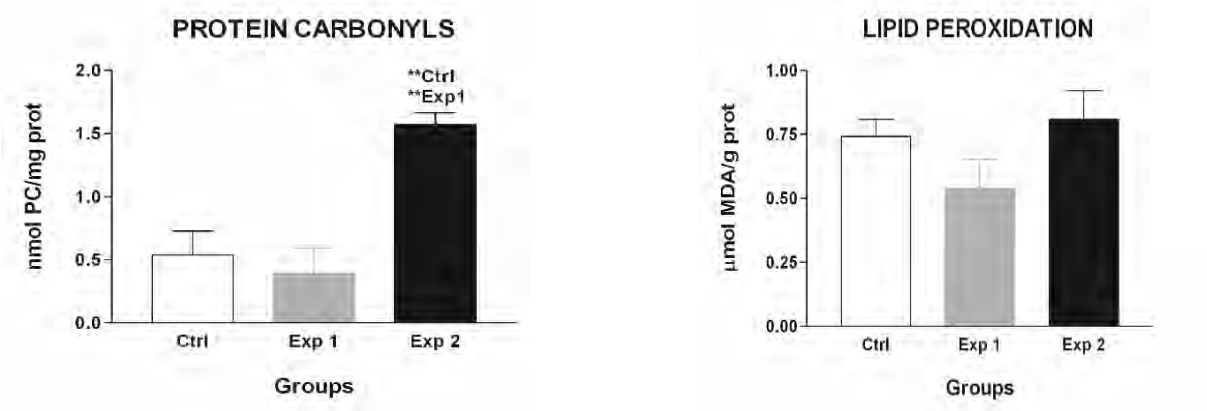
The chemiluminometric assessment revealed a non-significant decrease of the ROS generation in the Exp. group 1 (114.00 ± 6.50 RLU/s/g prot), administered with 0.6 mg/kg b.w. AMG ($P > 0.05$). On the other hand, a significant increase of ROS was detected in the tissue samples collected from the Exp. group 2, treated with 3.0 mg/kg b.w. AMG (258.30 ± 19.30 RLU/s/g prot) when compared both to the Control (145.70 ± 6.71 RLU/s/g prot) as well as Exp. 1 group ($P < 0.01$; Figure 1).

Our results revealed that AMG may have a dose-dependent activity on the testicular tissue, displaying an interesting dichotomy: low doses may improve the oxidative balance, yet high doses may compromise this delicate balance. On one hand, Heikkila and Cabbatt (1980) suggest that AMG may act as a good hydroxyl radical scavenger since it contains both a benzene ring and a sugar moiety and compounds with these groups have very high rate constants for reactivity with the hydroxyl radical. We may therefore speculate that while AMG is metabolized, glucose is the first component to be released and stimulates, to a certain extent the antioxidant mechanisms of the cell (Ceriello et al. 1996, Ruiz-

Roca et al. 2008). As it seems that thanks to the presence of glucose in AMG, this molecule may exhibit cell-protecting effects at lower concentrations. On the other hand, AMG is hydrolyzed by β-glucosidase, emulsin and amygdalase to gentiobiose and L-mandelonitrile. Gentiobiose is further hydrolyzed to glucose, whereas mandelonitrile is hydrolyzed to benzaldehyde and hydrogen cyanide (Conchie and Mann 1957).

Based on the ROS results, we decided to study the potential oxidative damage to the male reproductive tissue more deeply, by assessing the possible ROS-inflicted damage by AMG to testicuarproteins and lipids.

Figure 2 Protein oxidation expressed as the content of protein carbonyls and lipid peroxidation expressed as malondialdehyde content assessed in the testicular tissue of the Control and Experimental groups.



*Legend: Ctrl group: without amygdalin administration; Exp. 1 group: 0.6 mg/kg IM AMG; Exp. 2 group: 3.0 mg/kg IM AMG. Mean ± SEM. ** P < 0.01. Ctrl – vs. Control group; Expl – vs. Experimental 1 group.*

According to the DNPH assay 0.6 mg/kg b.w. AMG administration led to a non-significant decrease of the protein carbonyl content in the Exp. group 1 (0.39 ± 0.02 nmol/mg prot; P > 0.05) in comparison with the Ctrl group (0.38 ± 0.02 nmol PC/mg prot). Inversely, a significant increase of protein oxidation was recorded in the Exp. group 2 (1.57 ± 0.09 nmol PC/mg prot) when compared both to the Control as well as Exp. 1 group (P < 0.01; Figure 2).

Lastly, the assessment of LPO showed that AMG administration led to a non-significant decrease of the MDA content in the Exp. 1 group (0.54 ± 0.10 µmol MDA/g protein), while a non-significant increase of the lipid peroxidation was recorded in the Exp. 2 group (0.82 ± 0.13 µmol MDA/g protein) when compared to the control (0.74 ± 0.09 µmol MDA/g protein; P > 0.05; Figure 2).

AMG interacts in the presence of β-D-glucosidase secreted in the small intestine or obtained from food, to release cyanide or undergoes hydrolysis in the absence of enzymes (Strugala et al. 1995, Deng et al. 2002). Cyanide can also be produced from prunasin, the by-product of amygdalin breakdown (Dorr and Paxinos 1978, Newmark et al. 1984). In fact, cyanide is believed to be the main cancer-killing agent (Rauws et al. 1982). Although the anticancer effects of AMG was never conclusive, AMG treatment proceeds mainly in Mexico and northern Europe (Chang et al. 2006).

Although LPO is considered to be the primary mechanism of ROS-inflicted damage to the biological system, our data revealed that the primary target of ROS overproduced as a consequence of high AMG doses were the proteins. This surprising result may be explained by the occurence of the Dakin oxidation, an organic redox reaction in which an benzaldehyde or ketone reacts with hydrogen peroxide in base to form a benzenediol and a carboxylate (Dakin 1906). This reaction, where the carbonyl group is oxidized, has been proposed to play a pivotal role in the production of oxidized proteins, i.e. protein carbonyls. As such we may hypothesize that ROS produced as a consequence of AMG and subsequently benzaldehyde overload, may trigger proteins first before LPO takes place. We may speculate that when the concentration of benzaldehyde and hydrogen cyanide rises to a certain critical values, AMG acts as a pro-oxidant, switching the oxidative balance towards ROS overproduction. As stated before, Dakin reaction may take over the control, leading to protein oxidation, rather than LPO, which may offer a possible explanation on the assessment of the oxidative damage.

CONCLUSION

In conclusion we may summarize that particularly higher AMG doses may affect the oxidative balance of male reproductive structures. Changes in the oxidative profile of testicular tissue may be a consequence of the intricate metabolism of AMG in a complex biological system as well as of the natural sensitivity of the male reproductive system to the shifts in the oxygen tension. Our results obviously cannot provide a definitive answer to the behaviour of AMG in male reproductive system, which is why further examinations of the impact of AMG on the testicular histology, efficiency of spermatogenesis as well as a deeper assessment of the most notable components of the testicular antioxidant profile are highly recommended.

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CANINE INTERACTIONS IN TOWN PŘEROV

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Abstract: The increasing incidence of obesity is a problem in the Czech Republic as well as in some other countries. Regular walking may be important component of weight management. Effective approaches are necessary to promote this activity. Dog walking may be such a solution that can help encourage physical activity and improve the health of humans and dogs. Dogs can interact with other dogs in public places. Therefore, the aim of our study was to examine interactions between dogs in public places in town Přerov. Four hundred and eighty five canine dyads were observed. The sex of the dog and age of the dog had an effect on the initiation of interactions between dogs. The age of the dog and size of the dog had an effect on the termination of interactions between dogs. Further research will be necessary to fully understand dog interactions in public places.

Key Words: dog, interaction, walk

INTRODUCTION

Physical activities may be an important component of weight management in people and dogs. Dog walking can improve the health of humans (Epping 2011) and dogs (Degeling et al. 2012). Dogs are social animals, and therefore they need contacts with other dogs. However, little is known about the incidence of interactions between dogs in public places (Řezáč et al. 2011). This can be one of the reasons why nearly half of owners do not walk with their dogs regularly (Bauman et al. 2001). An understanding of dog interactions in public places can help better predict dog behavior on walks. The objective of the present study was to examine the effect of a leash, dog age, sex and size and human gender on interactions between dogs in public places.

MATERIAL AND METHODS

The interactions between dogs were observed in public places in the town Přerov in 2016. Four hundred and eighty five canine dyads were examined. The observation was conducted by focal-animal and all-occurrences sampling methods. Interactions were recorded when one dog initiated the interaction. The observation was ended when owners or dogs terminated the interaction. The initiation of interaction and termination of interaction were recorded. The use of a leash, dog age, sex and size and human gender were recorded. The behavior of dogs and their owners was not influenced by the observer. Data were stored in the Excel database. Off-leash dogs that were recalled by their owners during interactions were not included in further analysis. The statistical analysis of the frequency of canine interactions was performed by the chi-square test. Results were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Dogs off a leash interacted one another more often ($P < 0.05$) than when one or two dogs were on a leash (Figure 1). This indicates that the use of a leash considerably decreases the interactions between dogs. Similar results were reported in other studies (Westgarth et al. 2010, Řezáč et al. 2011). Male dogs interacted one another more often ($P < 0.05$) than female dogs (Figure 2). However, the reason is not known. Small dogs interacted one another more often ($P < 0.05$) than with larger dogs (Figure 3). Large dogs interacted one another more often ($P < 0.05$) than with smaller dogs (Figure 3). Medium dogs did not show these differences (Figure 3). These results indicate that small and large dogs prefer the

interactions with dogs of the same size. Dogs interacted one another more often ($P < 0.05$) when both owners were women than when they were men (Figure 4). One of the reasons may be that more dog owners were women than men. Another explanation may be that women allow the interactions between dogs more often than men.

Figure 1 The effect of the use off a leash on the frequency of dog interactions

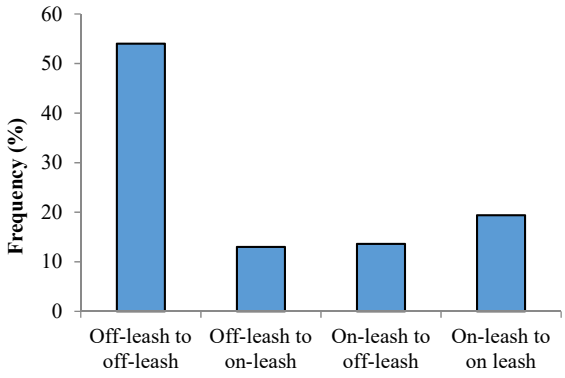


Figure 2 The effect of the sex of the dog on the frequency of dog interactions

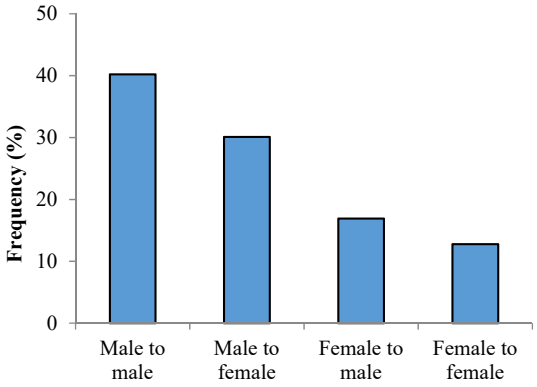


Figure 3 The effect of the size of the dog on the frequency of dog interactions

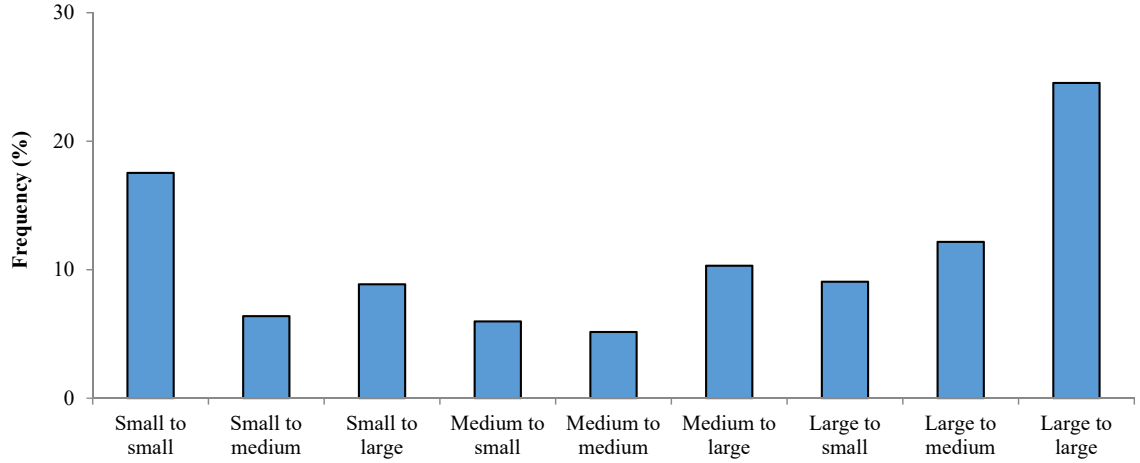
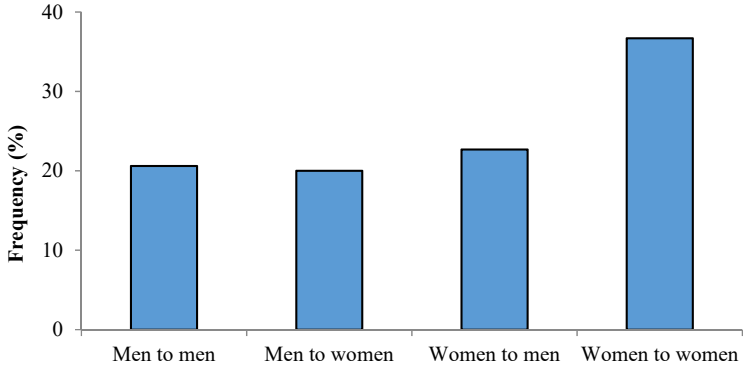


Figure 4 The effect of the sex of the owner on the frequency of dog interactions



Male dogs initiated interactions with female dogs nearly two times more often ($P < 0.05$) than vice versa (Figure 5). This suggests that the interactions between dogs with the opposite sex may have some association with the sexual behavior. Puppies initiated interactions with adult dogs more often ($P < 0.05$) than vice versa (Figure 6). The reason may be that puppies want to play with adult dogs more often than vice versa. Similarly, Řezáč et al. (2011) reports that puppies play more often than adult dogs.

Figure 5 The effect of the sex of the dog on the initiation of dog interactions

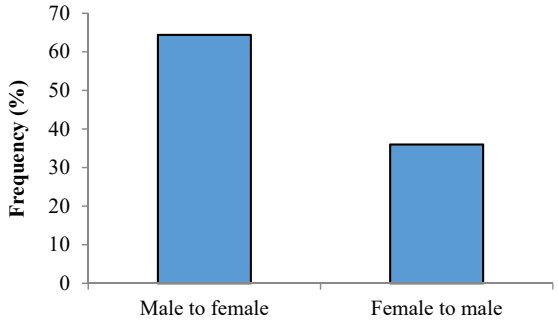
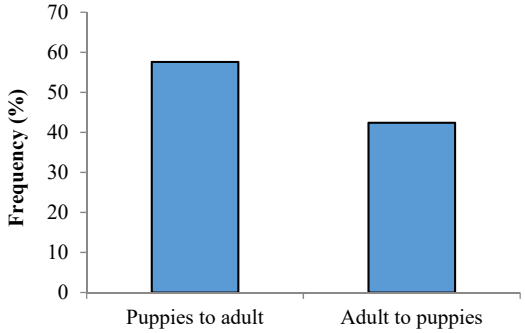


Figure 6 The effect of the age of the dog on the initiation of dog interactions



Adult dogs terminated interactions with puppies more than two times more often ($P < 0.05$) than vice versa (Figure 7). This suggests that adult dogs are not interested as much in the interactions with puppies than vice versa. Small dogs terminated interactions with medium dogs nearly two times more often ($P < 0.05$) than vice versa (Figure 8). This is in agreement with the finding that dogs probably prefer the interactions with dogs of the same size.

Figure 7 The effect of the age of the dog on termination of dog interactions

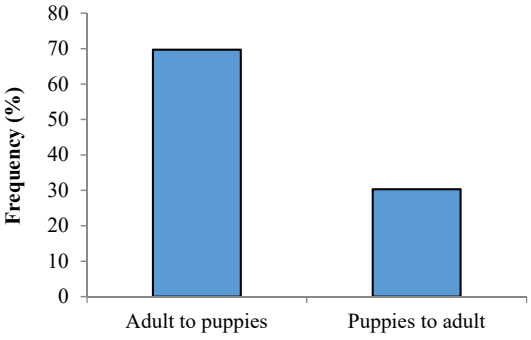
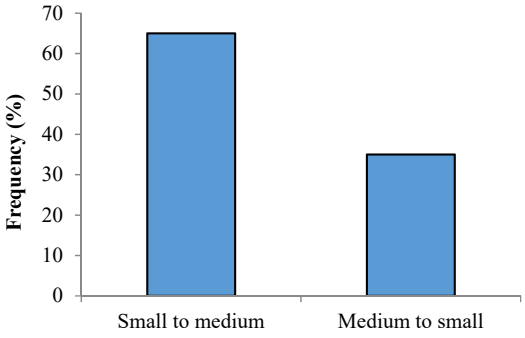


Figure 8 The effect of the size of the dog on the the termination of dog interactions



CONCLUSIONS

Our results showed that the initiation of interactions between dogs is affected by the sex of the dog and age of the dog. The termination of interactions between dogs is influenced by the age of the dog and size of the dog. Further research will be needed to fully understand the factors that affect dog interactions in public places.

ACKNOWLEDGEMENTS

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COMPARISON OF REPRODUCTION INDICATORS OF HOLSTEIN CATTLE

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Abstract: Aim of this study was to compare selected reproduction indicators of Holstein cattle at two farms. The data gained in 2014 a 2015 were compared. In the observed cow population values of the 1st inseminations, all inseminations, re-inseminations, cow pregnancies after the 1st insemination and all inseminations, interval, gestation period and conception rate were compared. The conception rate after all the inseminations at the both farms (32.16 to 39.57%) was almost in accord with the average rate in the Czech Republic. The gestation period values (103.83 to 144.26 days) and interval values (66.17 to 88.18) were of an average or even substandard level. These two herds shall be classified as of a low level of reproduction.

Key Words: cattle, Holstein, reproduction, insemination

INTRODUCTION

Fertility is a basic productivity and biological quality which significantly influences economics of the stock raising. It is considered to be superior to milk and meat productivity (Ježková et al. 2004). This quality is characterized by low heritability (0.05 - 0.2 = h^2). That means that fertility is highly influenced by external factors, e.g. stabling, nutrition, environment, quality of treatment, farming method etc. (Louda 2008, Slupka 2013).The level of reproduction is evaluated based on the reproduction indicators. Amongst these indicators belong non-return test, pregnancies after the first insemination, pregnancies after all inseminations, interval, gestation period, insemination index, cow birth rates, number of live born calves in 100 cows, and calving interval (Říha et al. 2000). From the genetic point of view we classify fertility and reproduction in general as quantitative markers which are more influenced by the genes. From the breeding point of view for good reproduction right choice of the parental pairs is important. Therefore it is possible to increase the productivity by mating the efficient parental pair. Concerning the paternal line the improvement can be reached if an insemination dose of a bull with positive fertility breeding value is used. In the maternal line the improvement of productivity can be reached if a cow with good pregnancy rates is chosen. For breeding for fertility it is beneficial to use selection and exclude individuals with deteriorative fertility and pregnancy rates. (Bezdíček et al. 2010).

Current trend in cattle breeding is getting a long-living cow of a good health condition, good fertility and excellent offspring. Cattle fertility has declined by 0.5% a year in the last four or five decades (Coufalík 2013).

Problems with Holstein cattle pregnancy have been increasingly frequent. These problems express themselves by increasing number of silent or indistinctive heat and cease of reproductive cycle of the cows. Problems with conception lead to prolonged gestation period, increasing usage of insemination doses and hormones in order to provide right reproductive cycle.

The aim of this article was to evaluate selected reproduction indicators of Holstein cattle dairy cows.

MATERIAL AND METHODS

The experiment was conducted at two farms breeding Holstein cattle. The first farm is situated in the Brno-Country District and the second in Třebíč District. The reproduction results in years 2014 and

2015 were compared. The evaluation of reproduction indicators was based on cumulative monthly and annual overviews of insemination and conception and from the databases of agricultural enterprises in the Czech-Moravian Union of Breeders information system.

The cows at the farm in Třebíč District (farm A) were stabled in loose boxes. An average number was about 263 cows. The average annual milk yield was 7500 kg of milk. As auxiliary detectors for heat identification pedometers and neck responders were used. The heat was also searched for by the cattlemen. Cows were let to rebreed 45 days after calving. Insemination was performed by the farm insemination technician. For the insemination sexed insemination doses were used. Early diagnostics were performed ultrasonographically at about 30th to 33rd day after the insemination, repeated pregnancy check was done 60 days after the insemination.

At the farm in Brno-Country District (Farm B) the stabling conditions and heat identification were the same. The average number was 350 cows and average milk yield was 8500 kg of milk. The insemination technician represented the different factor - insemination at this farm was performed by a privet veterinary doctor and there weren't used sexed insemination doses.

From the reproduction indicators the number of 1st inseminations, all inseminations, re-inseminations, cow pregnancies after the 1st insemination and all inseminations, interval, gestation period and conception rate were compared. The values of reproduction indicators were processed by the statistical software STATISTICA 10.

RESULTS AND DISCUSSION

At the above-mentioned farms data from the 1st inseminations, all inseminations, re-inseminations, gestation periods and conception rates in cows in 2014 and 2015 were compared (table 1). At the farm in Brno-Country District on average 37.83 ± 7.22 1st inseminations were performed per month in 2014 and 42.58 ± 11.24 per month in 2015. At the second farm there were less inseminations performed. In 2014 on average 26.58 ± 3.36 1st inseminations were performed per month and 23.33 ± 5.71 per month in 2015. In the results we evaluated statistically significant difference was discovered.

Czech-Moravian Union of Cattle Breeders (2016) declares that that in Holstein cattle there were 187 832 1st inseminations performed in 2014. In the following year the number of the 1st inseminations increased to 189 624. From the collected data it is evident that at farm A there was a decrease in the number of the 1st inseminations between the years 2014 and 2015. On the farm B there is increase in the number of the 1st inseminations in the same period apparent.

Table 1 Evaluation of cow insemination in years 2014 and 2015 at farms A and B

District	Year	Number of 1st inseminations (pcs)		Number of all inseminations (pcs)		Number of re-inseminations (pcs)		Conception rate	
		$\bar{x}$	s_x	$\bar{x}$	s_x	$\bar{x}$	s_x	$\bar{x}$	s_x
Farm B	2014	37.83	7.22	95.92	12.38	16.67	4.81	2.28	0.05
	2015	42.58	11.24	101.75	16.41	15.75	5.72	2.13	0.11
Farm A	2014	26.58	3.63	57.50	6.95	6.75	2.60	1.96	0.10
	2015	23.33	5.71	56.58	8.46	8.83	3.16	2.15	0.12

When comparing the values of all inseminations it can be established that at the farm B there were on average 95.92 ± 12.38 of all inseminations performed per month. In 2015 there were performed 101.75 ± 16.41 of all inseminations per month. On the contrary, at the farm A on average 57.50 ± 6.95 of all inseminations were performed per month in 2014 and 56.58 ± 8.46 of all inseminations per month in 2015. The number of inseminations is again higher at the farm B. In 2014 on average 16.67 ± 4.81 re-inseminations per month were performed and 15.75 ± 5.72 per month in 2015. At the farm B the conception rate was on average 2.28 ± 0.05 in 2014 and 2.13 ± 0.11 in 2015.

According to Říha et al. (2000) the lowest value of conception rate we discovered (table1) is unfavourable (1.96 ± 0.10) and the value of conception rate over 2.0 is unsatisfactory. At the farm B the conception rate decreased, however it did not reach the desired value under 2.0. In comparison the conception rate at the farm A increased.

Table 2 Evaluation of interval and gestation period length

District	Year	Interval (day)		Gestation period (day)	
		$\bar{x}$	s_x	$\bar{x}$	s_x
Farm B	2014	88.18	1.30	144.26	2.21
	2015	83.66	2.40	137.18	7.91
Farm A	2014	66.17	2.91	103.83	8.28
	2015	69.33	2.26	103.94	2.31

The interval was at the farm B 88.18 ± 1.33 days in 2014 and 83.66 ± 2.44 days in 2015. On the contrary the values at the farm A were lower. In 2014 the average interval value was 66.17 ± 2.91 days and 69.33 ± 2.26 days in 2015. The gestation period (GP) was also lower at the farm A, in 2014 the average value was 103.83 ± 8.28 days and in the following year the GP was 103.94 ± 2.31 days. At the farm B the average value of GP was 144.26 ± 2.21 days in 2015 and in 2015 it decreased to 137.18 ± 7.91 days (table 2). Norman et al. (2009) states that in 2006 the interval of Holstein cows was 86 days and the GP 144 days. Říha et al. (2000) states that level of reproduction interval between 66 to 76 days is considered average (sufficient) and the level of reproduction interval over 77 days is insufficient. The author also states that concerning the gestation period the average (sufficient) value is 91 to 110 days and the value over 110 days is insufficient. The results imply that the farm B belongs to the farms with insufficient level of reproduction. Kvapilík et al. (2015) declares that the interval value was 75.3 days and GP 118.8 days in 2014. From the obtained results we can deduce that the Třebíč district farm reached lower values in comparison to average values in the Czech Republic in 2014. Coufalík (2013) implies that the long GP is caused up to 60% by the nutrition, up to 30% by management and up to 10% by illnesses. The problems with high value of GP and low interval value can indicate problems related to reproduction of the heard of cattle or also with the insemination management (Louda et al. 2008)

Table 3 Evaluation of the percentage of conception in 2014 and 2015

District	Year	% of conception after the 1st insemination		% of conception after all inseminations	
		$\bar{x}$	s_x	$\bar{x}$	s_x
Farm B	2014	31.30	9.83	32.16	6.42
	2015	31.73	13.07	33.36	9.27
Farm A	2014	37.67	11.35	38.38	8.26
	2015	41.15	17.04	39.57	11.48

The percentage of conception after the 1st insemination at Brno farm is almost identical in 2014 and 2015. In 2014 the average percentage of conception was 31.30 ± 9.83% and in 2015 it was slightly higher (31.73 ± 13.07%) On the contrary at the second farm the average percentage of conception after the 1st insemination was 36.67 ± 11.35% in 2014 and 41.15 ± 17.04% in 2015. Kvapilík et al. (2015) state that the level of conception after the 1st insemination in Holstein cows in the Czech Republic is 34.9%. Similar values of conception presented also Syrůček and Burdych (2015). In comparison, Gilmore et al. (2011) claim the values of conception after the 1st insemination of Holstein cows 33.3%. When comparing our data it can be stated that the farm A has a better level of conception that the above-mentioned authors claim. On the contrary the farm B has a worse level of the conception in both years. Norman et al. (2009) claim that in the United States of America the conception rate after the 1st insemination was 31%. Říha et al. (2000) consider the conception after the 1st insemination reaching the level under the 40% as insufficient. The conception after the 1st insemination was except for one value in 2015 in the farm A insufficient. The percentage of conception after all inseminations was better also at this farm. In 2014 the values were 38.39 ± 8.26% and in 2015 they were 39.57 ± 11.48%. The resulting values are almost identical. On the contrary at the farm B the levels were significantly lower. In 2014 the average percentage of conception after all inseminations was 32.16 ± 6.42% and in 2015 it was 33.36 ± 9.27%. According to Norman et al. (2009) in 2006 in the United States of America the percentage of conception after all insemination was 30%. Syrůček and Burdych (2015) state that in 2014 the conception after all inseminations in Holstein cows was 33% with the yield of 9 to 10 thousand litres of milk. According to Coufalík (2013) quality management as well was quality work of the insemination

technician and veterinary doctor influence the conception the most. According to Kvapilík et al. (2015) the insufficient fertility is caused up to 60% by insufficient management and up to 40% by the nutrition of the cows.

CONCLUSIONS

The results of individual indicators of evaluated farms reached the values that are average or insufficient. At these farms the reproduction can be classified as a low level. At the Třebíč district farm the values of conception rate were insufficient and the values of GP, interval and conception percentage after the 1st insemination and after all inseminations were average. At the farm in Brno-Country district the values of conception rate, GP, interval and percentage of the conception after the 1st insemination were insufficient. Percentage of the conception after all inseminations is on average level. The level of reproduction at the two farms could be increased by increasing the quality of breeding management, either by improvement in heat detection or zoo-hygienic conditions.

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FORENSICALLY IMPORTANT MUSCIDAE (DIPTERA) ASSOCIATED WITH DECOMPOSITION OF CARCASSES AND CORPSES IN THE CZECH REPUBLIC

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Abstract: In years 2011 to 2015, three field experiments were performed in the capital city of Prague to study decomposition and insect colonization of large cadavers in conditions of the Central Europe. Experiments in turns followed decomposition in outdoor environments with the beginning in spring, summer and winter. As the test objects a cadaver of domestic pig (*Sus scrofa* f. *domestica* Linnaeus, 1758) weighing 50 kg to 65 kg was used for each test. Our paper presents results of family Muscidae, which was collected during all three studies, with focusing on its using in forensic practice. Altogether 29,237 specimens of the muscids were collected, which belonged to 51 species. It was 16.6% (n = 307) of the total number of Muscidae family which are recorded in the Czech Republic. In all experiments the species *Hydrotaea ignava* (Harris, 1780) was dominant (spring = 75%, summer = 81%, winter = 41%), which is a typical representative of necrophagous fauna on animal cadavers and human corpses in outdoor habitats during second and/or third successional stages (active decay phase) in the Czech Republic.

Key Words: Muscidae, Diptera, forensic entomology, pyramidal trap

INTRODUCTION

Forensic or criminalistic entomology is the science discipline focusing on specific groups of insect for forensic and law investigation needs (Eliášová and Šuláková 2012). Its main principle is to determinate the minimum time since death or so called post mortem interval (PMI) by establishing the time of colonization (or the time of the first oviposition) of a corpse by insects. Forensic entomology can also provide evidence whether the body was manipulated at the crime scene (e.g., after buried) or moved from a location to other as well as when the body was subsequently moved and it's still missing by genetic analysis of found larvae DNA profile of the host and therefore his/her identity can be obtained (Amendt et al. 2011). Forensic entomology with help of studies results can be consider as very effective and often crucial tool in explaining of criminal cases (Martins et al. 2013).

Muscidae is a large family of order Diptera with worldwide distribution. The family has about 4,500 species in 180 genera in the word (De Carvalho et al. 2005) and 562 species in 44 genera are known from Europe (Pont 2005). In the Czech Republic, 307 species is listed (Barták 2013). Several species of muscids are ubiquitous and synanthropic and therefore of medical importance because of their relationship with man. The synantropic habits ensure that some species are likely to become involved in medical and forensic cases (Byrd and Castner 2010, Smith 1986). Common members of this family, which can visit and colonized a body soon after death, are *Musca domestica* Linnaeus, 1758 and *Musca autumnalis* DeGeer, 1776 (Daněk 1990, Gennard 2012) or members of genus *Muscina* and *Hydrotaea* (Eliášová and Šuláková 2012, Šuláková 2014). Muscid flies tend to arrive at corpses after the blow flies (Calliphoridae) or later after the flesh flies (Sarcophagidae) (Byrd and Castner 2010, Eliášová and Šuláková 2012, Šuláková 2014). Muscid females often lay eggs in natural body openings, at wound, or blood-soaked clothing (Byrd and Castner 2010) as well as beneath the body into soil soaked by

decomposition liquid (Šuláková 2014). Larvae usually feed directly on the carrion but can also pray on the eggs and larvae of other necrophagous flies (Byrd and Castner 2010, Šuláková 2014) and in this way may affect the faunal composition (Byrd and Castner 2010).

MATERIAL AND METHODS

Description of the locality field experiments

Summer experiment

The summer experiment was situated in a fenced ground of the Police school in Prague 9 – Hrdlořezy, the eastern suburb of the capital. The research site consisted of bushy area with smaller, sunlit openings with grass and deciduous trees in vicinity. Geographic coordinates: 50°5’22" N, 14°30’19" E; altitude: 240 m amsl.

For purposes of the summer experiment, domestic pig (*Sus scrofa* f. *domestica*) weighing about 65 kg was killed by a single shot to the front of its head with a 0.22 calibre rifle. The cadaver was moved the trial site within 20 minutes, foil-wrapped during transport to avoid any earlier oviposition. On the experimental area carcasses was dressed in human clothing to more accurately mimic the decay of the human body. According Daněk (1990) clothing, among other factors, affects the rate of decomposition, acts in part as a shield. The experimental animal had a bleeding wound imitating injuries of a murder victim. The experiment lasted from 13 July 2011 and was completed 18 October 2012. Insects of the cadaver and its immediate surroundings were captured using am entomological net, tweezers and pitfall traps filled with a mixture of saline water with added detergent. Collection of insects was different according to the time from the start of the experiment: 1st to 17th day exposure was conducted trapping insects once a day, 17th to 62nd day every two to three days, 62nd to 195th day, once every ten to fourteen days and 195th to 464th day only once a month.

Spring experiment

The spring experiment was situated in a fenced experimental field of the Czech University of Life Sciences in Prague – Troja, the northern part of the capital. The area was on a west-facing slope near Vltava River, in a flooding zone, on grass-covered, sunlit opening with fruit trees and bushes around. Geographic coordinates: 50°7'16" N, 14°23'53" E; altitude: 185 m amsl.

For the spring experiment, domesticated pig weighing about 53 kg was used, which have died naturally 19 March 2012, the day before the start of the experiment. During the subsequent transfer on the experimental site the pig was placed in a sealed plastic bag to prevent earlier colonization by insects. This cadaver was also dressed in clothing. For collecting of the adults of the family Muscidae, which arrived on the cadaver or hatched on it, a pyramid trap was used. The trap consisted of soft polyester fabric placed over the carcasses shaped bottom open oblique pyramid (description Barták and Roháček 2011) which collects insect 24/24. The trap had base dimensions 2 m x 2 m to overlie the entire experimental animal, and has been placed 20–40 cm over the ground or vegetation, in order not to prevent access of insects to a test object. On the top of the trap, a collecting plastic bottle of capacity of 5 litres was placed and filled by a killing and preservative solution with its composition: 1.5 l of water, 2 ml 36–38% of formaldehyde and 1 ml of detergent. Experiment started 20 March 2012 by free exposition of cadaver and 6 June 2013 was terminated prematurely for reasons of flooding and damaging to the experimental area by the swollen Vltava River during the floods in Prague in 2013. Contents of the sampling vessel on the pyramid traps were collected at the following intervals: 1st–197th day of exposure once a week, 197th to 267th day once every two weeks, 267th to 393rd day once a month, and after two weeks until the end experiment.

Winter experiment

The winter experiment was also situated in a fenced experimental field of the Czech University of Life Sciences in Prague – Troja, on the west-facing slope near Vltava River, above the flooding zone, on sand-covered, sunlit opening among fruit trees. Geographic coordinates: 50°7'14.5" N, 14°23'56" E; altitude: 190 m amsl.

In the free exposition, the next dead domesticated pig weighing about 50 kg has been exposed. The pig was killed by a veterinarian by injection a month before the study and frozen to imitate a frozen

overdosed drug user. For the purpose, the carcass was dressed to men's shirts, sweatpants and socks. The winter experiment has been running from 9 December 2014 and has not been finished yet. For the purposes of this study, results of family Muscidae since the beginning, 9 December 2014, to 31 December 2015 are only presented. Trapping method coincides with the spring experiment, by the pyramidal trap. Samples were taken at the following intervals: at the beginning of the experiment, i.e. from December 2014 were sampled once per month. From January to early March 2015 every 14 days, from March samples were collected once a week until October. In October, the sampling interval is extended again for 14 days.

Material

The material was determined to species by Lt-Col. Ing. Hana Šuláková, Ph.D., Bc. Markéta Slobodová and prof. RNDr. Miroslav Barták, CSc. according to the monograph The Muscidae (Diptera) of Central Europe (Gregor a kol. 2002).

RESULTS

Summer

In the summer experiment, the total of 234 representatives of the family Muscidae belonging to these 8 species were collected and determined. These species, listed in alphabetical order, were: *Graphomya maculata* (Scopoli, 1763), *Hydrotaea armipes* (Fallen, 1825), *Hydrotaea dentipes* (Fabricius, 1805), *Hydrotaea ignava, Hydrotaea meteorica* (Linnaeus, 1758), *Hydrotaea pilipes* (Stein, 1903), *Muscina prolapsa* (Harris, 1780) and *Thricops simplex* (Wiedemann, 1817) (Figure 1). The first four stages of decomposition were: fresh 13 July to 14 July 2011, bloated 15 July to 16 July 2011, active decay 17 July to 1 August 2011, and dry remains 3 August to 12 September 2011.

Spring

From the obtained material of the field spring experiment, 19,910 specimens family Muscidae belonging to 38 species were collected and determined. The following species were recorded (in alphabetical order): *Azelia cilipes* (Haliday, 1838), *Azelia trigonica* (Hennig, 1956), *Azelia triquetra* (Wiedemann, 1817), *Azelia zetterstedti* (Rondani, 1866), *Coenosia humilis* (Meigen, 1826), *Coenosia nigridigita* (Rondani 1866), *Coenosia testacea* (Robineau–Desvoidy, 1830), *Coenosia tigrina* (Fabricius, 1775), *Graphomya maculata, Hebecnema nigra* (Robineau–Desvoidy, 1830), *Hebecnema umbratica* (Meigen, 1826), *Hebecnema vespertina* (Fallen, 1823), *Helina impuncta* (Fallen, 1825), *Helina Latitarsis* (Ringdahl 1924), *Helina reversio* (Harris, 1780), *Helina sexmaculata* (Preyssler, 1791), *Hydrotaea aenescens* (Wiedemann, 1830), *Hydrotaea armipes, Hydrotaea cyrtoneurina* (Zetterstedt, 1845), *Hydrotaea dentipes, Hydrotaea floccosa* (Macquart, 1835), *Hydrotaea ignava*, *Hydrotaea meteorica*, *Hydrotaea pilipes*, *Hydrotaea similis* (Meade, 1887), *Limnophora nigripes* (Robineau–Desvoidy, 1830), *Lispe tentaculata* (De Geer, 1776), *Musca stabulans* (Fallen, 1817), *Muscina levida* (Harris, 1780), *Muscina pascuorum* (Meigen, 1826), *Muscina prolapsa, Mydaea ancilla* (Meigen, 1826), *Mydaea Corni* (Scopoli, 1763), *Mydaea urbana* (Meigen, 1826), *Myospila meditabunda* (Fabricius, 1781), *Phaonia subventa* (Harris, 1780), *Potamia littoralis* (Robineau–Desvoidy, 1830) and *Thricops simplex* (Figure 1). The first four stages of decomposition were: fresh 20 March to 27 March 2012, bloated 3 April to 24 April 2012, active decay 2 May to 21 August 2012, and dry remains 28 August to 11 December 2012.

Winter

During the winter experiment, 9,093 specimens of the family Muscidae, belonging up to 41 species were capture and determined. The following species were recorded (in alphabetical order): *Azelia nebulosa (*Robineau–Desvoidy, 1830), *Azelia triquetra, Coenosia atra (*Meigen, 1830), *Coenosia humilis, Coenosia infantula (*Rondani, 1866), *Coenosia rufipalpis (*Meigen, 1826), *Coenosia testacea, Coenosia tigrina, Eudasyphora zimini* (Henning, 1963), *Graphomya maculate, Gymnodia humilis* (Zetterstedt, 1860), *Hebecnema vespertina, Helina depuncta* (Fallén, 1825), *Helina impuncta, Helina lasiophthalma* (Macquart, 1835), *Helina reversion, Helina setiventris* (Ringdahl, 1924), *Hydrotaea aenescens (*Wiedemann, 1830), *Hydrotaea armipes, Hydrotaea dentipes, Hydrotaea floccose, Hydrotaea ignava, Hydrotaea pilipes, Hydrotaea similis, Musca autumnalis* (De Geer, 1776), *Musca domestica* (Linnaeus, 1758), *Musca osiris* (Wiedemann, 1830), *Muscina levida, Muscina pabulorum*

(Fallén 1817), *Muscina prolapse, Muscina stabulans, Mydaea ancilla, Mydaea corni, Myospila meditabunda, Phaonia errans* (Meigen, 1826), *Phaonia subventa, Phaonia trimaculata* (Bouché, 1834), *Phaonia tuguriorum* (Scopoli, 1763), *Pyrellia vivida* (Robineau–Desvoidy, 1830), *Stomoxys calcitrans* (Linnaeus, 1758) and *Thricops simplex* (Figure 1). The first four stages of decomposition were: fresh 9 December 2014 to 3 March 2015, bloated 18 March to 1 April 2015, active decay 7 April to 1 September 2015, and dry remains 9 September to 8 December 2015.

Figure 1 Compared frequency of occurrence of muscids in all experiments

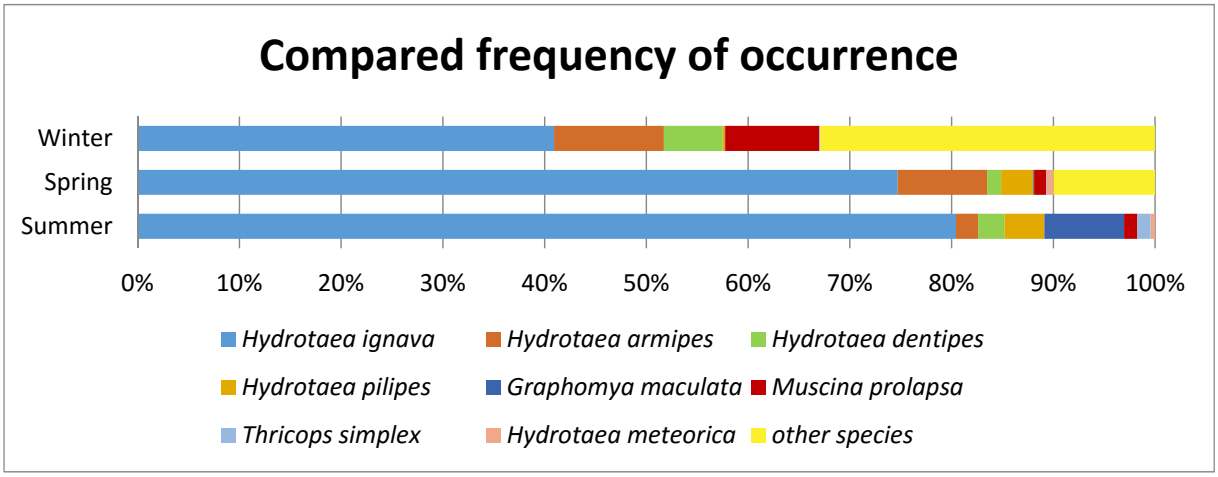


Figure 2 Summer – Quantity of three most common species according to decay phases

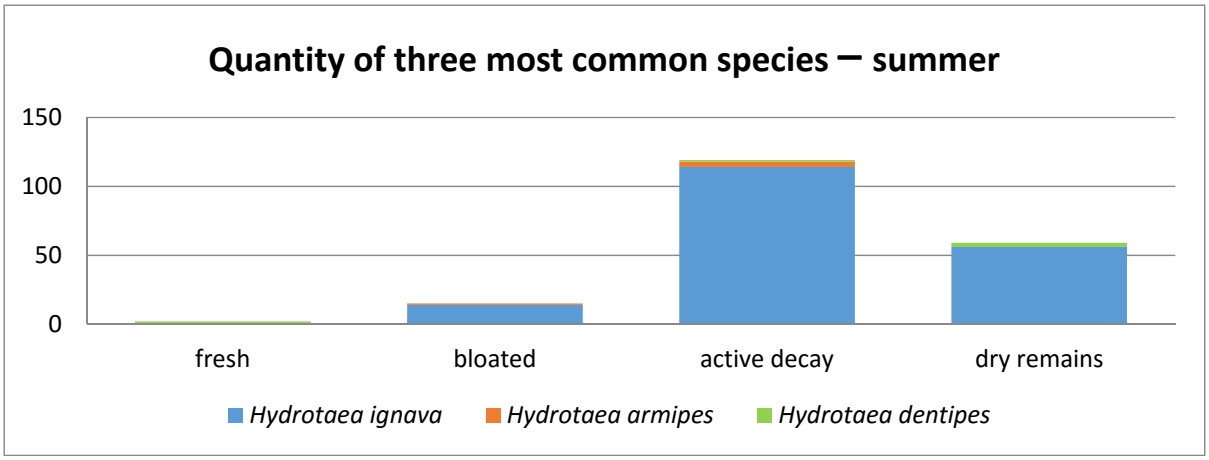


Figure 3 Spring – Quantity of three most common species according to decay phases

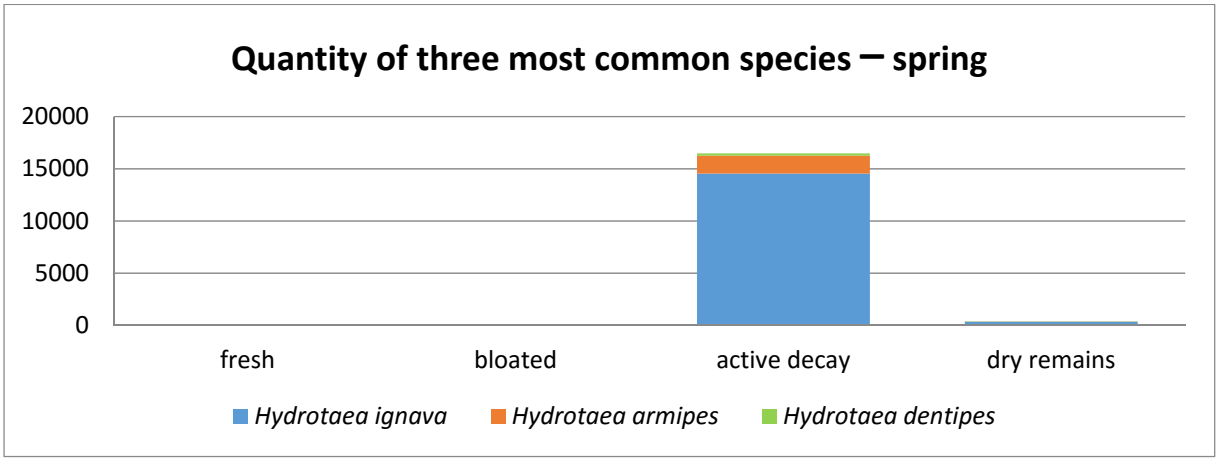
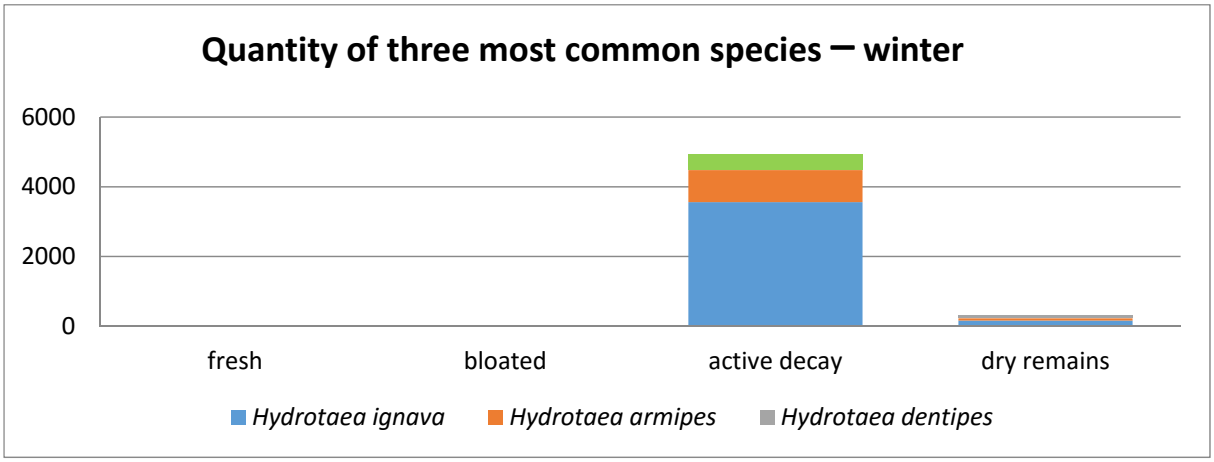


Figure 4 Winter – Quantity of three most common species according to decay phases



CONCLUSION AND DISCUSSION

During the experiments have been recorded 51 species of families Muscidae, which are 16.6% (n = 307) of the total number of species that can occur in the Czech Republic.

The most numerous species was *Hydroatea ignava*. Its abundance and importance discusses Daněk (1990) and Sukantson (2007) who confirm that 70% of all individuals detained on the corpse may be just *Hydrotaea ignava*. The most frequent species of the genus *Hydrotaea*, namely *Hydrotaea ignava*, *Hydrotaea armipes* and *Hydrotaea dentipes*, were related to third stage of decomposition, the active decay phase, in all three trials (see Figure 2, Figure 3 and Figure 4). These statements respond to published data (e.g., Byrd and Castner 2010, Eliášová and Šuláková 2012, Šuláková 2014) as well as our findings on human corpses in outdoor conditions (Šuláková, unpublished data). Other abundant species on the carcase of experiment Spring were also *Hydrotaea pilipes* and *Hydrotaea floccosa* – the literature dealing with forensic entomology not mention about them. The reason could be that species of *Hydrotaea pilipes* has been often confused with the species *Hydrotaea ignava* (females are very similar) and species *Hydrotaea floccosa* was probably due to poor recognisability confused with the species of *Hydrotaea armipes* (Klimešová et al. 2014).

Musca autumnalis and *Musca domestica*, which are usually mentioned as forensically important muscids, especially for the first or the second succession wave (Daněk 1990, Gennard 2012, Smith 1986), were not involved in Spring and Summer experiments at all, although, during Spring and Winter experiment (in Troja), near zoological garden and its stables were a potential source of them. In experiment Winter, there were present only in a small amount from the second to the beginning of the fourth stage of decomposition. We suppose that the occurrence of these species was probably caused by different (slower) processes of decomposition due to the beginning of the experiment in winter. Similar patterns are observed on human corpses in the Czech Republic; *Musca autumnalis* and *Musca domestica* are rare on dead bodies and their oviposition is usually qualified by both their presence in the locality and the appearance of faeces on or near the body (Šuláková, unpublished data). The result reflect that the genus *Muscina*, mainly *Muscina prolapsa*, which was recorded in all three trials but mostly in Winter (Figure 1), is more common and important for forensic practise then the genus *Musca*. These data confirm our observations from the human corpses, when muscids of the genus *Muscina* colonize dead bodies more frequently during winter, what time their make the best of reduction or absence of the usual first colonizers from the family Calliphoridae (Šuláková, unpublished data).

According our results, occurrence, frequency, and quantity of muscids are not affected by the reason of the death of the object (e.g., bodies with or without bleeding wounds). The family is associated mainly with third stage of decomposition. Nevertheless seasons can partly influence the species composition of muscids and the time their staying on the body.

In smaller amounts, many species that are commonly not found on corpses were recorded, for example: *Coenosia humilis, Coenosia testacea, Helina impuncta, Mydaea urbana* or *Azelia cilipes*. Their larvae are commonly feeding and growing on different types of decaying organic material, e.g.,

manure, meat, plants, and we assume they use carcases only occasionally. Some of them are not strictly necrophagous but predatory (the genus *Coenosia* are predatory also like adults). *Azelia trigonica*, recorded in twenty specimens in May 2012 and July 2012 in Spring experiment, is guided on the red list of endangered species of the Czech Republic as "endangered" (Farkač et al. 2005).

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THE EFFECT OF FEEDING EXTRACTED RAPESEED MEAL ON THE CONTENT OF IODINE IN MILK, URINE AND BLOOD PLASMA IN DAIRY COWS

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Abstract: The study was aimed at validating the influence of increased intake of extracted rapeseed meal (4.7 kg/cow/day) on the distribution of iodine in various body fluids (blood plasma, urine and milk) in groups of dairy cows. The feed ration contained 1.2 mg iodine/kg of dry matter (i.e. additive iodine – inorganic form) in the 1st stage of experiment, 0.6 mg iodine/kg of dry matter (i.e. additive iodine – inorganic and organic form) in the 2nd stage. The average contents of iodine in milk and urine were: 33.1µg/land 192.9 µg/l in 1st stage of experiment, 79.6 µg/land 376.3 µg/l in 2nd stage. Our results show that an increased intake of extracted rapeseed meal reduces utilization of iodine (iodine in milk) and increases urinary iodine excretion. Our results also show that organic form of iodine was effectively utilized.

Key Words: strumigens, iodine, milk, urine, blood plasma

INTRODUCTION

Screening the values of iodine in feed rations in farmed animals and also in animal products is important because of the risks that presents themselves from the abnormal intake of such. Not only in the past but also today, people as well as animals' suffering from the lack iodine has been a worldwide issue. This problem is solved by the International council for the control of iodine deficiency disorders (ICCIDD) and Interdepartmental commission for the solution iodine deficiency in the Czech Republic. The question of iodine deficit is still in the forefront of interest of not just medical professionals but also by the manufactures of mineral additives, feeding mixtures, cooking salt, food in general and last but not least the breeder animals. Schöne et al. (2009) recommend 0.5–1.5 mg iodine per kg dry matter (DM) feed ration.

Extracted rapeseed meal (ERM) is commonly used in animal nutrition as protein source. Limiting factor of this crop is the content of glucosinolates. Glucosinolates can be hydrolysed by the enzyme myrosinase to release products with goitrogenic effects that interfere with iodine metabolism and therefore affect the functioning of the thyroid gland and consequently with the animal performance (Mejicanos 2016). Tripathi and Mishra (2007) states that fission products of glucosinolates lead to the decrease of iodine secretion through milk and to the increase of urine excretion. Except strumingenous effect, these substances have a negative effect on the feed taste, which leads to the decrease in consumption. This is the reason why the content of rapeseed products is limited in feeding mixtures. For example, Šimek et al. (2001) states, that 3−5% share of rapeseed meal in feeding mixture does not affect the taste of the feed. Recommended maximum daily intake of ERM is 2.5 kg/cow/day with maximal glucosinolates content up to 20 mmol/kg (Zukalová and Vašák 2001).

The aim of this study was to validate the effect of higher share of extracted rapeseed meal on the content of iodine in individual samples of blood plasma, urine and milk in Holstein breed cows.

MATERIAL AND METHODS

The 5 months experiment was conducted on a farm in the district Klatovy. The experiment included a total of 9 Holstein Friesians cows in their second lactation (average age of cows: 42 months). The average milk production was 36 kg/day. The study was divided into 2 phases. In the first phase (two months) the inorganic iodine was supplemented as *Kalium iodatum* (1.2 mg iodine/1 kg DM of feed ration). In the second phase (three months), the inorganic iodine was reduced on 50% and the organic iodine (10%) was added. In the second phase was 0.6 mg iodine/1 kg DM of feed ration. In both phases of the experiment, the animals received higher amount of extracted rapeseed meal (4.7 kg/cow/day). The composition of feed ration and iodine concentration are shown in the Table 1 and Table 2.

Samples of body fluids (blood, milk and urine) were collected every month from January to May. The blood samples were taken 2 hour after morning meal. Blood was collected from the *Vena caudalis mediana* into heme sampling tubes with heparin. Urine samples were collected by catheterizing the bladder to sterile tubes. Individual milk samples were collected from the complete milking into a set of sterile tubes (100 ml) by milking directly into the tubes to avoid contamination and stored at -20 °C. In total, 45 blood samples, 45 urine samples and 45 milk samples were collected.

The iodine content in the body fluids was determined by a modified colorimetric method (Sandell and Kollthoff) after alkaline ashing of the material (Bednář et al. 1964). The variation was assessed by using the one-way analysis of variance (ANOVA). All data were analysed using STATISTICA CZ 12 software (StatSoft Inc.).

Table 1 Composition of the diet of dairy cows

Feed	Quantities of feed (kg)	DM (kg)
Grass silage middle blossom	34.00	12.58
Wheat grain	6.80	6.05
Corn grain cracked	0.50	0.44
Urea 45% N	0.12	0.12
Extracted rapeseeds meal [a]	4.70	4.42
Soybean meal 48	0.72	0.64
Limestone	0.35	0.35
Mineral supplement	0.35	0.33
Salt-white	0.08	0.08
Totals by weight	47.62	25.46

Legend: DM – dry matter; [a] glucosinolate concentration 18.1 mmol/kg;

Table 2 Concentration of iodine in various stages of the experiment

Stage of experiment	Iodine concentration (mg/kg DM)		Concentration of iodine in feed ration (mg/kg DM)
	Bulk and grain feed	Mineral supplement	
1st phase of experiment (2 months)	0.15	1.05	1.2
2nd phase of experiment (3 months)	0.15	0.45	0.6

Legend: DM – dry matter;

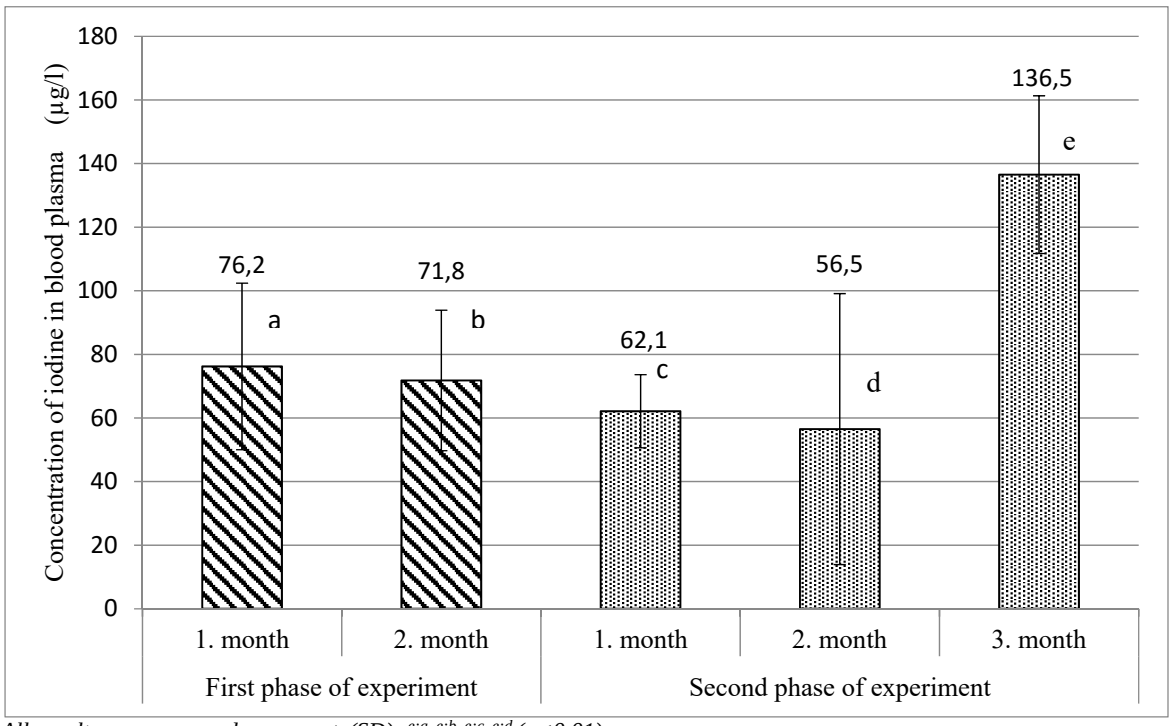
RESULTS AND DISCUSSION

The concentration of iodine in blood plasma samples

Physiological concentration of iodine in the plasma ranges from 40 µg/l to 110 µg/l (Špakauskas 2008). The average concentration of iodine in the blood plasma of dairy cows in our experiment

corresponds to sufficient iodine supply (Figure 1). The rise of iodine concentration ($p<0.01$) in the third month of the second phase of experiment (136.5 ± 24.8 mg/l) is propably related to the higher utilization iodine from organic sources (Bekeová 1998).

Figure 1 Concentration of iodine in blood plasma samples in 1st and 2nd phase of experiment

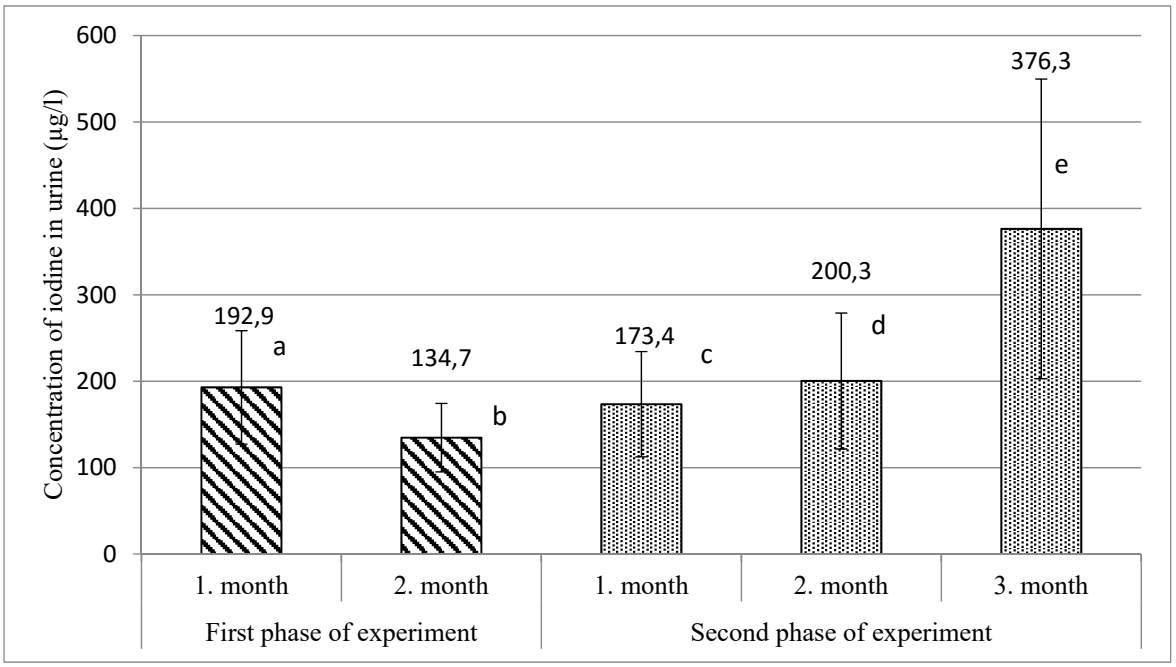


All results are expressed as mean ± (SD); e:a, e:b, e:c, e:d (p<0.01);

The concentration of iodine in urine samples

The dynamics of iodine concentration in urine is shown in Figure 2. The average iodine concentration in urine in the both phases of the trial (1st phase: 163.8 ± 61.5 µg/l, 2nd phase: 250.0 ± 146.4 µg/l), from diagnostic view, signalled satisfactory saturation of iodine in milking cows. According to Herzig et al. (1996) recommended values of iodine in excreted urine should be higher than 100 µg/l.

Figure 2 Concentration of iodine in urine samples in 1st and 2nd phase of experiment



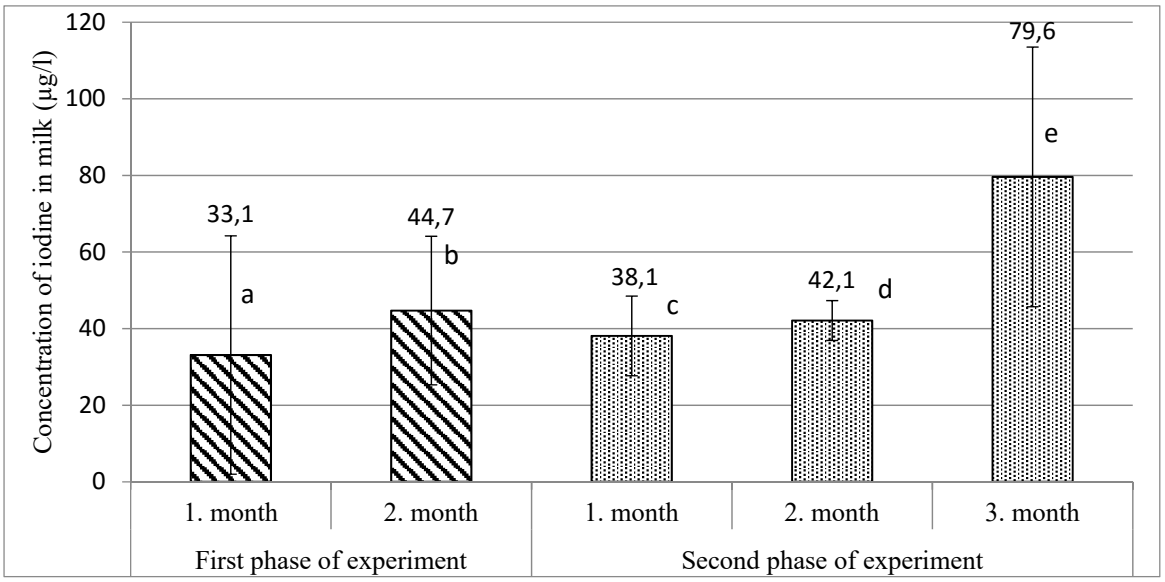
All results are expressed as mean ± (SD); e:a, e:c, e:d (p<0.05); e:b (p<0.01);

The concentration of iodine in milk samples

The physiological range of iodine concentration in milk is 100–200 µg/l (Schöne et al. 2009). The iodine milk concentration was under the physiological values during the whole experiment. According to Trávníček et al. (2011), the values of iodine concentration under 100 µg/l in milk related with low levels of the element intake. Low iodine values in milk indicate the negative effect of increased extracted rapeseed meal in feed ration on the iodine metabolism. Also Franke et al. (2009) proved that when feeding rapeseed oil, the iodine concentration in milk decreases by two thirds.

In the case of higher intake of rapeseed product in feed ration, it is recommended to increase the iodine to 2–3 mg/kg DM (Flachowsky et al. 2014). As our results show, it would also be possible to substitute inorganic iodine form by organic one (Figure 3).

Figure 3 Concentration of iodine in milk samples 1st and 2nd stage of experiment



All results are expressed as mean ± (SD); e:b(p<0.05); e:a, e:c, e:d (p<0.01);

CONCLUSION

Our results show that the increased intake of extracted rapeseed meal (glucosinolates) increases the secretion of iodine via kidneys instead of milk gland. For that reason, the diagnostic method of validation of the iodine concentration in urine or milk, as an iodine saturation parameter, level is considerably limited.

ACKNOWLEDGEMENTS

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SEPSIDAE (DIPTERA) ASSOCIATED WITH ANIMAL AND HUMAN DECOMPOSITION IN THE CZECH REPUBLIC

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Abstract: Applied research method is a combination of search retrieval and empirical part based on observing two experiments carried out between 2011 and 2012 and between 2012 and 2013. In all experiments model carcasses of pigs *Sus scrofa* f. *domestica* Linnaeus, 1758 were used. Pig carcasses meant to imitate the real crime scenes, substituting human corpses. Samples were collected from dead bodies and from nearby vegetation. The objective of the experimental part was to observe and annotate which species of Sepsidae family were present at given time and to elaborate on possible relation between their presence and phases of cadaver decomposition. During trials, altogether 15 195 adult specimens of family Sepsidae were collected which belonged to 15 species. The most abundant species was *Nemopoda nitidula* (Fallén, 1820) which larvae were collected from both carcasses. The experiment leads to broadening of the available knowledge about Sepsidae family and helped to verify applied data collecting methods.

Key Words: Sepsidae, forensic entomology, pyramidal trap, Acalyptratae, species identification

INTRODUCTION

Sepsidae family is currently understudied potential colonization interval indicator. Flies have small, ant resembling body and wing-waving habit, which is typical for the family. Species of Sepsidae family are located worldwide, there are 283 species in all (Meier 1996) and 31 of those are listed in the checklist of Diptera from the Czech Republic (Barták 2009). They are found mostly on excrements, cadavers and decaying vegetation which create environment for laying eggs and further larvae development (Pont and Meier 2002). Sepsidae family prefers bigger cadavers in advanced stage of decomposition to fresh cadaver (Wayne 1994, Byrd and Castner 2009). Smith (1986) states that Sepsidae spp. are occurred on approximately 3–6 months old body, but they could be found on the relatively fresh and on small cadaver (De Jong and Hoback 2006). There may be swarms of these flies at the crime scene, depending on its location (Gennard 2007).

In a forensic entomology context, several authors reported various Sepsidae species from animal decomposition studies and from human corpses. De Jong and Hoback (2006) documented *Themira putris* on rat carcass on day 7 of the trial. Adult Sepsidae flies were caught from rabbit and monkey carcass on 5th day of decomposition (Azwandi et al. 2013). Various species from Sepsidae family were collected during the study of the insects of buried human bodies (Motter 1898). Schoenly et al. (2007) compared colonization of pig carcass and human corpse. The results showed that Sepsidae flies were found equally on human bodies and on pig carcasses.

MATERIAL AND METHODS

Description of localities

The first experiment labelled as “Summer” took place in a fenced ground of the Police school in 9 – Hrdlořezy, the eastern suburb of the capital. The research site was open, sunlit place with grass, bushes and trees. Geographic coordinates: 50°5’22" N, 14°30’19" E; altitude: 240 m amsl.

The second experiment named as "Spring" was situated in a fenced experimental field of the Czech University of Life Sciences in Prague, in district Troja, the northern part of the capital. Research site was grass-covered, open and sunlit with fruit trees and bushes. The area was on west-facing slope near Vltava River, in a flooding zone. Geographic coordinates: 50°7'16" N, 14°23'53" E; altitude: 185 m amsl.

Both experimental sites were chosen as typical locations of founding dead human bodies in urbanized areas in the Czech Republic.

Description of experiments and collecting methods

The first experiment called "Summer" was based on using pig carcass as models for human decomposition. The pig was about 65 kg; it was killed on 13 July 2011 by a single shot to the front of its head with a 0.22 calibre rifle and moved to the research site within 20 minutes. Dead pig was foil-wrapped during its transport to avoid an earlier oviposition before reaching the site. Before exposition, the pig was dressed in a cotton shirt and overalls to imitate a common homicide crime scene. Pig was exposed on the research field on 13 July 2011; this day is day 1 of the experiment. The experiment was conducted from 13 July 2011 (day 1) to 18 October 2012 (day 464) until the carcass decomposition was finished. Samples were collected with insect net and pitfall traps. Control intervals were: day 1 to 17 once a day; day 17 to 62 once two to three days, day 62 to 195 once ten to fourteen days and day 195 to 464 once a month.

Pig used in the second experiment called "Spring" was about 53 kg and died by natural cases in the evening on 19 March 2012 and was stored in a cool room (at about 6 °C) to avoid an earlier oviposition. Day after the pig was transported to the research field and dressed in T-shirt and trousers. The experiment was conducted from 20 March 2012 (day 1) to 6 June 2013 (day 444) until a flood in Prague damaged the experimental site. Adult flies were collected over the pig with pyramidal trap (Barták and Roháček 2011) which was placed above the carcass for all time duration of the trial. The trap was situated 20 to 40 cm above ground or vegetation level to make accessible the carcass to insect, base of the trap was 2 x 2 m in size.

Head of the trap was filled with mixture: 1.5 l of water, 2 ml of 36–38% formaldehyde and 1 ml of detergent. Captured material was collected: day 1 to 197 once a week, day 197 to 267 every fourteen days, day 267 to 393 once a month and then until the end of the trial on day 444 again every fourteen days. The intervals corresponded with seasons.

Species identification

The identification of Sepsidae species was based on the identification key from monography The Sepsidae (Diptera) of Europe (Pont and Meier 2002), all specimens were identified by T. Olekšáková and M. Barták. Specimens of both families are deposited in collections of the Institute of Criminalistics in Prague (ICP).

Meteorological measurements

During both trials, daily (shade) air temperatures and ground surface temperature were measured. In Hrdlořezy ("Summer" experiment), air temperatures were determined with Volcraft DL-141TH (Volcraft) digital datalogger which was attached to an upright post approximately 1.5 m away from the carcass and 2 m above the ground level. It was enclosed in a waterproof casing and shielded from direct sunlight. During the experiment in Troja ("Spring"), data about air temperatures and ground surface temperatures were taken from a stationary weather station placed on the ground of the Czech University of Life Science which was located approximately 40 m away from the carcass.

RESULTS

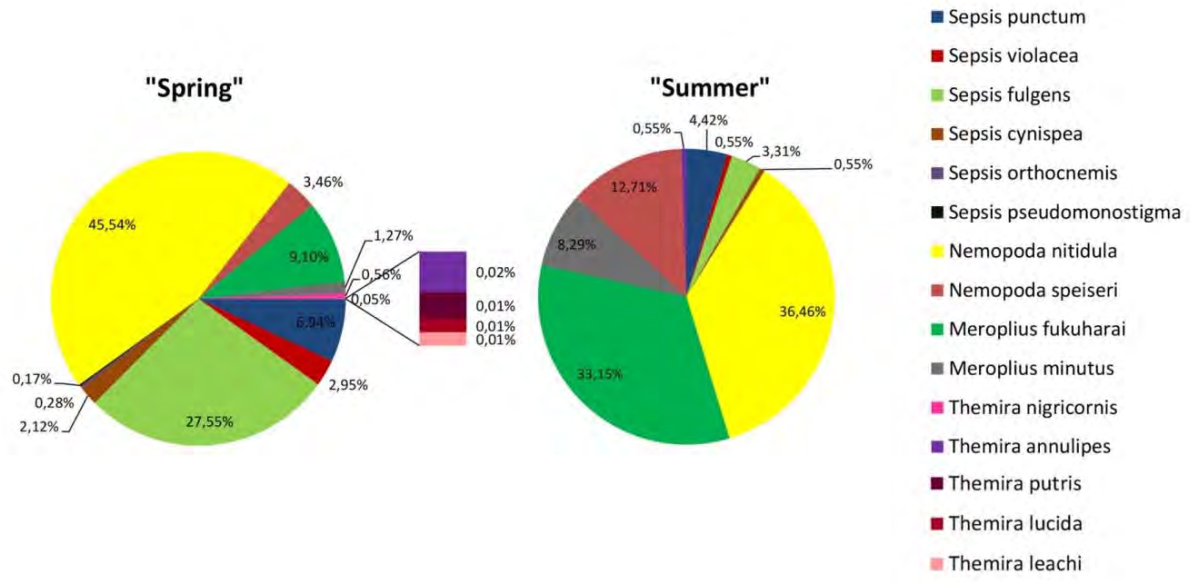
There were collected 181 adult specimens of the Sepsidae family during "Summer" experiment. In "Spring" trial 15 014 adult specimens were collected.

The "Summer" trial simulated colonization of a corpse, that started in the beginning of Summer. Altogether nine Sepsidae species were collected. These species were, in alphabetic order: *Meroplius fukuharai* (Iwasa 1984), *Meroplius minutus* (Wiedemann 1830), *Nemopoda nitidula* (Fallén 1820), *Nemopoda speiseri* (Duda 1926), *Sepsis cynipsea* (Linnaeus 1758), *Sepsis fulgens* (Meigen 1826), *Sepsis*

punctum (Fabricius 1794), *Sepsis violacea* (Meigen 1826) and *Themira annulipes* (Meigen, 1826). Most numerous species of the experiment was *Nemopoda nitidula*, which represented 36.46% (n = 66) of all samples (Figure 1). The first females arrived on the cadaver during the first day of the experiment and they were identified as *Sepsis punctum*. On the second day, *Nemopoda nitidula* females were found on the cadaver. Larvae collected from the carcass were reared out under laboratory conditions until adults, which were identified as *Nemopoda nitidula*.

The "Spring" trial simulated colonization of the corpse starting in early Spring. Fifteen species of the Sepsidae family were collected, in alphabetic order: *Meroplius fukuharai* (Iwasa 1984), *Meroplius minutus* (Wiedemann 1830), *Nemopoda nitidula* (Fallén 1820), *Nemopoda speiseri* (Duda 1926), *Sepsis cynipsea* (Linnaeus 1758), *Sepsis fulgens* (Meigen 1826), *Sepsis orthocnemis* (Frey 1908), *Sepsis pseudomonostigma* Urso 1968, *Sepsis punctum* (Fabricius 1794), *Sepsis violacea* (Meigen 1826), *Themira annulipes* (Meigen 1826) *Themira leachi* (Meigen 1826), *Themira lucida* (Staeger 1844), *Themira nigricornis* (Meigen 1826) and *Themira putris* (Linnaeus, 1758). Most numerous species of the experiment was *Nemopoda nitidula*, which represented 45.54% (n = 6838) of all samples (Figure 1). Various Sepsidae species were collected from the first day of the experiment. The larvae collected during the trial were reared out under laboratory conditions until adults, all specimens were identified as *Nemopoda nitidula*.

Figure 1 Percentage abundance of Sepsidae collected during "Spring" and "Summer" experiments



Mating behaviour was observed during both experiments. Mating on the cadaver was proved for following species: *Meroplius fukuharai*, *Themira nigricornis*, *Sepsis fulgens* and *Nemopoda nitidula*. The only species which developed entirely on the cadavers was *Nemopoda nitidula*.

DISCUSSION AND CONCLUSIONS

Altogether 15 species of Sepsidae family were collected during both experiments, representing 48.39% of all Sepsidae species known from the Czech Republic (Barták 2009).

There were a few species recorded during both experiments, which normally do not occur on cadavers, according to Pont and Meier (2002). Those species are: *Themira annulipes*, *Sepsis cynipsea*, *Sepsis violacea*, *Sepsis pseudomonostigma* and *Themira leachi*. All those were found during both trials, *Sepsis pseudomonostigma* and *Themira leachi* were found only during "Spring" trial. In any of the trials, there was no representative of *Nemopoda pectinulata*, which has similar distribution as *Nemopoda nitidula*, but *Nemopoda pectinulata* prefers colder environment (Pont and Meier 2002). *Sepsis orthonemcis*, *Sepsis pseudomonostigma*, *Themira annulipes*, *Themira putris*, *Themira lucida* and *Themira leachi* were detected in very small numbers (Figure 2, Figure 3); we assume that it was only a random capture.

Figure 2 Month by month percentage abundance of Sepsidae collected during "Spring" experiment

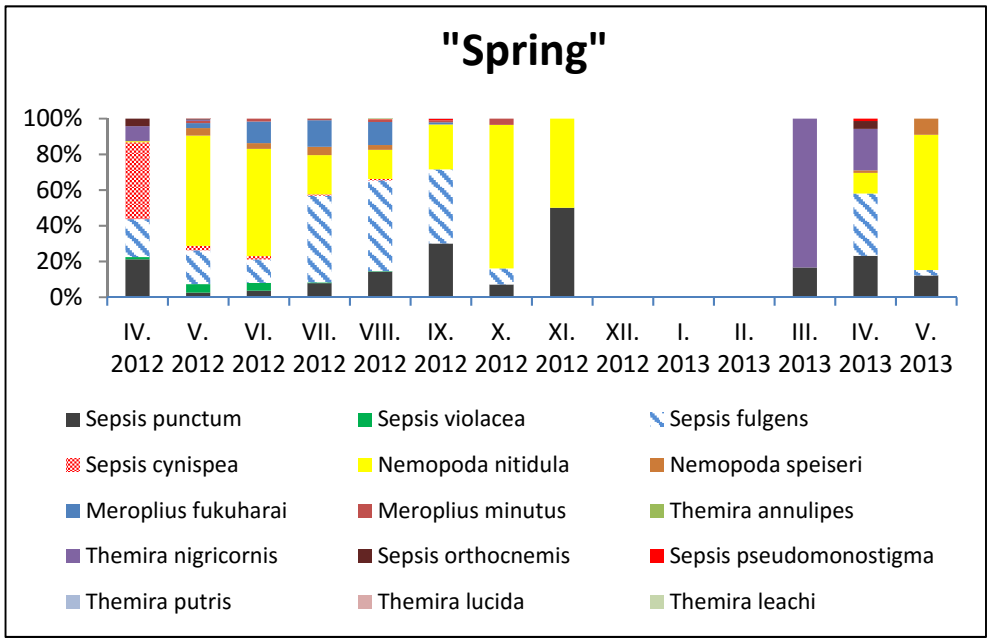
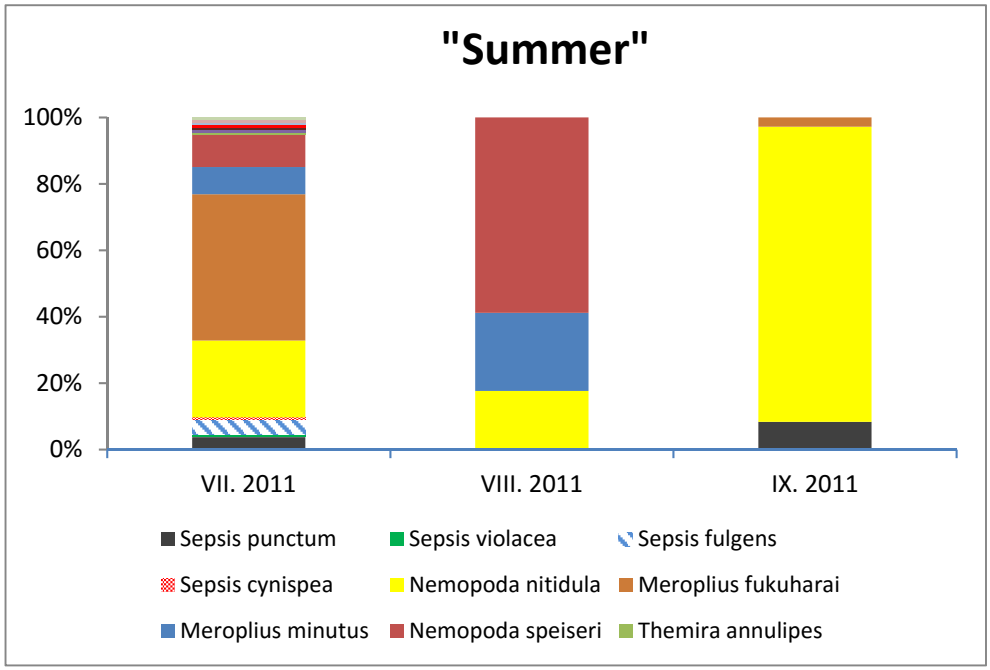


Figure 3 Month by month percentage abundance of Sepsidae collected during "Summer" experiment



Number of females and males for all species were compared. The highest difference between males and females showed *Nemopoda nitidula*. In "Spring" trial females represented 84.57% of all *Nemopoda nitidula* specimens, in "Summer" *Nemopoda nitidula* females represented exactly 75%. The highest incidence of females belonging to *Nemopoda nitidula* specie was recorded during the spring and early summer. In "Spring" trial, there was a periodic rise and fall of males, which we explains that for the most of the time was carcass visited by females for the purpose of laying eggs, and when the adults developed, the number of males temporarily increased (Olekšáková et al. 2014). For other species was proportion between males and females even, eventually males prevailed over females. *Nemopoda*

nitidula usually develops at vertebrate carrion (Van der Goot 1986) which was verified by successful rearing adults from puparia found on a dead pig.

Occurrence of mostly all species during the year corresponded with Pont and Meier (2002). In both trials, the most numerous species were *Nemopoda nitidula* (Figure 1), which is abundant in the Czech Republic (Barták and Vaněk 2009). Important is abundant occurrence of *Nemopoda speiseri*, which is considered as rare, according to Pont and Meier (2002), but it is occasionally collected from dead human bodies in the Czech Republic (Šuláková unpublished data). *Nemopoda speiseri* records are from June to August (Pont and Meier 2002). This does not correspond to results from "Spring" trial, when *Nemopoda speiseri* was presented from April to October (the first year of the experiment May to October, the second year April to May – see Figure 2) as well as to human corpses, when its living and active larvae were collected even at the end of December in an outdoor case with the beginning of the decomposition during July of the same year (Šuláková unpublished data).

In our opinion, most Sepsidae species are tightly bound to the season more than to the degree of cadaver decomposition, which was confirmed in "Spring" trial; a typical spring fly *Themira nigricornis* was captured first year of experiment in spring on fresh carcass, and one year later in the same period to nearly skeletonized carcass; similar incidence of spring species of Sepsidae confirmed Anton et al. (2011). The other Sepsidae species returned on the cadaver next year, regardless of its level of decomposition.

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EFFECT OF FISH OIL INTAKE ON PLASMA LIPIDS LEVEL IN RATS AND PIGS

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Abstract: The aim of the present study was to compare the effect of diet enriched with 2.5% fish oil (source of polyunsaturated fatty acids) and the effect of diet enriched with 2.5% palm oil (source of saturated fatty acids; control) on plasma lipids level. Two model animals were used: *Sus scrofa* and *Rattus norvegicus*. Levels of total plasma cholesterol, triacylglycerol, low-density cholesterol and high-density cholesterol were analysed by the enzymatic-colorimetric method. There were no significant differences between absolute values of the lipid fractions. Plasma lipid concentration in animals which was fed by diet enriched with fish oil was expressed like ratio of the plasma concentration in the other group of animals. In this case dietary fish oil decreased high-density cholesterol less ($P<0.01$), but low-density cholesterol and triacylglycerols more ($P<0.05$ and $P<0.001$) in rat plasma than in pig´s. However, used amount of fish oil added to diet was not able to improve plasma lipid markers in comparison with saturated palm oil.

Key Words: HDL-cholesterol, triacylglycerols, fish oil, *Sus scrofa*, *Rattus norvegicus*

INTRODUCTION

Dyslipidemia is one of the most serious risk factor of atherosclerosis which belongs among chronical civilization diseases that causes many premature deaths. Dyslipidemia is inter alia characterized by increased level of triacylglycerol (TAG), higher level of total plasma cholesterol (TC) and low-density cholesterol (LDLC) and decreased amount of plasma high-density cholesterol (HDLC). Levels of all stated lipids can be modified by dietary intake. There is great difference between two basic groups of fatty acids – saturated and unsaturated. Palm oil belongs to saturated fatty acids known due to its defective influence on health. On the other hand, polyunsaturated n-3 long chain fatty acids (PUFA n-3) have opposite effect. PUFA n-3 decrease plasma level of TAG and increase amount of HDLC (Balk et al. 2006). For example PUFA n-3 eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are known to decrease plasma TAG via activation of peroxisome proliferator-activated receptor α and inhibition of sterol response element-binding protein signalling pathways (Jump 2008). *In vivo* studies testing EPA and DHA effect on plasma lipids are usually carried out on rodents. However, rodents are not ideal models for humans according to differences in proliferation of peroxisomes (Komprda 2012). Our aim was to compare influence of fish oil intake on plasma lipids levels in pigs and rats. An intention was to carry out an experiment as similar to ordinary human conditions as possible.

MATERIAL AND METHODS

Animals, dietary interventions, analysed tissues

It was used to model animals: thirty-two male rats at the age of eight weeks with the average live weight of 312 ± 23 g (laboratory strain Wistar Albino; Meditox Konárovice, Czech Republic) and thirty-two pigs of both sexes (16 males, 16 females) at the age of eight weeks with the average live weight of 25.5 ± 1.15 kg (Large White x Landrace; Bioprodukt Knapovec a.s., Ústí nad Orlicí, Czech Republic).

The rats were bred in the plastic boxes of four animals in a room standard circumstances, temperature at 23 ± 1 °C, humidity of 60% and 12/12 h of light/dark cycle. The pigs were bred in floored indoor pens of four animals each.

The experiment was performed in compliance with the Czech National Council Act No. 246/1992 Coll. to protect animals against cruelty, the Amended Act No. 162/1993 Coll., and was approved by the "Commission to protect animals against cruelty" of the Mendel University in Brno and of the Ministry of Agriculture of the Czech Republic.

The rats and the pigs were divided into two groups of 16 animals: the experimental group was fed the basic feed mixture with 2.5% of fish oil (F) and the control group was fed the basic feed mixture with 2.5% of palm oil (P). An intention was to use dietary EPA and DHA in amount realistically achievable in human nutrition. The P-diet was used as a control. The animals had free access to the drinkable water and were fed daily *ad libitum* and the leftovers were weighed.

The fattening of rats and pigs lasted for ten weeks. Blood samples in rats were collected by cardiac puncture under anesthesia with isoflurane into the heparin-coated test tubes after the 12-h fasting at the end of the experiment. The pigs were anesthetized by the intramuscular application of the TKX mixture in the total volume of 0.2 mL/kg of the live weight and sacrificed by bleeding. Blood samples were collected (from the aorta) to the heparin-coated test tubes and the liver samples were taken. Both rat and pig blood samples were centrifuged at 200 g for 10 min at 4 °C to obtain plasma for the lipid fractions analysis.

Plasma lipids determination

TC, LDLC, HDLC and TAG values were determined by the enzymatic-colorimetric method using an automated chemical analyzer BS-200 (Mindray, Shenzhen, China) and commercial kits (Greiner Diagnostic GmbH, Bahlingen, Germany).

Statistical evaluation

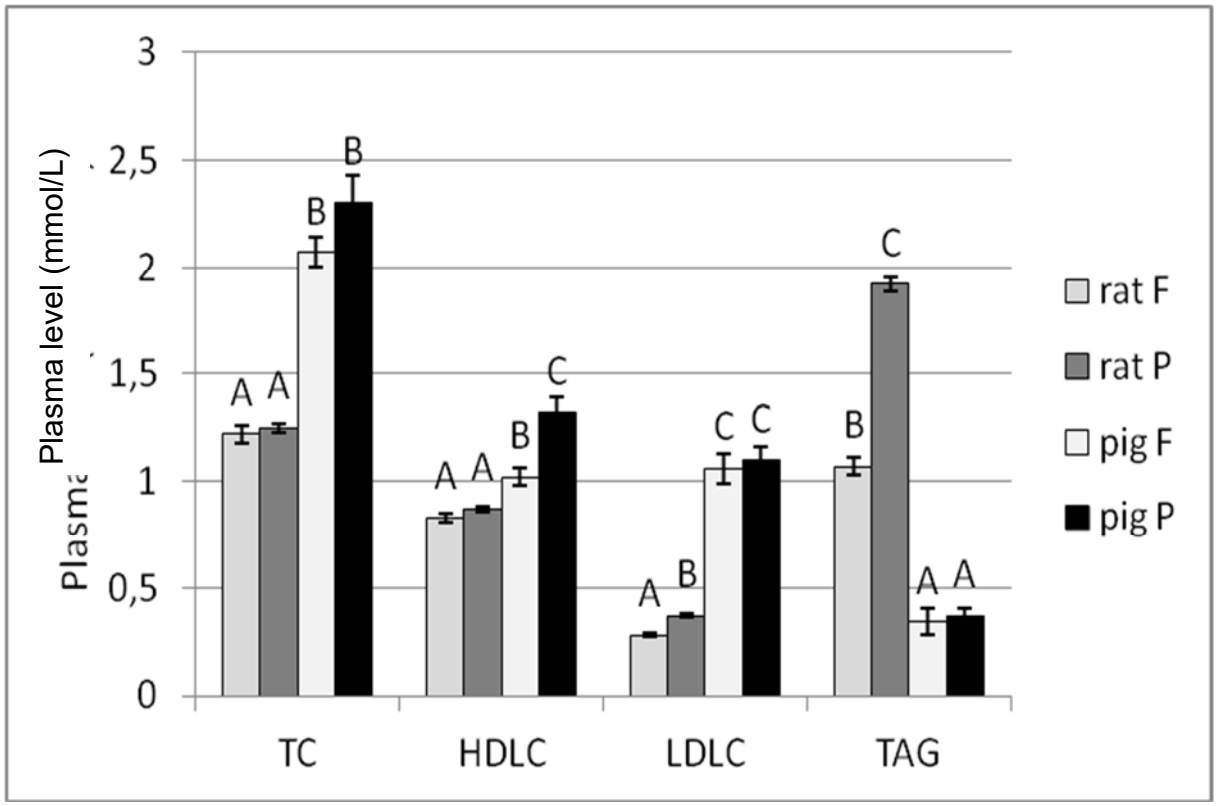
Normality of the data distribution was tested by Kolmogorov-Smirnov test. The comparison of the rat and pig model was realized on two levels. The contrasts in absolute amounts plasma lipid concentrations in rats and pigs fed the F- and P-diet were evaluated by one-way analysis of the variance ratio test, including *post-hoc* Tukey's test. In order to better recognize eventual inter-species differences, an effect of fish oil on plasma lipid concentrations was evaluated on a relative level. Values related the F-diet were expressed in a given species as percentages of the values measured using the control P-diet in this species. The rat and pig sets were compared using the independent samples t-test. For all evaluations, the STATISTICA 12 package (StatSoft, Tulsa, OK, USA) was used.

RESULTS AND DISCUSSION

The comparison of rats and pigs from the aspect of plasma cholesterol and TAG levels as influenced by the diet including either fish oil or palm oil is displayed in Figure 1. The pigs had higher concentration of total plasma cholesterol (TC) than the rats (nearly twice in average, $P<0.05$) regardless of the dietary intervention (the type of added oil in the diet affected TC neither in rats nor in pigs, $P>0.05$). As far as the absolute values of the lipid fractions in the rat plasma are concerned, fish oil did not affect TC in comparison with control ($P>0.05$) not only in the present study, but also in the experiments of Campioli et al. (2012) and Yamazaki et al. (2011).

A similar trend was also established as far as the cholesterol fractions are concerned. The pig samples had higher ($P<0.05$) concentration of HDL-cholesterol and LDL-cholesterol in plasma compare with the rats. Above that, fish oil in comparison with the control palm oil decreased ($P<0.05$) HDL-cholesterol in the pig plasma, but not in the rat plasma. Contrary result was established as far as LDL-cholesterol is concerned: dietary fish oil decreased ($P<0.05$) LDLC compared with the control palm oil in the rat plasma, but not in the pig plasma ($P>0.05$). No significant difference in HDLC between F- and control rats in the present experiment agrees with the results of Campioli et al. (2012) and Yamazaki et al. (2011), who used as a control a diet with olive oil and a standard feed mixture.

Figure 1 Concentration of total cholesterol (TC), high-density-lipoprotein cholesterol (HDLC), low-density-lipoprotein cholesterol (LDLC) and triacylglycerols (TAG) in plasma of rats and pigs fed the diet with 2.5% of fish oil (F) and palm oil (P); one-way analysis of the variance ratio test with post-hoc Tukey's test; n = 16; A, B, C – means with different letters within a given trait differ at P<0.05.



Contrary to our results, Takahashi (2011) reported a decrease of HDLC from 1.56 mmol/L in palm oil-fed rats to 0.58 mmol/L in the fish oil group. Dietary fish oil decreased both TC and HDLC in the rat plasma in comparison with control safflower oil also in our previous experiment (Komprda et al. 2015), where the diets contained 6% of the particular oil.

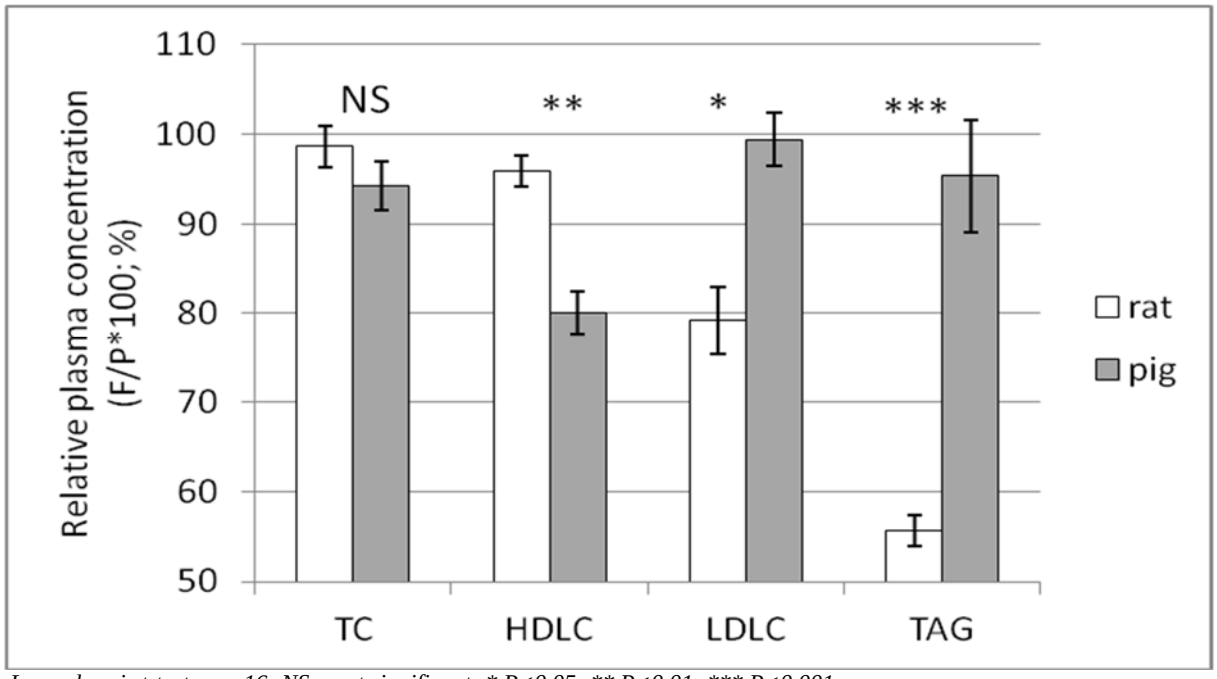
Contrary to rats, dietary fish oil in comparison with palm oil decreased ($P<0.05$) HDLC in pigs in the present experiment (Figure 1). Taken from the opposite viewpoint, a surprising ability of saturated fat to increase the favourable HDLC fraction in pigs reported also Puccinelli et al. (2015) after feeding a diet with 20% of lard either continuously or intermittently: HDLC increased from 0.62 mmol/L (control diet) to 1.19 and 2.28 mmol/L, respectively (compare with the values of 1.02 and 1.32 mmol/L in the F- and P-pigs in the present experiment; Figure 1).

Plasma TAG levels were in average more than four-times lower in pigs in comparison with rats ($P<0.05$). Moreover, type of dietary oil had varying effect on the tested animal species: dietary intervention did not affect plasma TAG in pigs ($P>0.05$), but fish oil in the diet decreased ($P<0.05$) plasma TAG in the rats to 56% of the established level when the control diet with palm oil was fed.

An indifference of plasma TAG to the type of dietary oil found in the present study in pigs (contrary to rats; Figure 1) confirms the results of Puccinelli et al. (2015), though the quoted authors used different fats than in the present study with different objectives.

A collation of the tested animal species concerning an effect of fish oil relative to an effect the control palm oil is presented in Figure 2. As far as total plasma cholesterol is concerned, no differences between rats and pigs were established ($P>0.05$). More favourable effect of fish oil on rats is apparent from Figure 2 concerning cholesterol fractions. Plasma lipid concentration in the F-animals was expressed as a ratio of the plasma concentration in the P-counterparts. In this case dietary fish oil decreased the fraction of HDL-cholesterol less ($P<0.01$), but the fraction of LDL-cholesterol more ($P<0.05$) in rats than in pigs. Moreover, fish oil relative to control palm oil decreased plasma TAG in rats substantially more than in pigs ($P<0.001$; Figure 2).

Figure 2 Concentration of total cholesterol (TC), high-density-lipoprotein cholesterol (HDLC), low-density-lipoprotein cholesterol (LDLC) and triacylglycerols (TAG) in plasma of rats and pigs fed the diet with 2.5% of fish oil (F), relative to the corresponding values established with feeding the control diet with 2.5% of palm oil (P), respectively;



*Legend: pair t-test; n = 16; NS – not significant; * P<0.05; ** P<0.01; *** P<0.001.*

Generally speaking, plasma lipid level established in the present experiment in pigs is more similar to humans than to rats; porcine model can therefore be considered superior in the given context. However, using this model, fish oil submitted at a reasonably achievable dose (2.5%; EPA+DHA ingested at an amount of ca 80 mg per kg of live weight and day) was not able to improve plasma lipid markers in comparison with saturated palm oil.

CONCLUSION

The purpose of the present study was to determine the effect of the diet enriched with 2.5% fish and palm oil, respectively on plasma lipids level of animal models (*Sus scrofa* and *Rattus norvegicus*). We focused on amount of total plasma cholesterol, triacylglycerol, low-density cholesterol and high-density cholesterol, all of them were analysed by the enzymatic-colorimetric method.

From the point of view of the absolute levels of plasma lipids no significant differences were found between diets, which is not unique result. The consumption of diet enriched with fish oil decrease of HDLC in pig plasma was observed but not in rat one. Diet enriched with palm oil increased levels of LDLC and TAG in rat plasma but not in pig plasma. Plasma lipid concentration in animals which was fed by diet enriched with fish oil was expressed like ratio of the plasma concentration in the other group of animals. In this case dietary fish oil decreased HDL-cholesterol less ($P<0.01$), but LDL-cholesterol and triacylglycerols more ($P<0.05$ and $P<0.001$) in rats than in pigs. The used amount of added fish oil is not able to improve levels of plasma lipids as compared with palm oil.

ACKNOWLEDGEMENTS

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THE STUDY OF COLOUR GENES SEQUENCES IN CHINCHILLA (*CHINCHILLA LANIGERA*) BASED ON HOMOLOGY OF HUMAN AND MICE SEQUENCES

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Abstract: In domesticated animals there are many different coat colors and mutations, often connected with pleiotropic effects. The aim of this thesis named The study of color genes sequences in chinchilla based on homology of human and mice sequences was describe by molecular-genetic principles of pigmentation, introduce genes involved in melanogenesis and influencing a melanin function, their structure, mutations and mention other mutations which change the phenotype. Information about alleles *TYR, TYRP1, TYRP2/DCT, agouti, AGRP*, gene group *MCR* gene group (*MC1R-MC5R*) and more are focused on human (*Homo sapiens*), mouse (*Mus musculus*) and chinchilla (*Chinchilla lanigera*). In these three species selected sequences of genes *TYR* and *TYRP2/DCT* were compared.

Key Words: chinchilla lanigera, coat color, color mutation, human, melanogenesis, mouse, *TYR, TYRP2/DCT*

INTRODUCTION

Wild animals in nature have a hair color that makes it as inconspicuous as it is possible and blend in with the surroundings for its safety. By domestication there were start to appeal animals with more noticeable hair color, more colorful or patterns and these populations expanded by their attractiveness (Alderton 2011, Ziegler 2014). Genetic variations and mutations which affect final phenotype also changed with this variance. Results of mutations can show attractive hair color or pattern of an animal, but which can may carry some negative consequences because of connection of some mutations with pleiotropic effects, frequently occurring in mammals (obesity, reproductive disorders, deafness, skin sensitivity etc.) (Reismann and Ludwig 2013, Snustad 2009). In human, gene mutations affecting pigmentation mostly cause albinism or red hair (Montoliu et al. 2014). In mice, these gene mutations in addition to albinism cause lighter hair color then wildtype phenotype, color patterns and numerous pleiotropic effects (Ito and Wakamatsu 2011, Beerman et al. 2004).

For location of these mutations of genes there were need to come through the basic molecular-genetic principles of mammal hair pigmentation and mention important genes included in melanogenesis, process of creation of melanins (eumelanin and phaeomelanin) and in metabolic pathways which affect these processes. These genes are mainly *ASIP, AGRP, TYR, TYRP1, DCT/TYRP2, MC1R, P/OCA2, PMEL17* and *SLC45A2*, which have homologues in mice (in the same order): agouti, Agrp, albino, brown, slaty, extension, pink-eyed dilute, silver and underwhite loci (Slominski et al. 2004, MGI 2016). Mouse is used as a model organism in general for research in different specializations and even in this thesis was used to compare sequences of genes affecting pigmentation in mice, human and chinchilla to make a conclusion, how much are genomes of these three mammals similar. Specifically investigated genes were tyrosinase (*TYR*) and *DCT*/*TYRP2* (dopachrome tautomerase/tyrosine related protein 2) because of their very high importance in all of the processes.

MATERIAL AND METHODS

In theoretic part of the thesis the molecular and genetic base of pigmentation, melanogenesis and major genes which affect hair and skin color in human and mice are extensively described. Experimental

part of the thesis compares sequences of genes tyrosinase (*TYR*) and dopachrom tautomerase (*DCT/TYRP2*) in three species of mammals: human (*Homo sapiens*), mouse (*Mus musculus*) and chinchilla (*Chinchilla lanigera*). There were found appropriate sequences, which were suitable for comparison, especially sequences of coding DNA (cDNA) in mRNA form in FASTA format using databases NCBI and Ensemble. In case of chinchilla, appropriate parts of whole genome sequence, where is probably located *Tyr* (ChiLan1.0 scaffold00013) and *Dct* (ChiLan1.0 scaffold00003) and predicted mRNA of these genes were found. Further, for chinchilla there was found exact location of all exons of each gene, which is part of a results. For this and for comparison were used databases of NCBI and Ensembl. Sequences of *TYR* and *DCT* in human and mouse were compared together gradually using program BLAST („The Basic Local Alignment Search Tool"), specifically a megablast method (finds highly similar sequences). It is an online tool located on NCBI server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for searching similar regions of biologic sequences. It compares nucleotide or protein sequences and calculates statistic indexes (similarity, gaps added by the program) of the accord lines. This tool can be used to assess functional and evolutional relationships between sequences or it can help to identify gene family's representatives. Program was evolved by Zhang et al. (2000) team. Multiple sequence alignment was made by program Clustal Omega on server EBI (http://www.ebi.ac.uk/Tools/msa/clustalo/), where has been made a survey how similar are the investigated sequences. Program was evolved by Li et al. (2009) team. Compared parts of genome were studied in detail, analogy of human, mouse and chinchilla sequences were carefully examined, further mutations and polymorphisms with influence on skin and hair pigmentation in human and hair color in mice were highlighted. NCBI was an excellent tool and source of information interwoven with Ensembl database, which provided a lot of information with great graphic elaboration and simple orientation in the system.

RESULTS AND DISSCUSION

In the experimental part of the thesis we compared sequences of *TYR* and *DCT* gene of the three studied species, first, gradually between them (human – mouse, human – chinchilla, mouse – chinchilla) to find out, how much the sequences similar to each other are. The program evaluated their similarity graphically and statistically. Finally, a multiple sequence alignment for all of the three studied species of mammals together to show the potential similarity was executed.

Gene *TYR* is by NCBI located on 11th chromosome in human, on 7th chromosome in mouse and in chinchilla is unknown, because of no determination of the genomic location. It was found that *TYR* gene contains 5 exons in all the three species of mammals, based on evaluation of the mRNA sequences by the program and there were added the gaps in the regions where were probably missing bases against the comparing sequence by the program. Result is shown in the Table 1. In Table 2 is exact location of *TYR* exons in whole genome chinchilla genome. It was found by comparing whole genome shotgun and predicted mRNA of *TYR* gene. Result of comparison shows that predicted mRNA is placed on the opposite strand against the whole genome shotgun. Finally, was made multiple sequence alignment of sequences of all the three kinds of mammals together, where is not made statistical result, but based on results of each comparing we can say, that the multiple alignment is identical to 83–85%. Further it was found, that match begins with starting codon AUG of the first exon in all the three species of mammals and continues by 4 following successive exons.

Table 1 Evaluation of TYR mRNA similarity in three studied species of mammals

Comparison	Similarity (%)	Added gaps
Human – mouse	84	21 (16 – human, 5 – mouse)
Human – chinchilla	85	9 (6 – human, 3 – chinchilla)
Mouse – chinchilla	83	21 (6 – mouse, 15 – chinchilla)

Table 2 Exact location of exons in TYR gene in chinchilla

Number of exon	Exon beginning	Exon ending
1	4314001	4313183
2	4302731	4302510
3	4286254	4286096
4	4254596	4254412
5	4244545	4244035

Gene *DCT* is by NCBI located on 13th chromosome in human, on 14th chromosome in mouse and in chinchilla is unknown as *TYR* gene. It was verified, that the gene contains 8 exons in all of three species of mammals. Process was the same like an analysis of *TYR* sequences similarity and result is shown in Table 3. There is the exact location of *DCT* exons in whole chinchilla genome in Table 4. Results show that gene is located on the same strand as whole genome shotgun, which is conversely than result of *TYR* gene. Finally, it was made multiple sequence alignment and, based on results of each comparing, we can assume, that the sequences are identical to 82–86%. Sequence of human and mouse gene are identical in the first exon including the non-coding part. Match of all the three sequences begins by the starting codon of coding part of the first exon and continuous by all 8 following successive exons.

Table 3 Evaluation of DCT mRNA similarity in three studied species of mammals

Comparison	Similarity (%)	Added gaps
Human – mouse	83	26 (10 – human, 16 – mouse)
Human – chinchilla	86	6 (3 – human, 3 – chinchilla)
Mouse – chinchilla	82	10 (9 – mouse, 1 – chinchilla)

Table 4 Exact location of exons in DCT gene in chinchilla

Number of exon	Exon beginning	Exon ending
1	15359402	15359696
2	15368621	15368919
3	15371477	15371577
4	15372255	15372420
5	15375371	15375550
6	15377101	15377245
7	15390063	15390264
8	15395358	15395556

CONCLUSION

Molecular-genetic basic principles of hair color are identical in all species of mammals. However, genes which affect pigmentation are located in different parts of the genome. Those genes which are the most included in processes of melanogenesis and operation of pigments are analyzed in the thesis. Result of gene analysis by online tools shows gene sequence similarity in the three studied species. Human and mouse genomes and sequences of genes are already well studied, thus localization of genes is known.

But in chinchilla nobody has analyzed the genome and genes for pigmentation yet, so there are available just predicted gene mRNA and probably localized genes. In chinchilla there are not known even mutations of the genes, which are plentiful in human and mouse and affecting final phenotype. Based on high similarity of the sequences of genes we can assume that even in chinchillas occur similar mutations like in human and mouse. After a rigorous detailed study of genes affecting hair color of chinchillas those polymorphisms could be identified in the future and then compared with mutations in mice, what can be the basis for determination of genetic principle of the color mutations in chinchillas.

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MORPHOLOGICAL STRUCTURES IN GERMINAL EPITHELIUM AFTER IMPROVAC APPLICATION IN PIG

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Abstract: Immunocastration of pigs is an alternative way to prevent the presence of boar taint, especially skatole which cause negative deviation of meat quality. The vaccine Improvac actively immunizes against gonadotropin – releasing hormone (GnRH). It triggers a cascade of hypotalamic – pituinary – gonadal axis actions during sexual maturation and contributes to the development of spermatogenesis. This inhibition leads to significant changes in testicular tissue, both structural and functional. Testes undergo changes effecting reproduction in addition to other indicators, such as boar taint. For comprehensive understanding of this topic is needed specifically specify changes in testis tissue and possible impacts in other economically interesting tissues. In the experiment 10 boars were treated with the vaccine Improvac with two subcutaneous applications. The testes were processed by standard histological techniques. Testes tissue have showed significant morphological changes in seminiferous tubules and interstitial space. Null or decreased spermatogenesis were performed in germinal tissue and atrophy of Leydig cells leaded to inhibition of steroidogenesis.

Key Words: immunocastration, testicles, histological structure, light micropscopy

INTRODUCTION

Today castration of pigs is a frequent topic professional public. There are three possible ways how to prevent boar taint in porcine meat: untimely slaughter, surgical castration or immunocastration by immunization against gonadotrophin – releasing hormone (GnRH). The most discussed has been surgical castration, which presents problem in term of welfare (Brunius et al. 2011). On the basis of physiological and etological parameters is known that surgical castration presents a painful treatment, even is done in young animals (Kubale et al. 2013).

Current European legislation (Council Directive 2008/120/EC laying down minimum requirements for the protection of pigs) authorize the implementation of castration only qualified persons, ie. veterinarians or trained persons (European union 2008). Surgical castration is performed in young animals – without anesthesia and can be performed within 7 days after birth, after this period must be used during surgical castration anesthesia and subsequent pain relief. Opinion of the Scientific Panel on Animal Health and Welfare Animal European Food Safety Authority (EFSA) in 2004, however, indicates that surgery castration is painful at any age. That's why many European countries have decided to surgery castration retreat and replace it with other, non-painful methods. One of the first steps is the introduction of analgesia or anesthesia to alleviate pain, which has been used since 1 January 2012. The next step, to which European countries committed themselves to the phasing out of surgical castration. This way prevention of boar taint should stop using until 1 January 2018 respectively (EFSA 2004).

For this reason, there are alternative ways to approach prevention of boar taint (Batorek et al. 2012). One of these methods is immunocastration with vaccine Improvac, which is beeing examined in this work. Introduction of new methods in practice requires comprehensive knowledge of the physiological status of the animal. The effect of the vaccine is directed to eliminate boar taint, but primarily affects the histological structure of testes (Brunius 2011). Therefore, the aim of this study was histological analysis of the germinal epithelium of the pig testes treated with the vaccine Improvac.

MATERIAL AND METHODS

Animals

The total of 10 male pigs was included in the study. Rearing animals were carried out in accredited stables of Veterinary Research Institute in Brno (VRI, Czech Republic).

Experimental design

Experimental boars were administered with two consecutive Improvac vaccine in the range of 43 to 46 days, wherein the second vaccine was administered 2–4 months before slaughter. Age at slaughter ranged from 22–29 weeks. Live weight before slaughter ranged from 78.8 kg to 100.2 kg.

Examination post mortem

The experimental material was collected by trained staff from authorized and registered slaughterhouse at VRI. Samples were collected from the testicle tissue among the *mediastinum testis* and *tunica albuginea* in the range of 10 mm x 10 mm x 10 mm to 10% formalin. The samples were processing according to standard histological techniques as are processing, embedding, sectioning and hematoxilin-eosin staining protocol (Bancfort et al. 2008). Sections were mounted in permanent preparations. The histological slides were evaluated using the light microscope (Olympus BH–2, Japan) a morphometry was determined after digitizing microscope slides (camera Canon EOS 1100D, Japan) using software (Quick Photo Micro 3.0, PROMICRA, Czech Republic).

The total of 20 histologic slides were analysed, each slide had 5 sections and evaluated in duplicate magnification 100x and 400x. Following parameters were evaluated : diameter of coiled seminiferous canal, height germinal epithelium, number and nucleocytoplasmatic index of Leydig cells, the presence of mature spermatids in the lumen of the seminiferous coiled duct (Kubale et al. 2013).

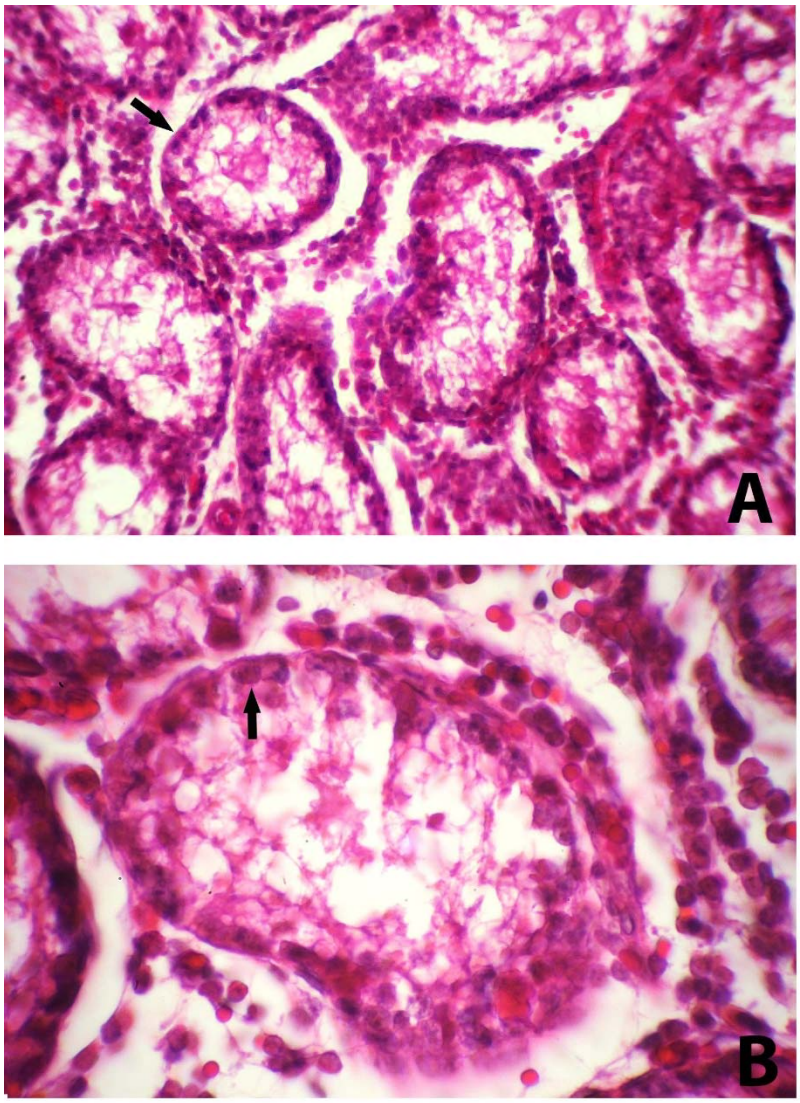
RESULTS AND DISCUSSION

The testicular histology in vaccinated male pigs was clearly affected. The data obtained from histological sections showed the distribution of the seminiferous tubules in the testes tissue in cross section, with a density 3–5 tubules per 100 μm^2. Individual variation was presented in testicular morphology among pigs. Important factor is length of period since vaccination to slaughter. Seminiferous tubules do not contain all the developmental stages of spermatogenic cells. Significant changes are noticeable in interstitial space and on morphology of Leydig cells.

Germinal epithelium

Conclusive decrease in area of seminiferous tubules was observed on diameter 50 µm ± 10 µm in the samples taken from animals administrated with immunovaccine 2 months before slaughter. Spermatogenesis was inhibited, as evidenced by the absence of higher stage of development of spermatogenic cells into the lumen of the seminiferous tubules. On preparations only spermatogonias and non distinctly Sertoli cells to the basement membrane were visible, see figure 1B. Lumen was not formed inthe center of seminiferous duct. Apical poles of Sertoli cells fill the entire ducts. From this description it is obvious that the seminiferous coiled tubules cannot contain spermatids, which undergo metamorphosis in mature sperm. For this reason it is difficult to measure height of germinal epithelium, even if it is not mature. On the other hand testicles tissue from animals administrated with vaccine 4 months before slaughter have showed recovery of spermatogenic function, the same conclusion reached Kubale et al. (2013). Their diameters were 150 µm ± 30 µm and all levels of developmental stadium were presented, see figure 2D. On the other hand in the study of Einarsson et al. (2011) was indicated long-lasting disrupting effect on histological status in male pigs vaccinated as early as at 10 and 14 weeks of age.

Figure 1 Histological structure of testes tissue after immunocastration with Improvac vaccine



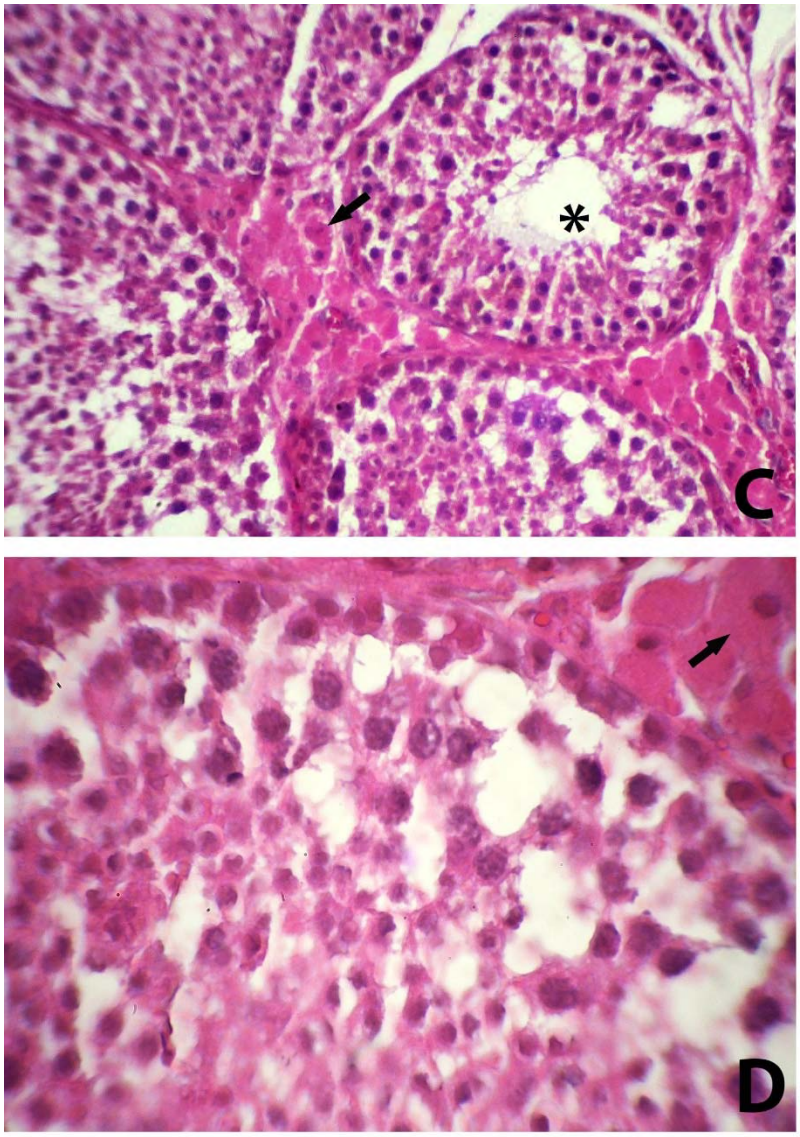
Legend: Light microscopy photographs of testicular tissue of immunocastrated pigs. Figure ***„A“*** *shows seminiferous coiled tubules of male pig administrated with second vaccine of improvac 70 days before slaughter in magnification 100x. No lumen of seminiferous tubuli and reduced interstitial space, number and size of Leydic cells were presented.* ***„B“*** *Spermatogenesis was disrupted here, only spermatogonias as a first developmental stadium and Sertoli cells were observable in magnification 400x.*

* *Structure of histological sections are slightly disrupted by processing of technical process.*

Interstitial space

In non – castrated pigs interstitial space contains Leydig cells, which are responsible for the production of testosterone and perform the main function of the trigger formation of male sex cells. Improvac vaccine clearly disrupted the number and morphology of the interstitial Leydig cells. Hilbe et al. (2006) have evaluated Leydig cells as well as atrophic. The size of the interstitial space in immunocastrated males has been significantly reduced. The Leydig cells lost their pycnotic-like nuclei and they were difficult to distinguish from the other interstitial structures. Nucleocytoplasmatic index of Leydig cells is noticeably altered. The volume of the cytoplasma decreased, see figure 1A, on the other hand tissue with renewed spermatogenesis has showed specific polygonal shape of Leydig cells which represent their activity, see figure 2C.

Figure 2 Histological structure of testes tissue after immunocastration with Improvac vaccine



Legend: All stadiums of spermatogenesis were showed at figure ***„C“*** *in magnification 100x and the lumen* * *of seminiferous tubuli is presented here. The tissue sample was teken from pig immunocastrated with the second vaccine 111 days before slaughter. The arrow marks Leydig cells with their specific polygonal shape.* ***„D“*** *Spermatogonia cells adjacent to the basement membrane, primary nad secondary spermatocytes and spermatids were distinguishable in magnicifation 400x.*

* *Structure of histological sections are slightly disrupted by processing of technical process.*

CONCLUSION

This histological study has showed clear signs of atrophy in the immunized animals. At the morphological level this alternative method of castration has proved the effectiveness and applicable in practice. Based on examination the significant effects of the vaccine is believed to be temporary as appeared spontaneous spermatogenesis in testicular tissue of pigs vaccinated with 4 month interval. The temporal boundaries of recovery process are not known. The question remains how long after immunocastration production of skatole is inhibited even if the spermatogenesis is resumed?

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STABILITY OF REFERENCE GENES ESTIMATED BY REAL-TIME PCR IN PORCINE LIVER

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Abstract: It is essential for the method real-time PCR to control variables by validation of normalization of the data. The aim of this study was to develop a set of reference genes for relative quantification of mRNA expression in the porcine liver. The mRNA stability of expression was studied for five genes: *GAPDH, HPRT1, PPIA, TOP2B, TBP1*, where for each gene the Ct value characterized the level of expression in liver. With the help of geNorm range of stability of analysed genes was (from the most stable to the least): *PPIA, TBP1, TOP2B, GAPDH, HPRT1.*

Key Words: real-time PCR, pig, gene expression, reference gene

INTRODUCTION

Real-time PCR is an efficient method for studying quantification of gene expression in tissue. This method is sensitive, fast and has precise measurement of examined material in the sample (Gachon et al. 2004). However, number of variables should be controlled such as precise determination of the amount of starting material, differences in transcriptional activity and presence of inhibitors in different samples (Nygart et al. 2007). One of essential steps in gene expression analysis is validation of normalization of real-time PCR data (Svobodová et al. 2008). Normalization of the data is usually gained by comparison of expression profiles of studied genes with constitutively expressed genes known as reference genes (Lee et al. 2007). Since the reference gene is exposed to the same preparation steps as the gene of interest, this normalisation adjusts for any differences in the amount of starting material, RNA isolation and cDNA synthesis (Nygart et al. 2007).

Five reference genes was selected for validation of their stability in porcine liver: *GAPDH, HPRT1, PPIA, TOP2B, TBP1*. The most commonly used reference gene is *GAPDH*, which brings good results in many studies but in others it is not recommended for its variability of expression (Kozera and Rapacz 2013). *HPRT1* gene is also commonly used reference gene, but doubted by some authors as stable reference gene (Erkens et al. 2006). *TOP2B* and *TBP1* and their gene expression stability was previously analysed by Erkens et al. (2006). Studies has shown suitability of *PPIA* as reference gene in other species (Pérez et al. 2007) and this gene is also used as a reference gene in pigs.

In the present study the expression stability of five reference genes has been compared in porcine liver. This has enabled to estimate the suitability of these genes for normalisation of gene expression in porcine liver.

MATERIAL AND METHODS

Samples collection and isolation

Samples were collected from four male hybrid pigs from the right part of liver. Samples were immediately submerged in RNAlater (Qiagen, Hilden, Germany). Total RNA was extracted using RNeasy Plus mini kit (Qiagen, Hilden, Germany). One µg of total RNA was reverse trascribed at 42 °C using Quantitec reverse transcription kit (Qiagen, Hilden, Germany) with elimination of genomic DNA.

Relative quantitative PCR with SybrGreen

Standard curve was measured for each primer pair individually. Reaction for qPCR was prepared using Power Sybr® Green master mix (ThermoFisher scientific, Waltham, USA) in triplicate for each

sample and for non-template negative control. Reaction consisted of 1 µl of cDNA, 10 pmol/µl of each primer, 10 µl of Sybr Green, 0.2 µl of AmpErase® Uracil N-glycosylase (UNG) (ThermoFisher scientific, Waltham, USA), 8 µl of RNase-free water in total volume of 20 µl. The qPCR was run on Rotor gene (Qiagen, Hilden, Germany) with cycling conditions 1 cycle of denaturation at 95 °C/10 min, followed by 40 cycles of 95 °C/10 min and 60 °C/1 min. This was followed by melting curve for verification of specifity of PCR products. Used primers are shown in table 1.

Table 1 Details of primers used for analysis.

Gene symbol	Oligo sequence (5´→ 3´)	Amp. length	E (%)	Ref. seq.	Author
GAPDH	CAGCAATGCCTCCTGCACCA GATGCCGAAGTGGTCATGGA	70	80	AF141959	Svobodová 2011
HPRT1	AAGGACCCCTCGAAGTGTTG CACAAACATGATTCAAGTCCCTG	122	85	NM_001032376	Svobodová et al. 2008
PPIA	CTGAGTGGTTGGATGGCAAA CCACAGTCAGCAATGGTGATCT	130	79	NM_214353	Svobodová 2011
TOP2B	CTAATGATGCTGGTGGCAAAC CCGATCACTCCTAGCCCAG	100	89	AF222921	Svobodová et al. 2008
TBP1	AACAGTTCAGTAGTTATGAGCCAG A AGATGTTCTCAAACGCTTCG	153	99	DQ845178	Nygart et al. 2007

Legend: Amp. length – amplicon length, E – primer efficiency, Ref. seq. – reference sequence

Data Analysis

Mean of *Ct* (cycle of threshold) values were measured using Rotor gene software and were converted into input data for geNorm application. The geNorm algorithm is based on the principle of identical expression ratio of the 2 most stable reference genes in all samples. The obtained *M* value is the average pairwise variation of a particular gene with all other control genes (Vandesompele et al. 2002).

RESULTS AND DISCUSSION

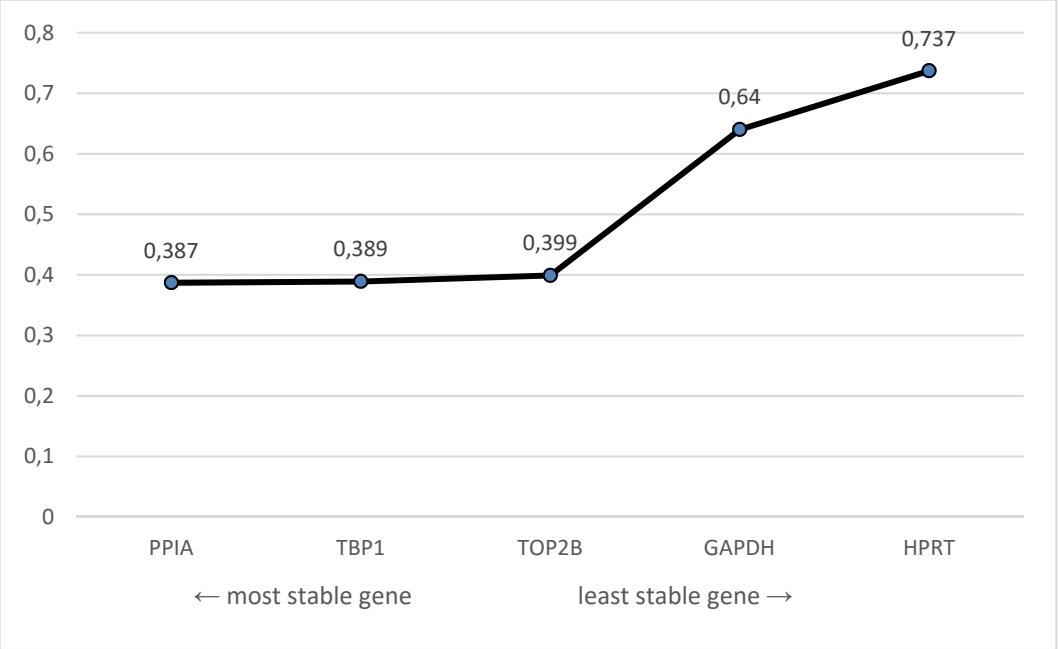
Five commonly used reference genes was selected (table 1). Efficiency of the qPCR amplification of analysed genes was between 79–99%. Average *Ct* values of *GAPDH, HPRT1, PPIA, TOP2B* and *TBP1* genes at each sample are shown in table 2.

Table 2 Average Ct values of analysed samples for genes GAPDH, PPIA, HPRT1, TOP2B and TBP.

sample	*GAPDH*	*PPIA*	*HPRT1*	*TOP2B*	*TBP1*
1	18.53	14.25	15.38	21.43	21.68
2	18.74	14.68	15.62	22.47	22.65
3	17.20	14.03	16.04	21.52	21.57
4	18.67	14.85	16.87	22.46	22.50

M values were obtained using the geNorm algorithm. The range of expression stability in analysed genes was (from the most stable to the least stable): *PPIA, TBP1, TOP2B, GAPDH* and *HPRT* with *M* values: 0.387, 0.389, 0.399, 0.640 and 0.737 (Figure 1).

Figure 1 Reference gene mRNA expression stability in porcine liver according to geNorm (M values)



The least stable gene in our study was *HPRT1*. This result is in disagreement with Nygart et al. (2007), who suggested that the gene *HPRT1* is good reference gene for low abundant transcript expression studies, but is in agreement with Erksen et al. (2006), who studied stability of gene expression in porcine back fat and *longissimus dorsi* muscle and found this gene unstable in his study. The second least stable gene is *GAPDH* witch result is in agreement with Svobodová et al. (2008), who studied stability of the reference genes in several porcine tissues, and Erksen et al. (2006). Our data suggest that the most stable reference genes for liver are *PPIA*, *TBP1* and *TOP2B*. These results are in agreement with findings in study of Erksen et al. (2006) who indicated for normalisation to use *TOP2B* and *TBP1*. Also Gu et al. (2011) recommends *TOP2B* as one of the most stable genes in several porcine tissue samples. Park et al. (2014) studied stability of reference genes in four pig breeds and several tissue samples and suggested as appropriate reference genes in three pig breeds genes *PPIA* and *TBP* and in fourth breed genes *PPIA* and *TOP2B*, which is in agreement with our findings.

According to Vandesompele et al. (2002) expression results are considerably more reliable when they are normalized using the geometric mean of multiple reference genes. Recommended number of reference genes for normalisations is three, because as GeNorm analysis indicates adding third reference gene to the normalisation factor had large impact on reducing variability (Erksen et al. 2006). Also expression stability of reference genes varies among different tissues and different breeds so it is important to test reference genes before analysing gene of interest.

CONCLUSION

Our study provides recommendation for the choice of endogenous control genes in mRNA expression studies in porcine liver tissue. We have investigated expression stability of five genes (*GAPDH, HPRT1, PPIA, TOP2B, TBP1*) in the porcine liver. Three of the investigated genes (*PPIA, TOP2B, TBP1*) were found to be the most stable in studied tissue, therefore we recommend using this genes as endogenous control in real-time reverse transcription PCR analysis for gene expression in porcine liver. Genes *GAPDH* and *HPRT1* were found out to be the least stable in our study.

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EFFECT OF DIETARY FISH OIL ON SELECTED MARKERS OF AN INFLAMMATORY STATUS IN PIGS

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Abstract: The objective of the experiment was to test a hypothesis that the biologically active substances present in fish oil (eicosapentaenoic acid, EPA, and docosahexaenoic acid, DHA) are able to stabilize inflammatory status in an organism. Thirty two pigs (Large White x Landrace) at the age of eight weeks with the mean live weight of 25.5 kg were used as a model organism. The pigs were divided into two groups with 16 animals each; the experimental and control group was fed the basic feed mixture with 2.5% of fish oil (F) and 2.5% of palm oil (P), respectively. The F – and P – pigs were randomly divided into two groups 70th day of fattening, and eight F – and eight P – pigs were treated with *E. coli* lipopolysaccharide (LPS). After anesthetizing, pigs were sacrificed by bleeding, the blood and liver samples were taken, and expression of the liver genes coding for 7 selected cytokines and plasma concentration of adiponectin and three cytokines was determined. No significant effect ($P > 0.05$) of dietary intervention on feed intake, live weight and live weight gain was found. Fish oil tended to increase ($P > 0.05$) relative expression of all tested cytokine genes, the effect being significant ($P < 0.05$) in the case of *IL – 6* and *TGF – β1* after LPS application. Fish oil also increased ($P < 0.05$) plasma concentration of *TNFα* in pigs treated with LPS. On the other hand, fish oil tended to decrease ($P = 0.22$) plasma adiponectin in comparison with palm oil. The present study did not confirm anti-inflammatory effect of fish oil.

Key Words: cardiovascular diseases, eicosapentaenoic acid, docosahexaenoic acid, cytokines, adiponectin

INTRODUCTION

Dietary docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), present in high quantities e.g. in fish oil, are able to modulate, among other things, chronic low-grade inflammation (Calder 2013), one of the hallmarks of atherosclerosis, which is a basis of cardiovascular diseases. EPA and DHA are endogenous ligands of the transcription factor peroxisome proliferator-activated receptor gamma (PPARγ). PPARγ ligation increases an amount of adiponectin, adipose tissue anti-inflammatory hormone (Siriwardhana et al. 2013). Anti-inflammatory effect of EPA/DHA is further mediated by a G - protein coupled receptor-sensor GPR120, whose activation leads to a repression of the macrophage induced inflammation (Flock et al. 2013). This repression is caused by inhibition of the signaling pathway of the transcription factor NF – κB (nuclear factor kappa B) (Calder 2012). Positive effects of EPA/DHA were mostly obtained by *in vitro* studies using higher-than-physiological EPA/DHA concentrations (Yates et al. 2014).

The objective of the present study was to use pigs as a model organism for testing a hypothesis that fish oil is able to stabilize inflammatory markers. Due to the fact that the length of the experiment was limited, it has not been possible to induce low-grade chronic inflammation status in experimental animals. Therefore a more robust intervention via lipopolysaccharide (LPS) intervention at the end of the experiment was necessary. The rationale behind the tested hypothesis was that fish oil (i.e. EPA and

DHA) increases plasma adiponectin and decreases nuclear fraction of the transcription factor NF – κB with a consequence of modulation of the pro- and anti-inflammatory cytokine plasma levels.

MATERIAL AND METHODS

Animals, dietary interventions, analyzed tissues

Thirty-two pigs of both sexes (16 males, 16 females; Large White x Landrace) (Bioprodukt Knapovec a.s., Ústí nad Orlicí, Czech Republic) at the age of eight weeks with the mean live weight of 25.5 ± 1.15 kg were used. The pigs were housed in an experimental stable in floored indoor pens (10 m^2) of four animals each.

The experiment was performed in compliance with the Czech National Council Act No. 246/1992 Coll. to protect animals against cruelty, the Amended Act No. 162/1993 Coll., and was approved by the "Commission to protect animals against cruelty" of the Mendel University in Brno and of the Ministry of Agriculture of the Czech Republic.

The pigs were divided into two groups with 16 animals each: the experimental group was fed the basic feed mixture with 2.5% of fish oil (F) and the control group was fed the basic feed mixture with 2.5% of palm oil (P) (amount which the animals are able to consume according to their weight). Both F and P diet contained in one kg 138 g of crude protein, 56 g of fat, 48 g of crude fibre and 758 g of nitrogen-free extractives. Metabolizable energy content was 13.6 MJ/kg.

Content of quantitatively and physiologically important fatty acids in the F and P diet was as follows (g/kg): 14:0 0.92 and 0.96; 16:0 6.79 and 14.78; 18:0 1.30 and 1.73; 18:1 9.56 and 13.97; 18:2n-6 11.55 and 10.61; 18:3n-3 0.88 and 0.77; 20:5n-3 2.83 and 0.05; 22:5n-3 0.58 and 0.04; 22:6n-3 4.34 and 0.05, respectively. The animals had free access to the drinking water and were fed daily *ad libitum*. The fattening lasted for 70 days.

Lipopolysaccharide (LPS) treatment

Last (i.e. 70th) day of fattening the F– and P – pigs, respectively, were randomly divided into two groups of eight animals each. *E. coli* LPS at an amount of 25 μg/kg of live weight (W) was applied *i.v.* (*vena auricularis*) to eight F – and eight P – pigs. Three hours after the LPS application (time sufficient for measuring the effect before dead of animals could occure), all pigs were anesthetized by the intramuscular application of the TKX (12.5 mg/ml ketamine, 12.5 mg/ml xylazine, 12.5 mg/ml tiletamine, 12.5 mg/ml zolazepam) mixture and sacrificed by bleeding.

Blood and liver sample collection

Blood samples were collected (from the aorta) to the heparin-coated test tubes and the liver samples (300 g) were taken. Blood samples were centrifuged at 200 g for 10 min at 4 °C to obtain plasma. Aliquots of the liver samples (100 g) were freeze-dried and stored at -20 °C for subsequent fatty acid analyses. Total RNA was immediately isolated from another liver aliquots (50 mg).

Fatty acid analysis

Fatty acids in the liver samples, and in the diet were determined (after total lipid extraction by the hexane/2-propanol mixture) using the procedure described in our previous study (Komprda et al. 2016a).

Quantification of cytokine genes expression

Total RNA isolation, reverse transcription and quantitative PCR was performed according to Komprda et al. (2016a).

Determination of plasma cytokines and plasma adiponectin

IL – 1β, IL – 10 and *TNFα* concentration in the pig plasma was measured by Milliplex^R MAP Porcine Cytokine/Chemokine Magnetic Bead Panel kit (Millipore Corporation, Billerica, MA, USA) according to the producer's recommendation.

Plasma adiponectin was determined by Porcine Adiponectin ELISA (BioVendor, Brno, Czech Republic) according to the producer's recommendation.

Statistical evaluation

Normality of the data distribution was tested by Kolmogorov–Smirnov test. The differences between dietary interventions were evaluated by the one-way ANOVA including the *post-hoc* Tukey's test and by the independent samples t-test (sets with a normal distribution, i.e. all data sets except gene expressions), and by the non-parametric Wilcoxon signed-rank test (data sets concerning relative expression of the liver and adipose tissue genes), respectively. For all evaluations, the STATISTICA 12 package (StatSoft, Tulsa, OK, USA) was used.

RESULTS AND DISCUSSION

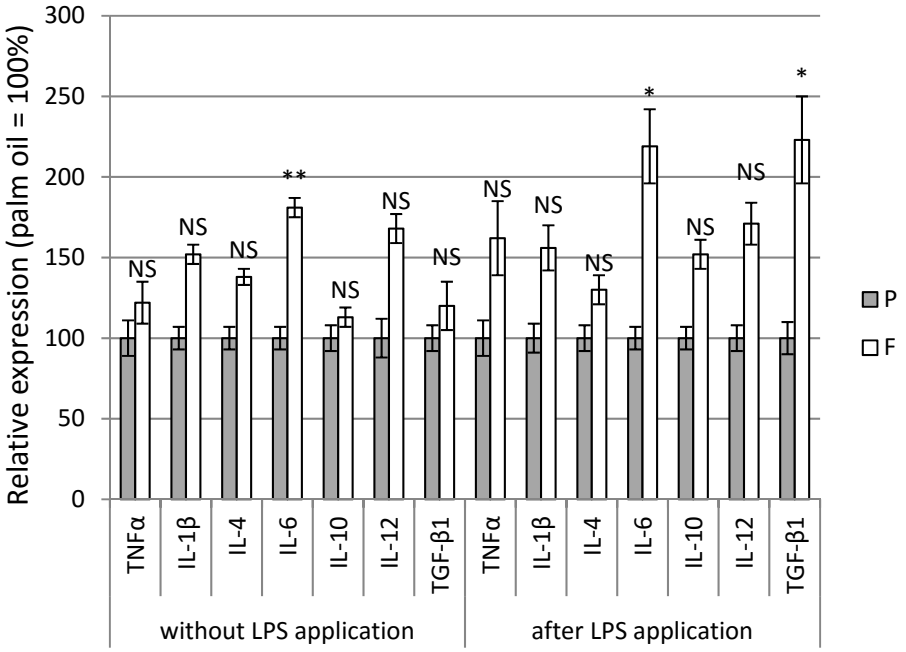
Feed intake, live weight, daily weight gain

Feed intake of pigs fed the F– and P – diet was 880 and 890 g/day; corresponding values expressed per kg of live weight (W) were 10.5 and 10.6 g · kg/W/d, respectively. Daily weight gain and the final live weight of the F– and P – pigs was 0.85 ± 0.05 kg/day and 0.86 ± 0.04 kg/day, and 83.64 ± 1.82 kg and 84.06 ± 3.35 kg, respectively. No significant effect of the type of dietary oil on any of the above-mentioned traits was established ($P > 0.05$).

Expression of the cytokine genes

Relative expression of the liver genes coding for selected pro- and anti-inflammatory cytokines is presented in Figure 1.

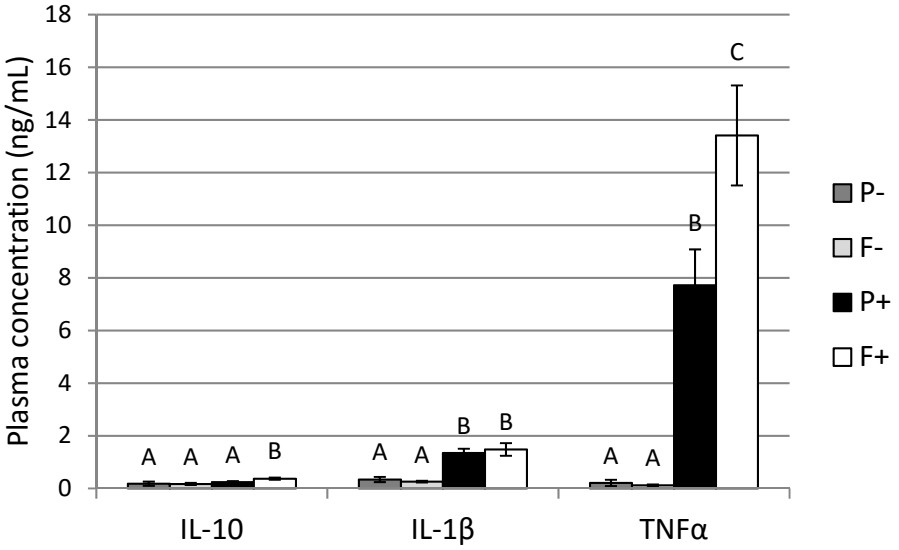
*Figure 1 Expression of the genes coding for selected pro- and anti-inflammatory cytokines in the liver of pigs fed a diet supplemented with 2.5% of fish oil (F) relative to expression of these genes in the control pigs fed a diet with 2.5% of palm oil (P) [mean ± standard error of the mean; n = 8]; NS – not significant; * P < 0.05; ** P < 0.01*



Plasma cytokine and adiponectin levels

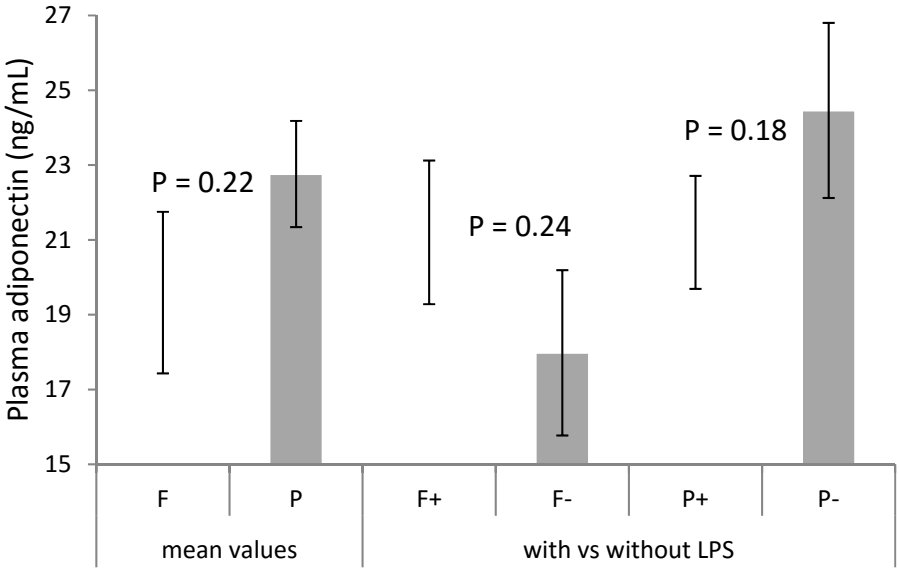
Plasma concentration of selected pro-inflammatory (IL – 1β; TNFα) and anti-inflammatory (IL – 10) cytokines is shown in Figure 2. An increase of TNFα, one of the acute phase proteins in the plasma of the F – pigs after LPS application is contrary to the hypothesis of a fish oil anti-inflammatory effect. In corresponding human studies, level of pro-inflammatory cytokines (IL – 6, TNFα) was low and not affected by EPA and DHA in obese patients in an experiment of Labonté et al. (2013); fish oil had no effect on TNFα and IL – 6 in overweight subjects (Bragt and Mensink 2012). No effect of EPA and DHA on IL – 1β, IL – 6 and TNFα in healthy volunteers reported Skulas–Ray et al. (2011).

Figure 2 Concentration of selected cytokines in the plasma of pigs fed a diet supplemented with either 2.5% of fish oil (F) or 2.5% of palm oil (P); values measured before (F-; P-; n = 8) and after (F+; P+; n = 8) the LPS application, respectively, are presented; A, B, C – means with different letters within a given trait differ at P < 0.05



Regarding adiponectin, fish oil tended (P = 0.22) to decrease plasma adiponectin in comparison with palm oil. Again, this finding is contrary to the tested hypothesis, and is also contrary to our previous finding in rats (Komprda et al. 2016b). On the other hand, as compared to the status before the LPS application, fish oil tended (P = 0.24) to increase plasma adiponectin after the LPS application, contrary to palm oil (Figure 3).

Figure 3 Adiponectin level in the plasma of pigs fed a diet supplemented with either 2.5% of fish oil (F) or 2.5% of palm oil (P); either mean values irrespective of the lipopolysaccharide (LPS) application (F; P; n = 16) or the values measured before (F-; P-; n = 8) and after (F+; P+; n = 8) the LPS application, respectively, are presented; NS – not significant



CONCLUSION

The tested hypothesis was the influence of fish oil, respectively biologically active substances EPA and DHA, on stabilization of inflammatory status in organism. In our study this hypothesis wasn´t proved.

ACKNOWLEDGEMENTS

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THE INFLUENCE OF ZINC NANOCOMPLEXES ON ANTIOXIDANT POTENTIAL OF THE ORGANISM

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Abstract: The aim of our experiment was to find out if a new forms of zinc based on nanotechnology, can increase the antioxidant state in laboratory rats. The male of outbred *Wistar albino* rats strain were used in this experiment. The rats were sorted out to the three groups. In each of group were stabled 6 males. The first control group (Control) (n = 6) was not fed with higher doses of zinc. The second group (ZnEDT) of rats (n = 6) was fed with zinc nanoparticles chelated by ethylenediaminetetraacetic acid (EDTA) (200 mg/kg of diet). The third group (ZnNTA) of rats (n = 6) was fed with zinc nanoparticles chelated by nitrilotriacetic acid (NTA) (200 mg/kg of diet). After 15 days of zinc nanoparticles exposure, the rats were sacrificed. Immediately, the samples of blood and liver were analyzed. As markers of oxidative potential of organism were used antioxidant activity determined using FR method, level of superoxiddismutase and methallothionein and finally, content of total zinc determined by atomic absorption spectrometry (AAS). The nanozinc supplementation shows the influence on studied parameters. Our results proved the rat organism is sensitive on the used zinc nanocomplexes.

Key Words: zinc nanoparticles, antioxidant status, rat, blood, liver

INTRODUCTION

Zinc (Zn) is an important essential trace mineral involved in protein synthesis, carbohydrate metabolism and many other biochemical reactions, it affects all cellular functions (Ao et al. 2011). It is necessary for growth, immune system and disease resistance, wound healing, fertility, metabolism (Zhao et al. 2014). Zinc is essential to the structure and function of numerous proteins which are classified as regulatory, structural and enzymatic (Swain et al. 2016). Zinc deficiency is considered to cause an increased oxidative stress that leads to damage of biomolecules including DNA (EFSA 2014). Zn is considered as a cofactor or component of more than 240 enzymes and influences oxidative processes. Cunningham-Rundles et al. (1990) showed that Zn acts as an antioxidant to reduce cell membrane damage due to free radicals. It has been demonstrated that Cu-Zn-SOD is involved in cellular scavenging of free radicals (Zhao et al. 2014).

In the last decade, nanotechnology has been widely used in animal husbandry to improve the utilization of trace elements in animal diets. Among metal nanoparticles annually produced, nano zinc oxide (nano-ZnO) is the third highest globally produced nano metal after nano SiO_2 and nano TiO_2 (Piccinno et al., 2012). In the animal body, nano minerals interact more effectively with organic and inorganic substances due to their larger surface area (Zaboli et al. 2013). Reports have pointed out the growth promoting, antibacterial, immuno-modulatory and many other beneficial effects of nano zinc. It may be used at lower doses in animal feed to provide better results than the conventional Zn sources due to highly bioavailable (Swain 2016). Compared with zinc oxide (ZnO), nano-ZnO has a stronger chemical activity, oxidation reactions and the permeability of nano-ZnO can help avoid adverse gastrointestinal reactions (Zhao et al. 2014). Nano minerals have the capability to cross the small intestine and further distribute into the blood, brain, lung, heart, kidney, spleen, liver, intestine and stomach (Wang et al. 2008).

The aim of our experiment was to determine the influence of new forms of zinc based on nanotechnology, to antioxidant state of *Wistar albino* rats.

MATHERIAL AND METHODS

Animals

The experiment was carried out in experimental facility on the Department of Animal Nutrition and Forage Production, Faculty of Agronomy, Mendel University in Brno. All test were done in accordance with the law to protect animals against cruelty (number 246/1992 Coll.).

Microclimatic conditions in laboratory limited by temperature were measured using DATALOGGER S 3120 (Comet system, Czech Republic). The temperature was kept at 23 °C ± 1 °C. The humidity was controlled using DATALOGGER S 3120 (Comet system, Czech Republic) and kept at 60%. The photoperiod was driven according to scheme: 12h/day and 12h/night with maximal intensity 200 lx.

The male of outbred *Wistar albino* rats strain were used in this experiment. The males are usually used in feed experiments because they have better growth performance then females. The average weight of each animal was 235 ± 3g. The male rats were stabled on plastic cages with grates. The experimental animals had free access to food and water *ad libitum.* The rats were sorted out to three groups. In each of group were stabled 6 males. The first control group (Control) (n = 6) was not fed with higher doses of zinc. The second group (ZnEDT) of rats (n = 6) was fed with zinc nanoparticles chelated by ethylenediaminetetraacetic acid (EDTA) (200 mg/kg of diet). The third group (ZnNTA) of rats (n = 6) was fed with zinc nanoparticles chelated by nitrilotriacetic Acid (NTA) (200 mg/kg of diet). All tested groups of rats were fed a mono-diet containing kibbled wheat (total amount of zinc 32.2 mg/kg/DM.). After 15 days of zinc nanoparticles exposure, the rats were sacrificed. Immediately, the samples of blood and liver were analyzed. According to the available literature 15 days is sufficient for the measured parameters.

Preparation of zinc nanoparticles

Zn-EDT

$ZnCl_2 + H_4EDTA + 4NaOH$

The solution of zinc chloride was added H_4EDTA under stirring on a magnetic mixer. The pH was adjusted to 7 by addition of NaOH. The solution was diluted to the volume of 100 ml (0.5 g Zn/100 ml).

Zn-NTA

$ZnCl_2 + Na_3NTA$

The solution of zinc chloride was added nitrilotriacetic acid disodium salt under stirring on a magnetic mixer. The pH was adjusted to 7.16 by addition of NaOH. The solution was diluted to the volume of 100 ml (0.5 g Zn/100 ml).

Determination of oxidative potential

As a markers of oxidative potential of organism were selected antioxidant activity determined using FR method, level of superoxiddismutase and methallothionein and finally content of total zinc was determined by atomic absorption spectrometry (AAS) according to Horký et al. (2016), Horký et al. (2013).

Statistical analyses

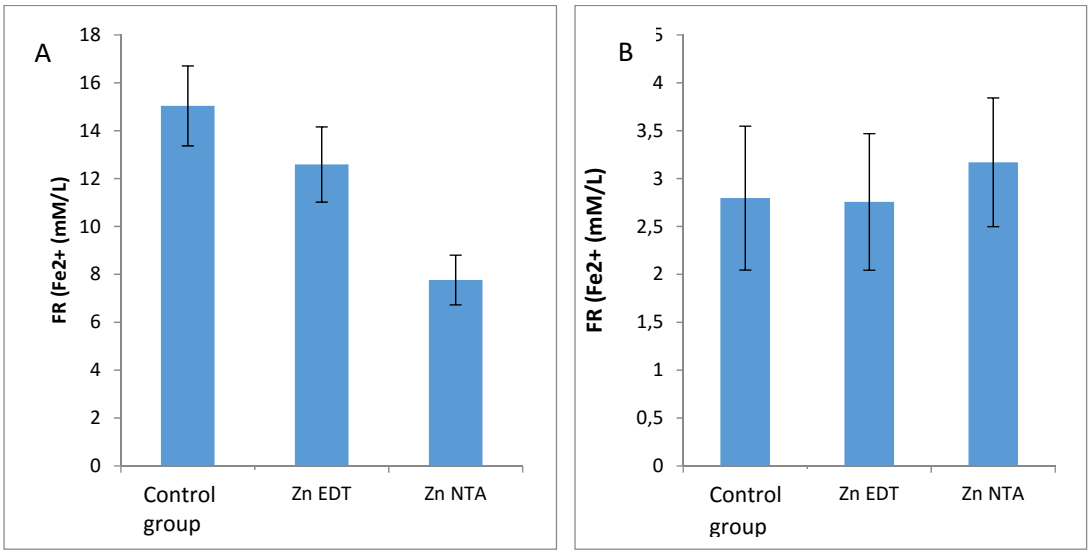
The data were statistically processed using STATISTICA.CZ, version 10.0 (Czech Republic). The results were expressed as a mean from 6 measurements ± standard deviation. Statistical significance was determined by the examining of basic differences among groups using ANOVA and Scheffé's method for the parameters FR; SOD; MT; AAS. The differences with $P < 0.05$ were considered as significant.

RESULTS AND DISCUSSION

In the experiment, the influence of nano-Zn on the antioxidant status of the rats was observed. Whole blood and liver were analyzed.

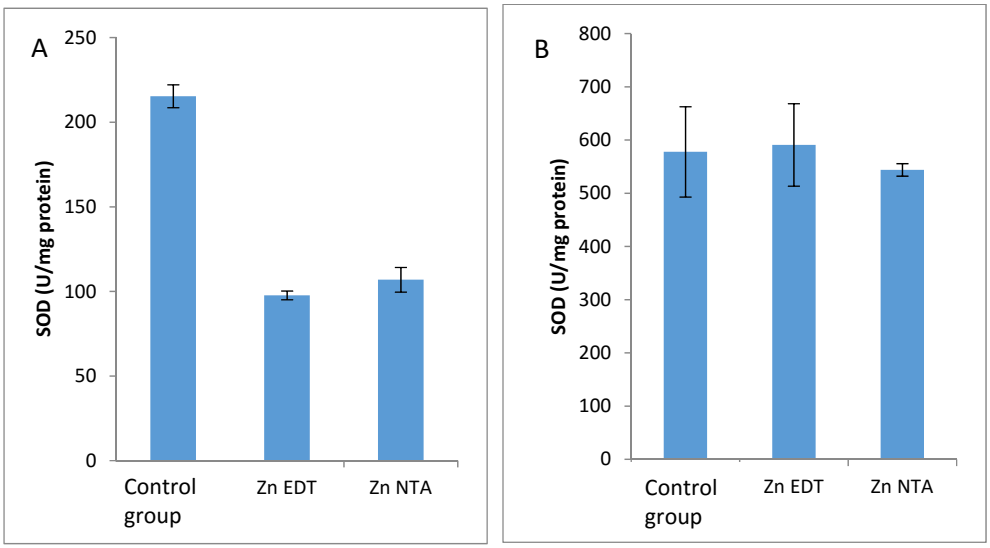
Antioxidant activities were measured by FR method. The antioxidant activity in blood samples (Figure 1A) decreased of 16% in the case of ZnEDT group and 48% in the case of ZnNTA in contrary with control group. In liver (Figure 1B) there were not estimated differences between ZnEDT and control group. The level of antioxidant activity was increased up to 13% in contrary to control group. Similar results have been achieved in the study of antioxidant capacity evaluated by FR method. The antioxidant activity was increased in the trial Horký et al. (2013). The evaluation of antioxidant activity in the blood samples was estimated significant differences between experimental ZnNTA group and control group. However, the significant differences between control group and ZnNA group were not estimated. Similar results have been achieved in experiment Horký et al. (2016a).

Figure 1 The influence of Zn nanocomplexes in A) blood, B) liver on antioxidant activity determined by FR method



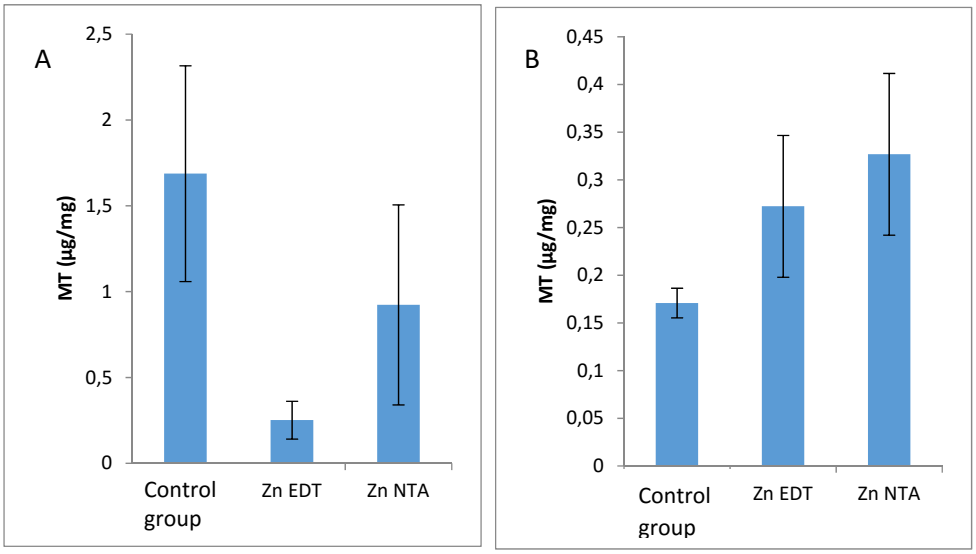
The activity of Superoxiddismutase (SOD) in the blood samples (Figure 2A) significantly decreased in both experimental groups. The decrease of blood SOD level in the case of ZnEDT group was estimated to 55 % and in the case of ZnNTA to 50%. The differences of SOD level in the liver (Figure 2B) samples was not estimated in the case of all tested groups. These results are in agreement with Horký et al. (2016b) a Horký et al. (2013).

Figure 2 The influence of Zn nanocomplexes in A) blood, B) liver on SOD level determination



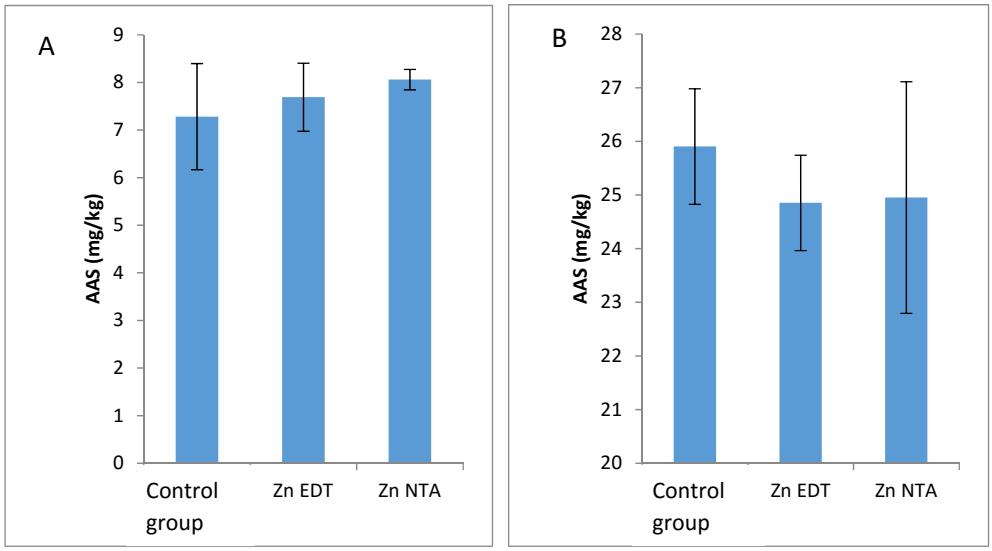
The concentration of metallothionein (MT) in blood in both experimental groups decreased (Figure 3A). In the tested group ZnEDT was estimated significant decrease of 85%, whereas the concentration of MT in liver samples (Figure 3B) increase in both of groups. In the group ZnNTA was significant increase up to 94%. These results are in agreement with results of Horký et al. (2016a).

Figure 3 The influence of Zn nanocomplexes in A) blood, B) liver on SOD level determination.



The level of zinc in the blood and the liver of the male rats were evaluated in all of examined groups. No significant differences were detected. Whereas the concentration of zinc in the liver was decreased (Figure 4A), in the case of zinc concentration in the blood there were estimated significant increase in both tested groups in comparison with the control group (Figure 4B). These results are in agreement with Horký et al. (2016a).

Figure 4 The influence of Zn nanocomplexes in A) blood, B) liver on zinc concentration.



CONCLUSION

This experiment is the first study focused on monitoring the influence of zinc nanocomplexes Zn-EDT and Zn-NTA in the diet of rats on antioxidant activity of monogastric animals. The zinc supplementation shows the influence on tested parameters such as SOD, MT and zinc concentration. According these results is obvious the animal organism is sensitive to the used zinc nanocomplexes. The significant difference was estimated in these parameters: FR, SOD, MT.

ACKNOWLEDGEMENT

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EFFECT OF SMM SEMEN EXTENDERS ON RABBIT SPERMATOZOA MOTILITY AND VIABILITY

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Abstract: SMM mediums were invented to increase turkey spermatozoa motility. Effect of SMM extenders were previously tested on turkey spermatozoa with outstanding results in *in vitro* conditions what lead testing these mediums on rabbit spermatozoa with emphasis on motility and viability parameters. Semen samples of New Zealand White rabbits were assessed in this study. The aim of this study was to evaluate selected parameters of rabbit spermatozoa motility in *in vitro* conditions using different semen extenders (SMM1, SMM2, SMM3) at different times, cultured at temperature of 5˚C. Using CASA analysis, the effect of selected extender (SMM1, SMM2, SMM3) on rabbit spermatozoa motility parameters *in vitro* during the incubation period and at 5 ˚C was determined. The total spermatozoa motility was in experimental samples in the range of 90.59–31.28% (SMM1), 85.94–28.15% (SMM2) and 89.48–24.66% (SMM3) compared to control sample – 87.82–20.51%. Even though motility decreased along with rising time, some significant differences between control sample and samples enriched with tested SMM extenders were found. Significant stimulation effect of SMM extenders was preferably manifested in samples which contained medium SMM1.The percentage of progressive motile spermatozoa showed significantly positive effect of added extender, furthermore after one hour of incubation all experimental samples reached the level of significance ($P < 0.001$). No significant difference in velocity parameter VCL was detected between control and experimental samples, except for the one hour interval where positive effect of semen extender was monitored ($P < 0.001$ and $P < 0.01$). Long term semen storage (24 hours) resulted in significantly ($P < 0.01$ and $P < 0.001$) decrease of path in samples with mediums SMM2 and SMM3. The spermatozoa viability was tested using MTT assay at intervals of 0, 1, 2, 3, 4, 5 and no significant difference compared with control was detected. Results of this study confirm that the newly developed extenders for turkey semen have also positive effect on rabbit spermatozoa *in vitro*.

Key Words: semen extender, ejaculate, rabbit, CASA, motility

INTRODUCTION

Semen extenders has been modified and improved for over 30 years. The role of extenders is to enhance the volume of ejaculate and to stimulate spermatozoa motility before insemination. Viability of sperm cells can be maintained by hypothermic storage (5–10 °C) under conditions of lowered pH of medium and aerobic environment (Chenoweth and Lorton 2014). Adequate semen extenders have to provide the source of energy for spermatozoa. Since the seminal plasma represents the most optimal environment for spermatozoa, extenders are created with composition familiar to composition of seminal plasma (Siudzińska and Łukszewicz 2008). Standard component of semen extenders is glutamic acid, which is the most efficient anion compound of seminal plasma. Source of energy in semen mediums is often represent by carbohydrates as glucose, fructose and other substances (citrate, glutamate, acetate) with potential to provide energy (Christensen 1995, Thurston 1995).Highly stimulation effect on respiration and motility of ejaculated spermatozoa was determined in caffeine which acts as inhibitor of cAMP-dependent phosphodiesterase (El-Gaafary 1994); however Mao et al. (2005) describe caffeine induced polyspermy when high doses of caffeine are added to ejaculate. Slowed down growth and even abortion of embryos are also caused by higher concentration of caffeine in medium (Tatham et al. 2003). Glutathione may play an important role in cell physiology and metabolism so its use in semen extenders is advised (Zhandi and Ghadimi 2014). According to Crisol et al. (2012),

glutathione affects cell metabolism by detoxication and prevention of free oxygen radical production in spermatozoa. The use of glutathione in semen extenders resulted in improved motility, viability and plasmatic membrane properties of ram spermatozoa. Glutathione in Bucak and Tekin's (2007) experiment offered spermatozoa protection from free radicals what elevated the amount of viable sperm cells after 6 hours of storage in 5 °C. Oxidative stress protective properties were demonstrated also by Tirpak et al. (2015) when bull ejaculates diluted in TRIS-based egg yolk extender were treated with taurine and motility of thawed spermatozoa significantly overcome motility of spermatozoa in conventionally used extender. The aim of this study was to analyze selected newly developed extenders on rabbit spermatozoa *in vitro* – mainly motility and viability.

MATERIAL AND METHODS

Semen collection and processing

Nine rabbit ejaculates of New Zealand White breed were used in this experiment. Semen collection was accomplished by use of artificial vagina which had been pre-warmed. Consequently, semen was stored at 5 °C. Fresh ejaculate (10 µl) was diluted in ratio 1 : 5 with physiological solution (NaCl 0.9% Braun, B. Braun Melsungen AG, Germany) and with three different semen extenders (SMM1, SMM2, SMM3) – groups KS1, KS2, KS3 according to Slanina (2015). Control sample (K) was presented by semen diluted only in physiological solution. Samples were continuously cultivated at 5 °C. Spermatozoa motility parameters were analyzed in various time intervals: 0, 1, 2, 3, 4, 5 and 24 hrs.

Motility analyses

Semen analyses were performed using the CASA method with SpermVision software (Minitub, Tiefenbach, Germany) and the microscope Olympus BX 51 (Olympus, Japan). Semen samples were placed into Makler counting chamber (10 µm, Sefi-Medical Instruments, Germany) (Tirpák et al. 2015). Measurements of spermatozoa motility were repeated 5 times - every hour (Time 0, 1, 2, 3, 4, 5) and also after 24 hours. Tested samples were stored in fridge at 5 °C. The following spermatozoa characteristics were assessed: motility (MOT), progressive motility (PRO) and velocity curved line (VCL) (Kročková et al. 2012, Slanina et al. 2015). Every single output of the CASA system is the result of 7 diverse sub-measurements of 7 different fields of Makler Counting Chamber.

Mitochondrial toxicity test

The MTT tetrazolium salt (Sigma, St. Louis, USA) was dissolved in PBS (Dulbecco´s Phosphate Buffer Saline, Sigma, St. Louis, USA) at 5 mg/ml. 10 µL of the solution was added to the cells (in 100 µl medium per well). After a 2 h incubation (shaker, 37 ºC, 95% air atmosphere, 5% CO_2), the cells and the formazan crystals were dissolved in 150 µl of acidified (0.08 M HCl; Centralchem, Bratislava, Slovak Republic) isopropanol (Centralchem, Bratislava, Slovak Republic). The optical density was determined at a wavelength of 570 nm against 620 nm as reference by a microplate ELISA reader (Anthos MultiRead 400, Austria). Data were expressed in percentage of the control (Knazicka et al. 2013).

Statistical analyses

For the comparison of the CASA and MTT results in certain time intervals with the focus on effect of extenders on spermatozoa, ANOVA and Dunnett´s comparative test were applied using GraphPad Prism 5 (GraphPad Software Inc., USA). All statistical tests were carried out at levels of significance at $P < 0.05$, $P < 0.01$ and $P < 0.001$.

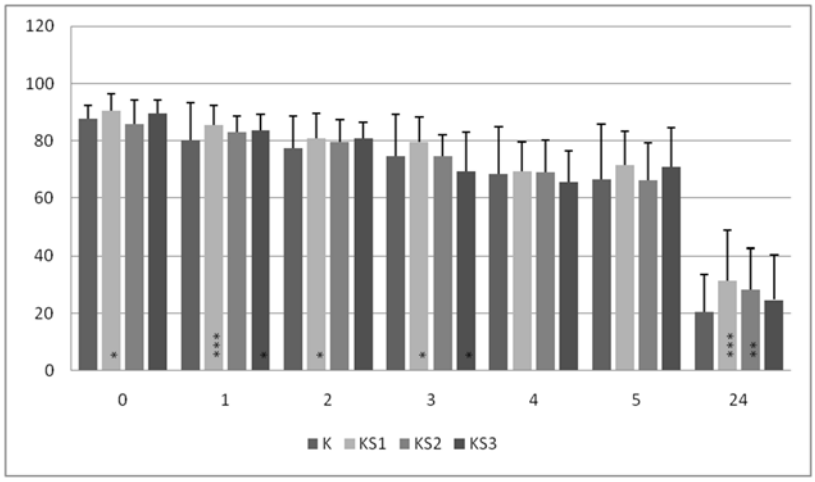
RESULTS AND DISCUSION

Effect of SMM1, SMM2 and SMM3 mediums were tested *in vitro*. Analyses of CASA and MTT represent overall impact of tested extenders on spermatozoa motility parameters and viability status.

At the beginning of cultivation process significant difference ($P < 0.05$) was detected only in KS1 sample which implied positive stimulation properties of extender. After one hour of incubation all experimental samples signified improved motility; samples KS1 and KS3 were statistically significant at levels $P < 0.001$ and $P < 0.05$. Two hours of incubation resulted in significantly higher ($P < 0.05$)

motility in sample extended with SMM1 medium compared to control. Another 60 minutes showed the same significance difference between the control sample and KS1, moreover positive impact of SMM3 extender was observed in sample KS3 ($P < 0.05$). No significant difference was detected neither in fourth or fifth time interval. Experimental samples analyzed after 24 hours of incubation showed positive effect of SMM extenders on spermatozoa, in samples KS1 ($P < 0.001$) and KS2 ($P < 0.01$) even with statistical significance.

Figure 1 MOT (%) – spermatozoa motility in different semen extenders at various time intervals (hrs)

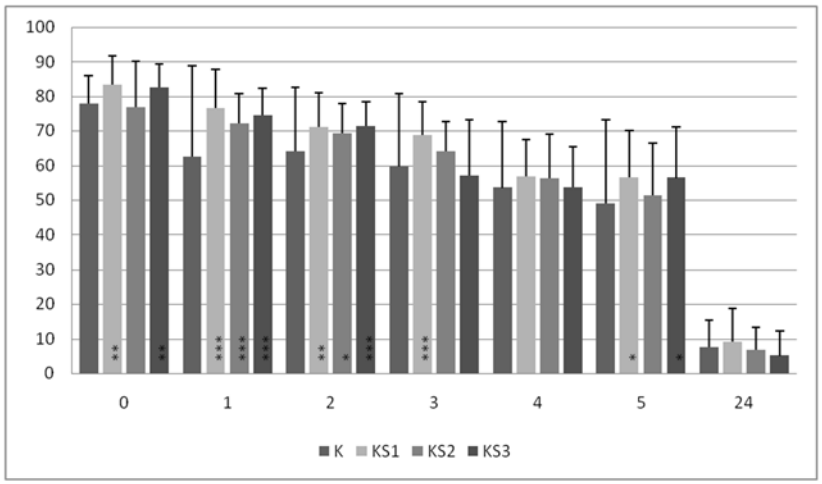


*Legend: K – control sample; KS1 – semen extender SMM1, KS2 – semen extender SMM2, KS3 – semen extender SMM3. Significant difference: *P < 0.05; **P < 0.01; ***P < 0.001.*

Progressive motility in inceptive time interval was significantly elevated in KS1 sample ($P < 0.05$) in comparison to control sample (K). First hour of incubation resulted in increased progressive motility in all experimental samples, positively significantly in samples KS1 ($P < 0.001$) and KS3 ($P < 0.05$). Positive effect of SMM extenders was manifested in samples KS1 ($P < 0.001$), KS2 ($P < 0.05$) and KS3 ($P < 0.001$). Analyses realized in 3 hours interval showed significant difference only between the control sample and KS2 sample.

Although no significant difference was noticed after 4 hours of cultivation, spermatozoa in experimental samples reached higher percentage of progressive motility than the control one. Assessment of progressive motility after 5 hours of storage showed positive significance in samples KS1 and KS3 ($P < 0.05$). 24 hours of incubation caused decrease in progressive motility under 10% with no significant difference between the control and experimental samples.

Figure 2 PRO (%) – spermatozoa progressive motility in different semen extenders at various time intervals (hrs)

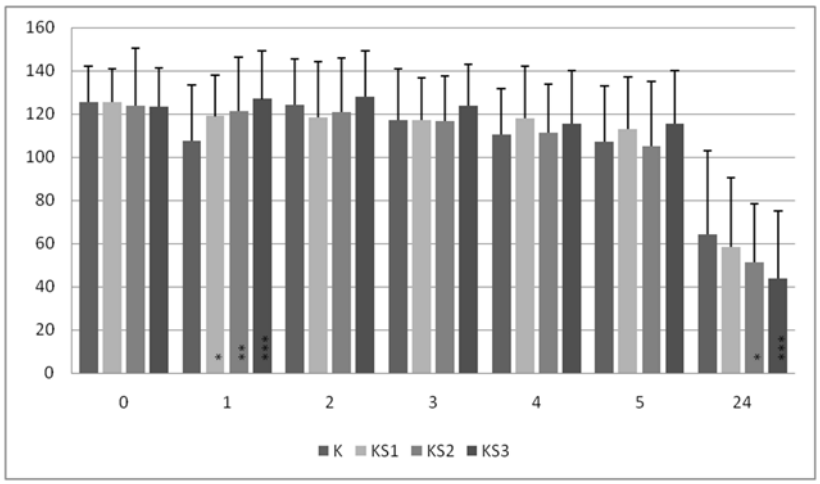


*Legend: K – control sample; KS1 – semen extender SMM1, KS2 – semen extender SMM2, KS3 – semen extender SMM3. Significant difference: *P < 0.05; **P < 0.01; ***P < 0.001.*

Considering velocity parameter VLC, beginning time of cultivation was described with an absence of statistical significance between samples. The highest positive differences were observed following one hour incubation, when compared to the control all experimental samples were significant – KS1 ($P < 0.05$), KS2 ($P < 0.01$) and KS3 ($P < 0.001$).Time intervals 2, 3, 4 and 5 hours, when velocity curved line were evaluated did not showed any significance.

Long term incubation (24 hours) resulted in lowered velocity of spermatozoa treated with SMM extenders. When compared to the control, SMM2 caused significant decrease at the level of significance $P < 0.05$, while spermatozoa in KS3 sample differed at the level of significance $P < 0.001$.

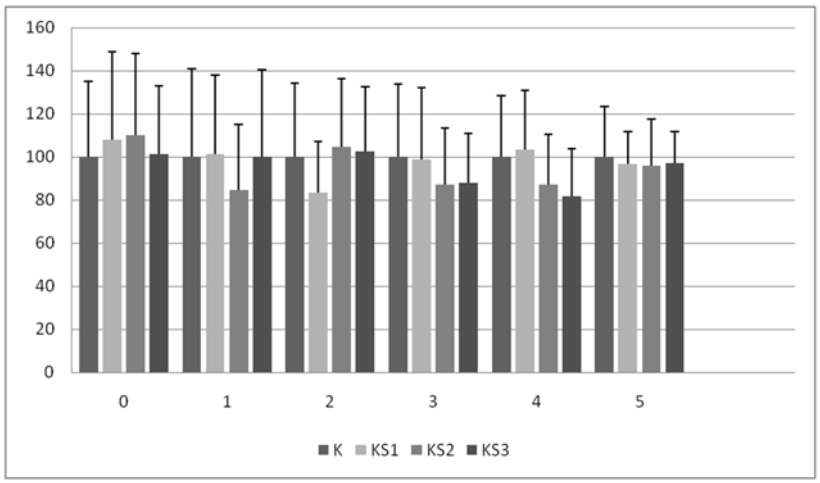
Figure 3 VCL (μm/s) – velocity curved line of spermatozoa in different semen extenders at various time intervals (hrs)



*Legend: K – control sample; KS1 – semen extender SMM1, KS2 – semen extender SMM2, KS3 – semen extender SMM3. Significant difference: *P < 0.05; **P < 0.01; ***P < 0.001.*

Mitochondrial activity was assessed by colorimetric test which spectrophotometrically measures the conversion of yellow formazan to blue formazan deposits. This conversion is induced by succinate dehydrogenase which is produced by intact mitochondria within living cells. Viability of spermatozoa in tested samples was varying from sample to sample in dependence with time interval, however without statistical significance.

Figure 4 MTT – mitochondrial activity of spermatozoa in different semen extenders at various time intervals (hrs)



*Legend: K – control sample; KS1 – semen extender SMM1, KS2 – semen extender SMM2, KS3 – semen extender SMM3. Significant difference: *P < 0.05; **P < 0.01; ***P < 0.001.*

Our results suggest significant effect of three different semen extenders (SMM1, SMM2 and SMM3) on motility of rabbit spermatozoa *in vitro*. The use of previously mentioned extenders resulted in improvement of motility parameters; however spermatozoa were not so affected as described by

Slanina et al. (2015), who tested SMM extenders on turkey spermatozoa. El-Kelawy et al. (2012) studied viability and fertilizing ability of diluted rabbit spermatozoa at 5 °C. Fresh ejaculates were diluted in ratio 1:5 in three variations: fresh semen and physiological solution (0.9% NaCl); fresh semen and glucose, egg yolk, citrate; fresh semen and fructose, egg yolk and Tris. Samples were stored at 5 °C for 24 hours after dilution. Consequently, 65 rabbits were randomly divided in two groups. First group was inseminated with semen diluted with physiological solution and the other half was inseminated with other two variants of insemination doses. Significantly ($P < 0.05$) positive effect of experimental semen extenders was observed. Comparison of successful fertilization rates was almost without any difference. Caffeine as an additive to fresh bull semen was described by Rafajová (2011). Caffeine used in this study stimulated spermatozoa activity. Time intervals 2, 3 and 4 hours showed increased spermatozoa motility what corresponds with SMM mediums in which caffeine was also used.

CONCLUSION

Tested extenders (SMM1, SMM2 and SMM3) in *in* vitro conditions showed the potential to be used in rabbit semen processing before artificial insemination. Significant differences between spermatozoa extended with SMM mediums and control samples recommend further research with focus on elevation of amount of viable spermatozoa. Our study determined that the most efficient SMM semen extenders for the use in rabbit breeding may be SMM2 and SMM3 mediums.

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BIOIMAGING OF BIOLOGICAL TISSUES BY MEANS OF LASER ABLATION WITH INDUCTIVELY COUPLED OF PLASMA AND MASS SPECTROMETRY

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Abstract: Bioimaging using laser ablation connected with inductively coupled plasma and mass spectrometry (LA-ICP-MS) offers the ability of imaging of elements in the different types of biological tissues with very good spatial resolution from units to hundreds μm. This method is used in biomedical research such as cancer diseases. A wide range of application also provides information about distribution of essential elements in organs (liver, kidneys).

Key Words: laser ablation, tumour, cancer, imaging, biological tissues, inductively coupled of plasma, mass spectrometry

INTRODUCTION

Bioimaging or mapping of element/isotope distribution in the thin sections of biological tissues and its determination of concentration is of increasing interest in the life sciences (Becker et al. 2008).

One of many approaches which provide this analytical technique is investigation of distribution of three type platinum-based cytostatics (cis-, carbo- and oxali-) in prostatic tumour tissues. Cis-platin (cis-$[PtCl_2(NH_3)_2]$ is one of the most effective chemotherapeutic agents and it is used for treatment of a variety of solid human tumours but there exists a major problem (Crone et al. 2015). During the drug therapy it can appear development of tumour resistance. This problem can occur with carbo- and oxali-platin, as well. The first one is more suitable for treatment of prostatic tumours, the second one is better for therapy of breast tumours. Oxali-platin effectively operates at tumours which is resistant to cisplatin (Misset et al. 2000). The reason and mechanism of tumour resistance is still unknown.

Besides bioimaging can help to understand the different nephrotoxic behaviour of kidney (Moreno-Gordaliza et al. 2011) because we are able to observe the distribution of these type platinum in organs (kidney and liver). Consequently it may provide information about accumulation Pt in organs or tumours.

Aim of this study is distribution of elements of interests in prostatic tumours and organs (kidney and liver) tissues treated by different type of platinum-based cytostatics.

MATERIAL AND METHODS

LA-ICP-MS measurement

All samples were analysed by LA-ICP-MS which consists of laser ablation system UP 213 (New Wave, USA). Ablated material was carried out by He (1.0 l/min). Prior entrance into ICP-MS was admixed Ar (0.6 l/min) into He flow. The ICP-MS Agilent 7500ce (Agilent, Japan) was used for analysis of ablated material. LA-ICP-MS parameters were optimized with respect to reach best S/N ratio and minimal oxide and double-charged ions formation. The combination of laser beam diameter and scan speed was optimized to get sufficient lateral resolution, limit of detection and time of analysis according to (Vaculovic et al. 2015). The LA-ICP-MS parameters are listed in (Table 1).

Sample preparation

The prostatic tumours were grown on nude mice train Nu/Nu. After that the mice were treated by various cytostatics drugs containing three type of complex of Pt – oxali-, carbo- and cis-Pt. The fourth group was not treated. The tumour were put out from mouse, frozen in medium then were cut by means of cryotom device and the resulting slices were put to the slide and frozen. The resulting thickness of slices was 30 µm. The same procedure of preparation of thin sections was used for organs as are kidney and liver.

Quantification

For the quantification purpose agarose gels were used as calibration standards. It was doped by known amount of elements of interest to get following contents: Cu, Zn – 1, 10 and 100 mg/kg, Pt – 0.1, 1 and 10 mg/kg. Ablation was performed using single line of one cm long in each gel.

Table 1 Overview of determinated isotopes and parameters of technique.

Determinated izotopes	
^{63}Cu,^{66}Zn, ^{208}Pb, ^{195}Pt	
PARAMETRS	**VALUE**
Carrier gas (He)	1 l/min
Distance between lines	110/70 µm
Speed of scan	200 µm/s
Beam diameter	100/65 µm
Frequency	10 Hz
Fluence	8 J/cm^2
Integration time ^{63}Cu, ^{66}Zn	0.1 s
Integration time ^{208}Pb, ^{195}Pt	0.3 s

RESULTS AND DISCUSSION

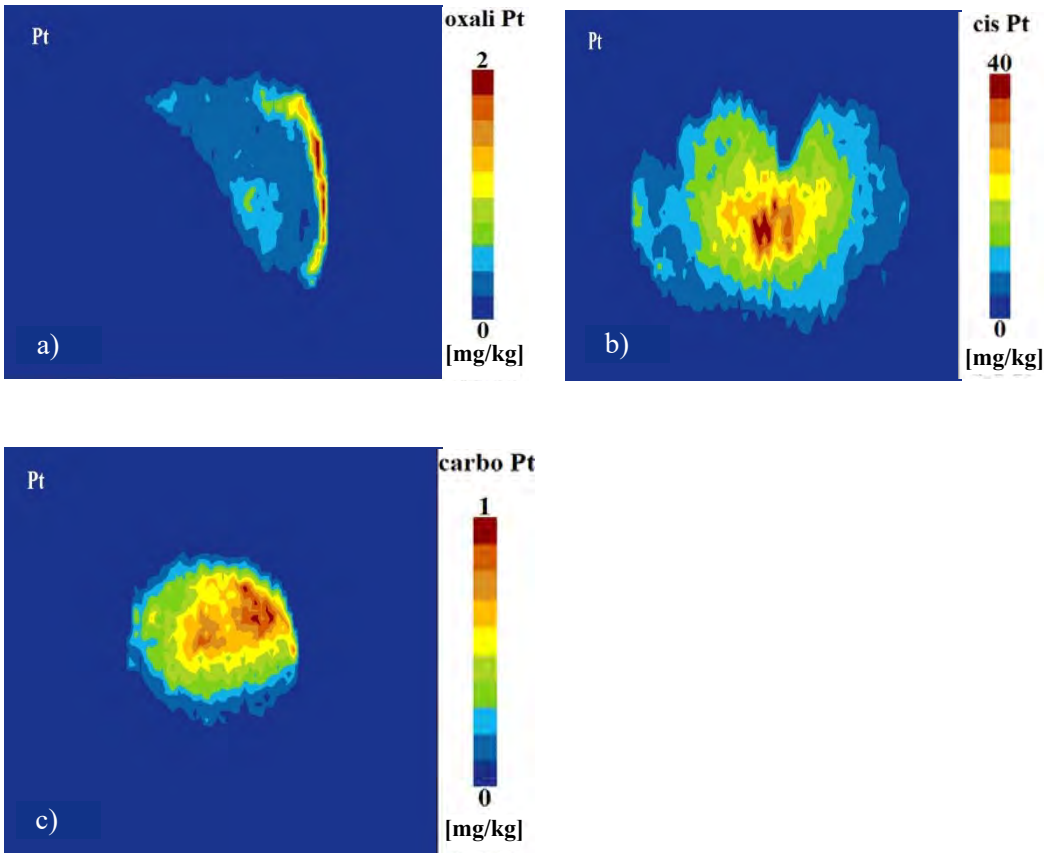
Tumour tissue – prostatic tumour

The examined samples of mice tissue consists of 4 different groups. 3 groups were treated by 3 types of Pt-based cytostatics as are oxali-, carbo- and cis-Pt. Fourth group was non-treated and served as control samples. All presented elemental maps are quantified. For quantification purposes the agarose gels doped by elements of interest were used.

Distribution of Pt

Figure 1 shows distribution of Pt in prostatic tumour tissue treated by cis-, carbo- and oxali-Pt. The highest content of Pt is observed in tumour treated by cis-Pt (more than 40 mg/kg). The Pt content is significantly lower in case of tumours treated by oxali- and carbo-Pt, respectively. Distinctive difference is observed when the distribution is compared. In case of cis-Pt the strong enrichment is visible in the central part of tumour. Whereas, in case of oxali- and carbo-Pt the Pt is accumulated in the edge part of the tumour tissue.

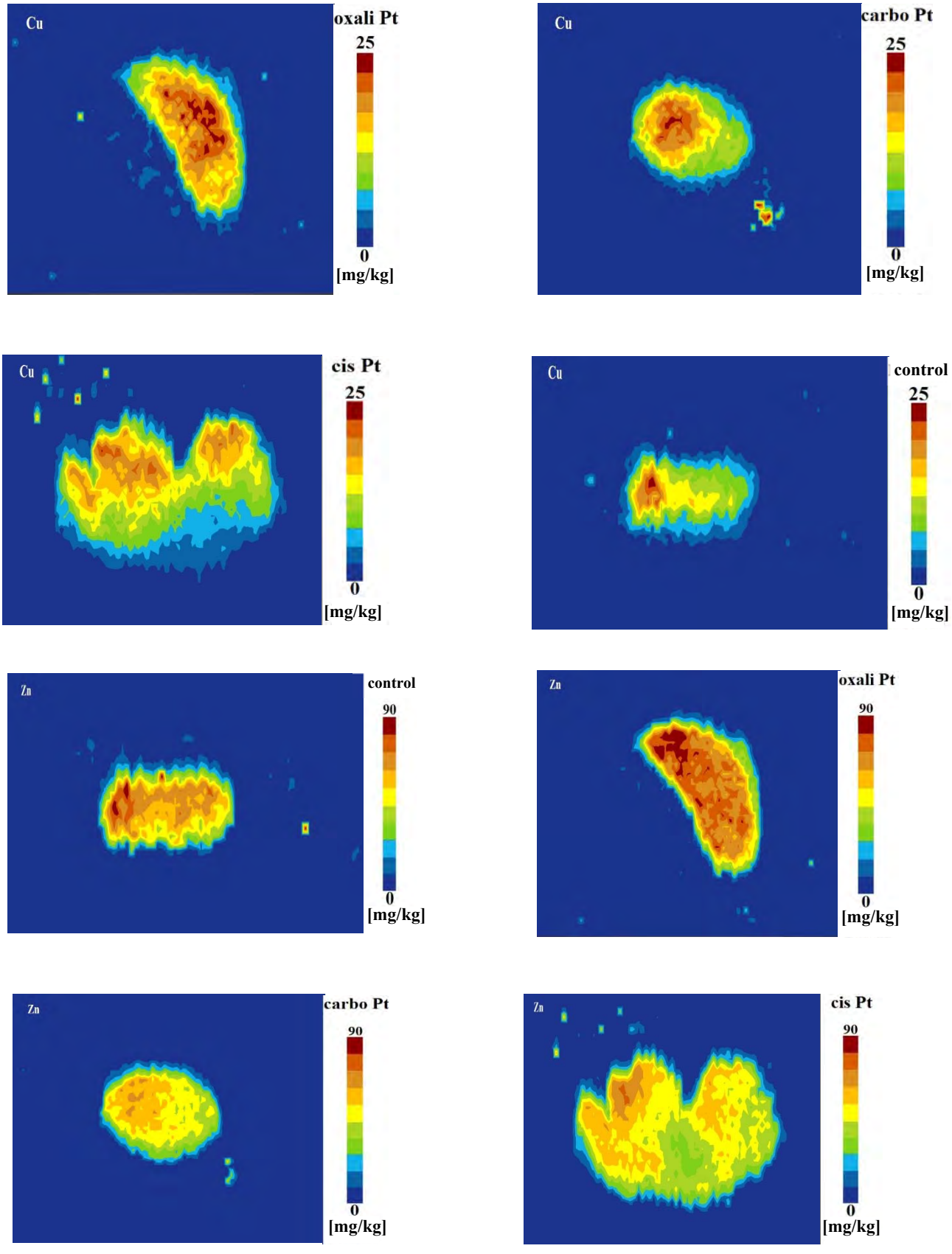
Figure 1 Distribution of three type Pt in prostatic tumour tissue.



Distribution of Cu and Zn

Figure 2 shows distribution of Cu and Zn in 4 groups of tumour tissues (cis-, carbo-, oxali-Pt and control sample). The distribution and amount of Cu in thin tissues is very similar for all four types of tumour tissues including even control sample (untreated). The Zn shows discrepant behaviour in comparison with Cu. Distinctively higher amount of Zn is observed in case of oxali-Pt and non-treated samples. Moreover, the distribution is more homogeneous in comparison with Cu.

Figure 2 Distribution of Cu and Zn in 4 groups of tumour tissues (cis-, carbo-, oxali-Pt and control sample).

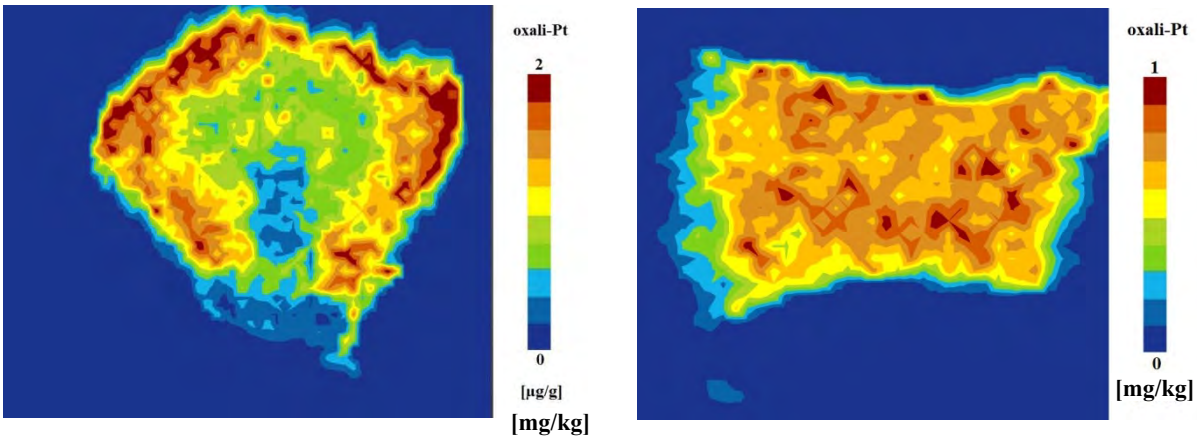


Organs tissue – kidney and liver

Distribution of Pt

Figure 3 shows distribution of oxali-Pt in d) kidney and e) liver. Both organs have different behaviour. Oxali-Pt in liver is evenly distributed compared to kidney, where it is observed inhomogeneity, particularly higher amount occurs at the edges of this organ.

Figure 3 Distribution of oxali-Pt in d) kidney e) liver tissue.



CONCLUSION

The present study shows quantification imaging of elements of interest. It was determined significant inhomogeneity in the distribution of elements in tumour tissues, especially between Pt and Zn. The zones with enriched content of Pt are depleted in Zn content. In case organs tissues distribution of oxali-Pt is also different. Inhomogeneity in case kidney is obvious in contrast with liver, where is oxali-Pt evenly distributed.

The results with combination immunohistochemistry can give novel information about transport processes elements through proteins (especially metallothionein) that is associated with tumour progression.

Because there are no relevant works in literature it can´t be here a confrontation of our results with other authors.

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EFFECT OF DIETARY FISH OIL ON EXPRESSION OF LIVER GENES CONTROLLING CHOLESTEROL HOMEOSTASIS: COMPARISON OF TWO ANIMAL MODELS

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Abstract: The aim of this study was to compare the effect of the fish oil, respectively its main component, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on the expression of genes *PPARα, SREBP-2, Insig-1, Hmgcr and Ldlr* which control cholesterol homeostasis in the liver of rats (Wistar Albino; n = 32) and pigs (Large White x Landrace; n = 32). Rats and pigs were randomly assigned into two groups of 16 animals and fed ten weeks by the diet with either 2.5% of fish oil (group F; source of eicosapentaenoic and docosahexaenoic acid, EPA+DHA) or 2.5% of palm oil (group P; high content of saturated fatty acids; control group). Dietary fish oil relative to palm oil increased *PPARα* and *SREBP-2* gene expression much strongly ($P<0.01$) in the pig liver in comparison with the rat liver, but expression of *Insig-1* and *Hmgcr* genes in the liver of the F-pigs relative to the expression of these genes in the liver of the P-pigs was substantially lower ($P<0.01$ and $P<0.05$, respectively) as compared to rats.

Key Words: Eicosapentaenoic acid (EPA), Docosahexaenoic acid (DHA), Gene expression, Cholesterol homeostasis, *Sus scrofa*, *Rattus norvegicus*

INTRODUCTION

This study focuses on the effect of n-3 polyunsaturated fatty acids, respectively eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on the expression of liver genes which control cholesterol homeostasis. Assumed, that cholesterol is situated in the hepatocytes. The liver is the central organ of cholesterol homeostasis.

Polyunsaturated fatty acids (EPA and DHA) significantly affect gene transcription by regulating the activity of a number of transcription factors, including nuclear receptors such as PPARs (peroxisome proliferator activated receptor), or a group of transcription factors SREBP (Afman et al. 2012). Expression of the genes coding for the key proteins controlling cholesterol homeostasis, hepatic 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA-R) and low-density lipoprotein receptor (LDL-R), is stimulated by the transcription factor SREBP-2, whose activation is affected by the INSIG protein (insulin-induced gene), product of the *Insig* gene (Sato 2010). Moreover, SREBP-2 activation is presumably related to PPARα ligation by EPA/DHA (Luci et al. 2007).

In vivo studies are usually carried out on rodents. However, rodents (in this context "proliferating" species) are not ideal models for humans ("non-proliferating" species) opposed to pigs which are "non-proliferating" species as humans.

The objective of the present study was to compare two animal models, rat and pig, regarding an effect of fish oil (the most common source of EPA+DHA) on expression in the liver of the genes presumably affecting cholesterol homeostasis. An intention was to carry out an experiment as similar to usual human conditions as possible and to use dietary EPA+DHA (fish oil) in the amount realistically achievable in human nutrition. On the other hand, palm oil, containing a high percentage of saturated palmitic acid, and which is currently included in a broad spectrum of foods, can be mentioned in this context as a negative example.

In the present study was tested the hypothesis (Chatterjee et al. 2009, König et al. 2007) that PPARα activation by EPA/DHA increases the expression of the *Insig-1* gene leading to the retaining of SREBP-2 precursor protein in endoplasmic reticulum, to the decrease of the SREBP-2 active form and consequently to the decrease of *Hmgcr* and *Ldlr* gene expression. Therefore the expression of the genes of two transcription factors (PPARα and SREBP-2) and three subordinate genes (*Insig-1*, *Hmgcr* and *Ldlr*) was measured.

MATERIAL AND METHODS

Animals, dietary interventions, analyzed tissues

As a model animals for this study were used thirty-two male rats (laboratory strain Wistar Albino; Meditox Konárovice, Czech Republic) and thirty-two pigs of both sexes (16 females, 16 males; Large White x Landrace; Bioprodukt Knapovec a.s., Ústí nad Orlicí, Czech Republic). The rats were at the age of eight weeks with the mean live weight of 312 ± 23 g and pigs were at the age of eight weeks with the mean live weight of 25.5 ± 1.15 kg. The animals were divided into two groups (experimental and control) of 16 individuals (the same number of males and females was in each group in the case of pigs). The experimental group was fed for ten weeks with the basic feed mixture with 2.5% of fish oil (F) and the control group was fed with the basic feed mixture with 2.5% of palm oil (P). The P-diet was used as a control in order to keep the diet not only isocaloric but also iso-lipidic within the given animal species. The rats were fed daily *ad libitum* and pigs were fed twice daily (7 a.m. and 2 p.m.). The animals were weighed in weekly intervals. After the experiment, the rats were sacrificed using inhalation anesthetics Isoflurane and the pigs were anesthetized by the intramuscular application of the TKX mixture (12.5 mg/ml of ketamine, 12.5 mg/ml of xylazine, 12.5 mg/ml of tiletamine, 12.5 mg/ml of zolazepam) in the total volume of 0.2 ml/kg of the live weight and sacrificed by bleeding. Total RNA was immediately isolated from liver aliquots (30 and 50 mg in rats and pigs).

Quantification of the gene expression in the liver

Total RNA was isolated from the liver using RNeasy® Mini Kit (Qiagen GmbH, Hilden, Germany); concentration was measured on NanoDrop 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific, Waltham, USA) and isolated RNA was stored at -80 °C. One µg of the isolated RNA was reverse transcribed using M-MLV reverse transcriptase system (Invitrogen, Paisley, UK) and oligo-dT primers. Obtained cDNA was used for quantitative PCR with specific primers characterized in Table 1. Different housekeeping genes were used in the rat (*Actb*) and pig (*TBP1*) liver samples.

Quantitative PCR was carried out using the Nanodrop II liquid dispenser (Innovadyne Technologies, Rohnert Park, CA). RNA expression was quantified in triplicate reactions in a final volume of 3 µl in 384-well plates using QuantiTect SYBR Green PCR master mix following the manufacturer's recommendations (Qiagen, Hilden, Germany) on a LightCycler 480 (Roche Applied Science; https://www.roche.com) under the following conditions: denaturation at 95 °C for 15 min and 45 amplification cycles at 95 °C for 15 s, 58 °C for 30 s and 72 °C for 30 s. The reaction mixture consisting of 0.5 µL of cDNA, 1.5 µL of SYBR® Green PCR Master Mix and 10 pmol of each couple of primers (Generi Biotech, Hradec Kralove, Czech Republic) was used.

Table 1 Primers used for quantitative PCR

Gene*	Species	Forward primer	Reverse primer
PPARα	**Rat** **Pig**	GCCTTCTCCCCACATATTCG AGACCGCAGATCTCAAGTCTCTC	AGAGGAGAGTTCCGGAAG ATGACGAAAGGCGGGTTATTGC
SREBP2	**Rat** **Pig**	ATCCGCCCACACTCACGCTCCTC CTGCCTACCGCAAGGTGTTTC	GGCCGCATCCCTCGCACTG AGGCTGTGCTCTAATAGCTGGTG
Insig1	**Rat** **Pig**	TCTTCCCGGACGAGGTGATAG GAAAATGGGATCTCTCTGCACTTTG	AGCTGCACATTATTGGCGAAAT AAAGGACCAATGACTGCTTTCGC
Hmgcr	**Rat** **Pig**	AAGGGGCGTGCAAAGACAATC CTACATTGCCTGTGGTCAGGATG	ACACGGCACGGAAAGAACCATAGT CCGATCTCTATGGATGGCATGGT
Ldlr	**Rat** **Pig**	GGACAAGTCGGACGAGGAGAA AACCCTGTGTACCAGAAGACCAC	AGCTGATGCACTCCCCACTGT TCTGTCTCGAGGGGTAGGTGTAG
Actb	**Rat**	AGAGGGAAATCGTGCGTGAC	GTTTCATGGATGCCACAGGATT
TBP1*	**Pig**	AACAGTTCAGTAGTTATGAGCCAGA	AGATGTTCTCAAACGCTTCG

*Legend: * PPARα = peroxisome proliferator-activated receptor α; SREBP2 = sterol response element-binding protein 2; Insig1 = insulin-induced gene; Hmgcr = 3-hydroxy-3-methyl-glutaryl-CoA reductase; Ldlr = low-density lipoprotein receptor; Actb = β-actin; all designed; **TBP1 = TATA box-binding protein, reference: Nygard et al. 2007*

Statistical evaluation

All data were statistically analyzed using STATISTICA 12 package (StatSoft, Tulsa, OK, USA). For testing the differences between animal species in the relative expression of the liver genes was used Wilcoxon signed-rank test.

RESULTS AND DISCUSSION

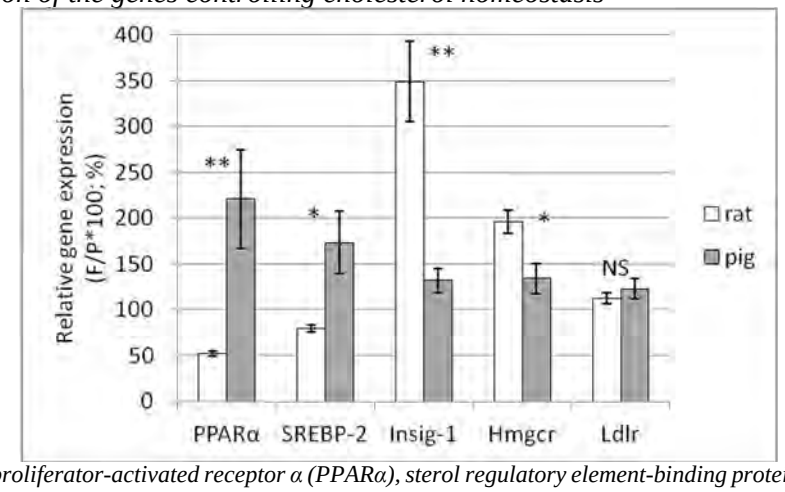
Live weight, daily weight gain

Daily weight gain of rats was 3.04 ± 0.14 g/day for F-diet and 3.32 ± 0.18 g/day for P-diet. Daily weight gain of pigs was 0.85 ± 0.05 kg/day for F-diet and 0.86 ± 0.04 kg/day for P-diet. The final weight of the F-rats was 524.8 ± 13 g and P-rats 544.4 ± 11 g and final weight of the F- and P-pigs was 83.64 ± 1.82 kg and 84.06 ± 3.35 kg. Despite a slight tendency (P = 0.12) of fish oil to decrease daily weight gain in rats, type of oil in the diet affected (P>0.05) neither daily weight gain nor the final weight in either tested animal species.

Liver genes expression rate

Substantial differences between the tested species in an extent of the relative expression of the genes presumably controlling cholesterol homeostasis are apparent from Figure 1. Dietary fish oil relative to palm oil increased *PPARα* gene expression much strongly (P<0.01) in the pig liver in comparison with the rat liver. The same result was obtain in *SREBP-2* gene (P<0.05). On the other hand, the expression of *Insig-1* and *Hmgcr* in the liver of pigs fed the diet with fish oil relative to the expression of these genes in the liver of the palm oil-fed counterparts was substantially lower (P<0.01 and P<0.05, respectively) in comparison with rats. The only gene whose relative expression in the liver did not differ (P>0.05) between pigs and rats was *Ldlr*.

Figure 1 Expression of the genes controlling cholesterol homeostasis



*Legend: peroxisome proliferator-activated receptor α (PPARα), sterol regulatory element-binding protein 2 (PPAR-2), insulin-induced gene 1 (Insig-1), 3-hydroxy-3-methyl-glutaryl CoA reductase (Hmgcr) and low-density lipoprotein receptor (Ldlr) in the liver of rats and pigs, respectively, fed the diet with 2.5% of fish oil (F) relative to the expression of the corresponding liver genes of the animals of the same species fed the diet with 2.5% of palm oil (P); Wilcoxon signed-rank test; n = 16; NS – not significant; * P<0.05; ** P<0.01*

In this study was measured the expression of the genes of two transcription factors (PPARα and SREBP-2) and three subordinate genes (*Insig-1*, *Hmgcr* and *Ldlr*).

As far as PPARα activation is concerned, fish oil caused a substantially lower increase (P<0.01) of relative expression of *PPARα* gene in the rat liver in comparison with pigs. Our results agree with the data of Arai et al. (2009) and Cheon et al. (2005) who reported higher expression of *PPARα* in pigs than in rodents after the administration of PPARα ligands.

Regarding SREBP-2, we have measured the same results as *PPARα*. Woo et al. (2005) mentioned that SREBP-2 is a weak transcription activator by itself and needs a presence of additional transcription factors.

Cheon et al. (2005) reported that PPARα activation does not influence SREBP-2 controlled transcription of genes involved in cholesterol homeostasis. According to Luci et al. (2007), an effect of

PPARα activation on SREBP-2-dependent cholesterol synthesis may be different between various species.

The hepatic *Insig-1* gene was up-regulated in rats and pigs in the present study (349 and 132% of the control in rats and pigs). But in the study of König et al. (2007), the suggested signal pathway via *PPARα* activation was confirmed only in pigs (up-regulation of the *PPARα* gene to 221% of the control; contrary to rats, where the gene was down-regulated to 52% of the control).

Despite the fact that pigs and rats differed in the expression of the *Hmgcr* gene but not in the expression of the *Ldlr* gene, both *Hmgcr* and *Ldlr* genes were up-regulated in the present experiment by the fish oil diet both in rats (196 and 113% of the control) and in pigs (134 and 123% of the control), which is contrary to the suggested signal pathway. However, this up-regulation of *Hmgcr* and *Ldlr* genes after administration of relatively low doses of fish oil (2.5%) confirms our previous results in rats consuming much higher fish oil doses (6% of the feed ration; Komprda et al. 2015).

CONCLUSION

The pigs as "non-proliferating" species are more ideal models for humans than rats ("proliferating" species) so we expected better results in pigs.

According to our hypothesis, dietary fish oil relative to palm oil increased PPARα and SREBP-2 gene expression much strongly ($p < 0.01$) in the pig liver in comparison with the rat liver. However, the expression of Insig-1 and Hmgcr genes in the liver of the F-pigs relative to the expression of these genes in the liver of the P-pigs was substantially lower ($p < 0.01$ and $p < 0.05$ respectively) as compared to rats.

Most of our results exactly match the results of other studies in the available literature. However, it would be appropriate to carry out further studies on this issue.

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The experiment was performed in compliance with the Czech National Council Act No. 246/1992 Coll. to protect animals against cruelty, the Amended Act No. 162/1993 Coll., and was approved by the "Commission to protect animals against cruelty" of the Mendel University in Brno and of the Ministry of Agriculture of the Czech Republic.

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Section – Techniques and Technology

MONITORING THE QUALITY OF WORK OF IRRIGATION MACHINES WITH THE DESIGNED SPEEDMETER SM2 DEVICE

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Abstract: As we could see in the last few years, climatic conditions indicated a very low level of atmospheric precipitation. For this reason, it is necessary to deal with the issue of supplementary irrigation. On selected crops, we have to apply several doses of spray. We then focus on the quality of work of the used technology. Longitudinal spray uniformity is among the factors of the quality of work and it is also associated with the continuous and stable hose winding speed. To be able to assess the quality of the longitudinal uniformity of the speed of hose winding on the reel, we needed to design a digital device. The hardware design itself is not sufficient; therefore its software part needs to be added. The microcontroller PIC16F877 with the MOL30 tachometer was used as the main logic unit. The port RS232 was used for communication. After solving and constructing this device, we verified its functionality directly in the working conditions of a particular hose reel irrigation machine. The designed device Speedmeter SM2 enables saving of the data into the internal memory. It enables to store up to 122 values with setting up the time interval from 1 to 32768 sec. This device was verified in practical measurements, where an absolute error of the 0.34 m/h irrigator was found (relative error was 2.7% to the set value).

Key Words: hose, reel, irrigation machine, coefficient of uniformity, quality of work

INTRODUCTION

For application of irrigation and its uniform distribution, we apply Wide-band and reel hose irrigation machines. To a certain extent, it is also possible to use micro-spray irrigation machines (Jobbágy et al. 2016). Reel hose irrigation machines are designed as wheeled machines with a supported chassis on which a reel with a polyethylene hose with a diameter of 25–140 mm and the length of 200–750 m is placed. The capacity of reel hose irrigation machines depends on the speed and uniformity of hose winding (Jobbágy et al. 2013). Other authors were also interested in the influence of the change of the speed of hose winding on the reel. They conducted practical measurements using reel hose irrigation machines equipped with Irrigamatic 300 (Matermacc/Italy) and Rain 9 (Nortoft Electronic/Denmark) microcomputers. An irrigation console was attached to the reel hose irrigation machine. A square grid of rain gauge vessels with a side length of 1 m was used for measuring the uniformity of spray. When changing the winding speed from 24 m/h to 40 m/h (in the area of 1–2 meters), the amount of spray decreased from 28 mm to 16 mm (in the area of 15 meters, Al Karadsheh 2003). As we can see from the results, the amount of water that is applied to the surface decreases with the increase in the travel speed (Rhoades et al. 1989). The programming languages are languages used for creating computer applications. Programming is a process of algoritmization of a given task, i.e. creating processes that will lead to solving the task (Hylmar 2012). Measurements were performed on the first designed device, which, however, had a number of disadvantages. There was no integrated memory in the device which means that we had to observe it and write correct times during measurements. For measurements we

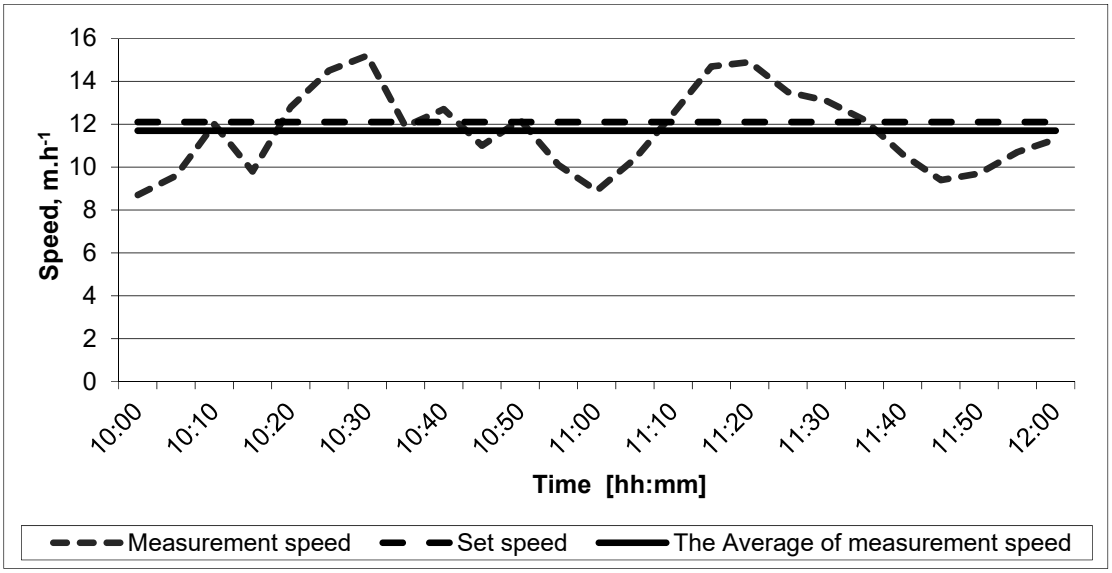
used the Bauer 90/300 reel hose irrigation machine with the hose length of 300 m and hose diameter of 90 mm. The winding speed was set up to 12.1 m/h (Jobbágy et al. 2013). The average value of the measured speed was 11.7 m/h (deviation from the set was 0.4 m/s). The graphical display of the results is shown in Figure 1 and the technical parameters of the Bauer Rainstar irrigators are shown in Table 1. A coefficient of variation in monitoring the winding speed amounted to 16.49%.

Table 1 Technical parameters of Bauer Rainstar TX Plus 90/300 reel hose irrigation machine

A, mm	B, m	D, m	E, m	F, mm	G, m^3/h
90	300	340	76	16–30	17–65
H, MPa	I, kg	J, kg	K, mm	L, mm	M, mm
3.5–10	1850	3270	5350	3700	3060

A- diameter of PE tube, B- PE tube length, D- max. length of irrigated strip, E- max. width of irrigation, F- range of nozzle diameter, G- the extent of the flow of water, H- bonding pressure (min.–max.), I- total weight without water, J- total weight with water, K- total length including the length of the machine, L- length without the irrigation truck, M- total high

Figure 1 Measurement of the longitudinal spray uniformity



In many contributions, authors focus more on testing the longitudinal uniformity and less attention is paid to examining the variability of the speed of hose winding on the reel. Therefore, we have decided to highlight their variability.

MATERIAL AND METHODS

The issue of irrigation and monitoring the quality of work is not easy. When using reel hose irrigation machines, it is possible to change the hose winding speed by changing the main gear and by a smooth change in water bypass in the turbine. The change can be performed in two ways – either mechanically or digitally with the use of a microcomputer. Therefore, when designing the device, it is necessary to focus on the precise solution to the issue of monitoring the speed (revolutions on the tachometer touching the hose) and the transfer of this parameter to the speed of linear motion. The device ***Speedmeter SM2*** for measurements was developed at the Department of Machines and Production Systems and it was designed in 2012 (Figure 1). As the basis, Speedmeter SM1 (Figure 2) was used. A certain modification was done in order to attach a frame with a tachometer MOL30 (MEGATRON Electronic, Germany). An incremental rotary encoder transforms the rotary motion to electrical impulses. The transfer of mechanical motion to electrical impulses is performed in a photoelectric contactless way.

Figure 2 Frame of the device – Speedmeter SM2

RESULTS AND DISCUSSION

The aim of this entry was to design and construct an independent device for monitoring the speed of hose winding on the reel – ***Speedmeter SM2*** (Figure 3). This device carries out the measurement without the presence of a human being (entering the data into the memory) and it enables the export of measured values to the computer. Other requirements for the device are the following:

- The ability to set up the time interval of monitoring in the range of 1 to 1800 s,
- measurement of the speed in the range of 0 to 40 m/h,
- the ability to set up the perimeter of the measuring cylinder for the construction of the device in the range of 50–300 m,
- independent power supply from a battery,
- displaying the actual speed on the display.

The design of the device can be divided into two main parts (with only a slight modification of the frame):

- hardware,
- software.

Hardware part

When designing the hardware part, it was important for the device to store the measured values during the entire measuring process. The design itself depends on the software part. The frequency reader is placed in the construction part of the device and it is described in the methodology of the entry. The microcontroller PIC16F877 (Microchip Technology Inc.) was used as the main logic unit. As an auxiliary logic unit, IC2 microcontroller also made by the American company Microchip Technology Inc. was used for their compatibility. Its task is, with the use of a counter, to count the impulses from the sensor and after a request from the main logic unit send the actual value. The displaying part consists of four 7–segment LED displays, which are controlled by the main logic unit. The display has four digits, the two of which are decimal digits.

Figure 3 Speedmeter SM2 – hardware part

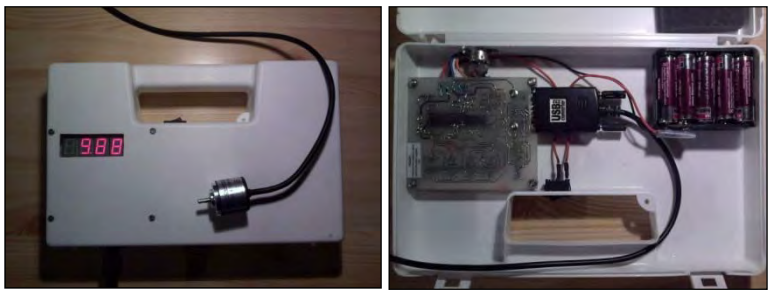
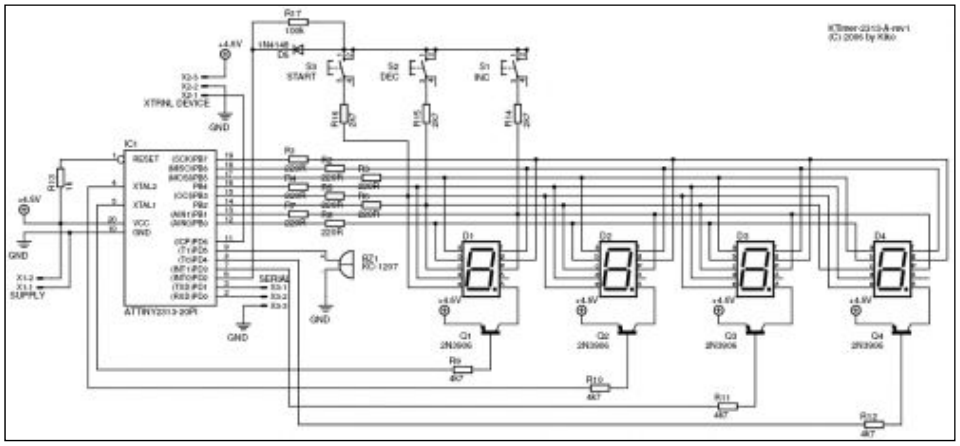


Figure 4 The scheme of frequency reader



The communication unit serves for communication between the monitoring device and the PC for transfer of the measured values to the PC. The communication device is also used for setting up the number of impulses of the sensor during one cycle, the cylinder perimeter, the interval of saving the measured value into the memory and deleting the memory. The serial port RS232 is used for communication.

Software part

When designing the software part, we relied on the principle of frequency counter and the materials for logic unit. The programming language C was used. For correct programming of the counter, it is particularly important to express mathematical formula that expresses all the relationships between parameters that arise and are needed for the calculation of the speed of the logic unit.

$$v = \frac{n_1.O_v.3600}{k.n_2.1000}, \text{ m/h (1)}$$

where:

v – hose winding speed, m/h

n_1 – the number of impulses recorded by the counter in a specified time limit

n_2 – the number of impulses per one cycle

t – time unit in the formula, t = 1

The time is an important parameter in this formula. The value of the time unit is 1 because the current speed is measured every second. The next measured value in the following second is added to that value and the sum is divided by 2 and we get the average value. The third value is again added to the average value and divided by 2 to get the average value again. This procedure is programmed in the logic unit; the number of repetitions depends on the setting of the interval of saving the data into the memory. In the formula 2, the values of calculations and the time value are combined because of the optimization during programming.

$$v = \frac{n_1.O_v.3{,}6}{n_2}, \text{ m/h (2)}$$

This application was also written in the C programming language for Windows operating system. The application serves just as a superstructure of terminal communication to make the user interface easier and it has an integrated support for the export of memory right into a TXT file and its storage in the PC. Besides that, the application automatically reads the set values right after connecting it to the monitoring device. In the application, there is also an integrated support for a quick change of COM port. The flow diagram shows the function of the programmed monitoring device and the command processing sequence according to the occurrence of events (Figure 5).

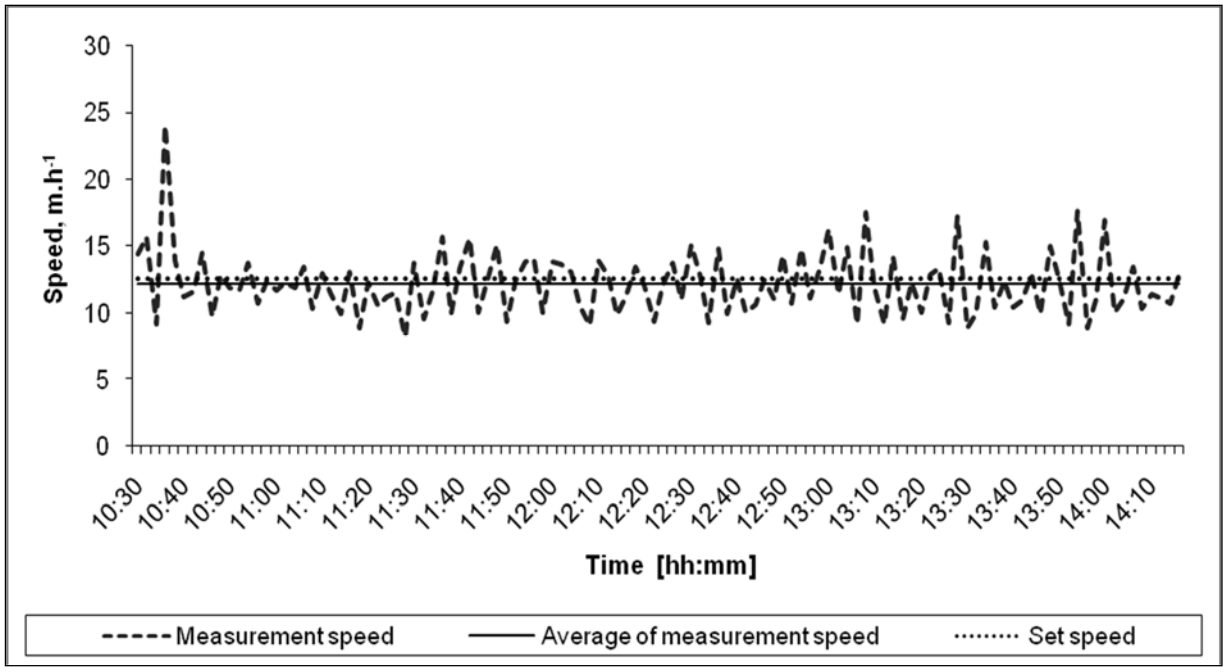
Table 2 Descriptive statistics of measured hose winding speed results

Parameter	Value, m/h
Average value, m/h	12.26
Standard deviation, m/h	2.41
Difference max.-min., m/h	15.84
Minimum, m/h	8.27
Maximum, m/h	24.11
Sum, m/h	1397.17
Number of measurements	114
Reliability (95.0 %)	0.45
Coefficient of variation, %	19.64
Average value, m/h	12.26

The measurement was performed on the property of AGROCOOP IMEĽ, ltd. near the village of Imeľ. The Bauer Rainstar 90/300 irrigation machine was equipped with BAUER ECOSTAR 4000 S electronic controller, set to the value of 12.6 m/h. The technical parameters are identical with the parameters shown in Table 1. The Speedmeter SM2 was placed approximately 150 m from the reel. Weather conditions were suitable for irrigation - clear sky, the temperature at the beginning of measurement was more than 30 °C in the shade. Before the practical measurement itself, we had to set up the input parameters such as the cylinder perimeter (143.8 mm), number of impulses of the sensor (1024 impulses/cycle) and the interval of saving values (120 s). The graphic process of the hose winding speed variability is shown in Figure 5. In this flow diagram, we marked both the average and the set values. The average value of working speed was 12.26 m/h. The absolute error of the measurement was 0.34 m/h.

In the past few decades, several coefficients of uniformity were developed to express the uniformity of water distribution for different sprinkler irrigation systems. Christiansen's uniformity coefficient seems to be the most popular uniformity coefficient used by researchers on the global scale. Finally, the results of study of quality of work in the Kurdistan Province emphasised the fact that various coefficients of uniformity depend on the field conditions and one is not allowed to use a given uniformity coefficient for any other field conditions (Maroufpoor et al. 2010).

Figure 5 Measured and evaluated results, Bauer Rainstar 90/300 irrigation machine



CONCLUSION

In this paper, we introduced the design and testing of the measuring device SPEEDMETER SM2 for the speed of hose winding. This device enables monitoring of changes in the quality of work and subsequent storage of the results in database. From the results, we can draw consequences for better quality of work of irrigation machines. By modernization of the monitoring equipment, we eliminated the need of a worker handling the device and recording the measured values. The device therefore enables monitoring of the speed of hose winding on the reel and it is able to store up to 122 values. It is limited only by the memory of the used logic unit. The export of the measured values to the PC is performed by connection to RS 232 or USB. The interval speed of saving the measured values into the internal memory of the logic unit may be in the range of 1 sec to 32768 sec (approx. 9.1 hours).

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EFFECTS OF ZINC ON ANAEROBIC FERMENTATION OF SEWAGE SLUDGE AND BIOGAS PRODUCTION

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Abstract: Toxic metals can be present in municipal wastewaters sludge and may inhibit the process of anaerobic fermentation. According to literature zinc is one from toxic metals with the strongest inhibitory effect. Therefore, this article deals with effect of zinc on anaerobic fermentation of sewage sludge and biogas production. Inhibitory effect of zinc on anaerobic stabilization of sewage sludge was studied using batch anaerobic fermenters at temperature 42 °C ± 1 ºC. Hydraulic retention time was 21 days. As toxic substance was used zinc chloride ($ZnCl_2$) in three different amounts: 75, 312 and 625 mg/l which represent 12, 50 and 100 mg/l Zn, respectively. Biogas and methane yield after 21 days hydraulic retention time were used as comparative parameters of inhibitory effect of zinc. There were no significant differences between biogas yields from all tested concentrations of zinc and blank. There was only one significant difference between methane yields from tested concentrations of zinc and blank. The reduction of 6.3 ± 2.5% in the cumulative methane production can be observed after addition of 100 mg/l Zn (625 mg/l $ZnCl_2$).

Key Words: anaerobic stabilization, municipal wastewaters sludge, inhibitory effect, zinc chloride, methane yield

INTRODUCTION

Municipal wastewater treatment plants (WWTP) produce sewage sludge as a by-product of the physical, chemical and biological processes used during treatment. Current daily production of sewage sludge ranges from 60 to 90 g of dry solids per population equivalent (PE) in EU (Apples et al. 2008). This sludge must undergo some treatment in order to reduce its volume, to transform organic matter into a relatively stable or inert organic and inorganic residue and to reduce amount of pathogenic microorganisms.

The disposal of sludge may represent up to 40% of the capital costs and 50% of the operating costs of WWTP (Spellman 2009). There are many possible ways how to handle with sewage sludge. Because of energy-rich biogas production, anaerobic fermentation is economically sustainable way of sludge stabilization.

The anaerobic fermentation involves a complex interaction of several groups of bacteria. Methanogens being the final group of microorganisms, which converts acetate and carbon dioxide with hydrogen into methane (Codina et al. 1998). Optimal conditions for microbial community are important to achieve the highest possible yield and quality of biogas. Toxic metals can be present in municipal wastewaters sludge and may inhibit the process of anaerobic fermentation. According to literary sources (for example Sarioglu et al. 2010) the relative toxicity of metals, obtained by using the inhibition of methanogenic activity assay, is Cu > Ni ≈ Zn > Pb. According to other authors inhibition effect of toxic metals is quite different: Zn > Cr > Cu > Cd > Ni > Pb (Mudhoo and Kumar 2013) or Zn > Cr > Ni ≈ Cd (Altaş 2009). Therefore, this article deals with effect of zinc on anaerobic fermentation of sewage sludge and biogas production.

MATERIAL AND METHODS

Sludge samples were taken at the WWTP Brno - Modřice, 513 000 PE, Czech Republic. Sludge samples were collected directly from the anaerobic stabilization tank at the WWTP, according to Czech

Standard Method CSN EN ISO 5667-13. After the collection, the sludge samples were transported to the laboratory immediately.

To determine the sludge dry matter (DM) content according to Czech Standard Method CSN EN 15934 fresh samples were dried at 105 °C ± 5 ºC, the laboratory oven EcoCELL 111 (BMT Medical Technology Ltd., Czech Republic), was used. Organic dry matter (ODM) content was determined by incineration of the samples in a muffle furnace at 550 °C ± 5 ºC according to Czech Standard Method CSN EN 15169, using a furnace (LMH 11/12, LAC, Ltd., Czech Republic). Sludge's pH, redox potential and conductivity were determined by using pH/Cond meter 3320 (WTW GmbH, Germany) in accordance with CSN EN 12 176 standard. The content of zinc in dried sample was determined by handheld spectrometer and metal analyser DELTA PROFESSIONAL (BAS Rudice, Czech Republic).

Biogas yield and quality was measured using batch anaerobic fermenters at temperature 42 °C ± 1 ºC, according to German Standard VDI 4630. Two systems, which each consists of eight batch fermenters of volume 5 dm^3, were used. All sixteen batch fermenters were filled up with 3 dm^3 of sludge samples from anaerobic sludge stabilization. In this research, glycerine (7 ml) was used as a co-substrate for carbon and energy source for microbial growth. In both systems, two batch fermenters were used as a blank. Into remaining fermenters three different amounts of zinc chloride ($ZnCl_2$) were added to achieve required concentration of zinc (Table 1). All tests were done in duplicate in both systems.

Table 1 Tested amounts and concentrations of zinc

Concentration of Zn [mg/l]	Amount of $ZnCl_2$ [mg/l]
Blank	0
12	75
50	312
100	625

The biogas produced was collected in wet gas meters over a defined period of 21 days and was measured daily. Methane (CH_4), carbon dioxide (CO_2), hydrogen (H_2) and hydrogen sulphur (H_2S) content was measured during the batch fermentation tests using gas analyser COMBIMASS® GA-s (BINDER GmbH, Germany). Biogas production was converted to standard conditions (T_0 = 273 K, p_0 = 101 325 Pa). The volume of biogas and methane produced by a sample was converted to biogas yield and methane yield, by expressing them as m^3 per kg of organic dry matter (ODM) of the substrate. All measurements were done in triplicate. All measured values are expressed as arithmetic mean ± standard deviation.

RESULTS AND DISCUSSION

Results of the toxicity effect of heavy metals on sewage sludge stabilization are quite different in literature. There are some possible reasons of this difference. For example the carbon sources used for anaerobic metabolism (glucose, volatile fatty acids etc.), measured evaluation parameter (methane or hydrogen production, chemical oxygen demand removal etc.), used reactors (batch or continuous), characteristics of anaerobic sludge, binding strength of a heavy metal ion to the anaerobic sludge (sorption, precipitation) (Sarioglu et al. 2010). The potential toxicity of heavy metals is significantly controlled by the physical and chemical environment in which they are present, and this is correlated to different ion-specific physicochemical parameters, e.g. standard redox potential, electronegativity, the solubility product of the corresponding metal-sulfide complex, the Pearson softness index, electron density and the covalent index (Workentine et al. 2008 in Chen et al. 2014). Moreover the operating solids level significantly impacts the heavy metal toxicity in anaerobic digesters by providing protection from metal inhibitory effect (Hickey et al. 1989 in Chen et al. 2008). Unfortunately, most of the literature only reported the inhibition concentration values in mg/l (Chen et al. 2008).

For the above reasons it is necessary to specify characteristics of tested sewage sludge, which are shown in Table 2.

Table 2 Sewage sludge sample characteristics

Sample	pH [-]	Redox potential [mV]	Conductivity [S/m]	Dry matter [%]	Organic dry matter [%]	Content of Zn [mg/l]
Sewage sludge	7.32 ± 0.01	-38.30 ± 0.40	0.70 ± 0.01	3.50 ± 0.05	57.11 ± 0.45	56.00 ± 3.60

Specific biogas yield generated after 21 days hydraulic retention time is shown in Figure 1 and Table 3. There are no significant differences between yields from all tested concentrations of zinc and blank.

Figure 1 Cumulative biogas yield during fermentation

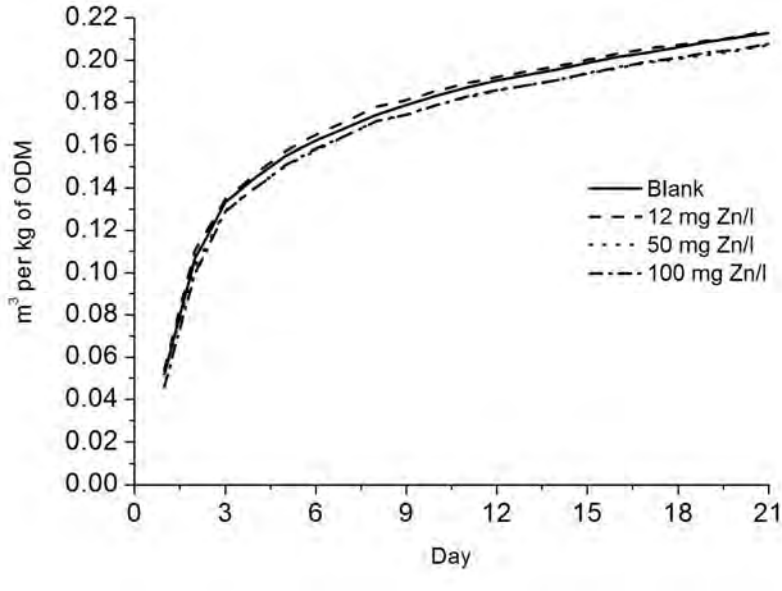

Table 3 Biogas yield after 21 days hydraulic retention time

Sample	Specific biogas production [m^3 per kg of ODM]	Relative biogas production [%]
Blank	0.212901 ± 0.004586	100.0 ± 2.2
12 mg Zn/l	0.213972 ± 0.001260	100.5 ± 0.6
50 mg Zn/l	0.207165 ± 0.005432	97.3 ± 2.6
100 mg Zn/l	0.207841 ± 0.000941	97.6 ± 0.4

Biogas composition generated after 21 days hydraulic retention time is shown in Figure 2. Specific methane yield after same time is shown in Table 4. There is only one significant difference between methane yields from tested concentrations of zinc and blank. The reduction of 6.3 ± 2.5% in the cumulative methane production can be observed after addition of 100 mg Zn/l (625 mg/l $ZnCl_2$).

These results are quite different from findings of other researches. For example when Altaş (2009) used unacclimatized (no adaptation to zinc) granular anaerobic sludge as a substrate, the inhibiting concentration of zinc that causes a 50% reduction in the cumulative methane production relative to the control sample over a fixed period of exposure time (24 h) was 7.5 mg/l. With whey as a substrate, Zayed and Winter (2000) observed 50% inhibition of methanogenesis in the presence of $ZnCl_2 \geq 40$ mg/l. According to their research, addition of zinc chloride at concentrations of 60 mg/l, 120 mg/l and 200 mg/l led to an inhibition of methane formation of 80%, 90% and 94%, respectively.

On the other hand, Sarioglu et al. (2010) used anaerobic sludge taken from an up-flow anaerobic sludge blanket reactor treating the wastewaters of Pakmaya Yeast Factory, cumulative methane gas

production decreased to 55 and 43%, respectively for 500 and 1000 mg/l Zn. Lin and Chen (1999) tested sludges that were obtained from an up-flow anaerobic sludge blanket reactor treating winery wastewater, the concentration at which zinc caused 50% inhibition of methane production was 690 and 270 mg Zn/l, respectively at hydraulic retention time 1 and 2 days.

At these examples is shown that the dosage of zinc for a 50% inhibition of methanogenesis during wastewater sludge anaerobic stabilization is not clear from the literature. Precipitated chemical forms of heavy metals, as sulfides, carbonates and hydroxides, may be sorbed onto either biomass or inert particulate matter and forming of complexes in system. Therefore, it is difficult to understand and interpret the heavy metal behaviour in anaerobic stabilization of sewage sludge (Sarioglu et al. 2010).

Figure 2 Methane and carbon dioxide concentration in biogas

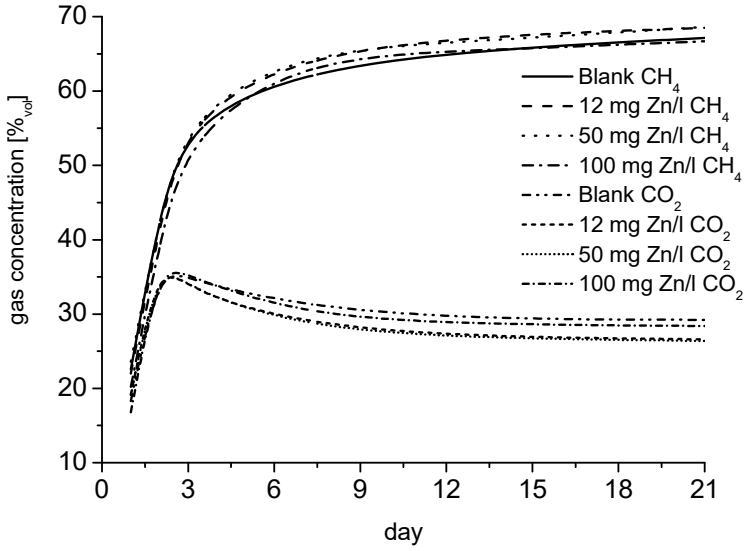

Table 4 Methane yield after 21 days hydraulic retention time

Sample	Specific methane production [m^3 per kg of ODM]	Relative methane production [%]
Blank	0.057319 ± 0.001151	100.0 ± 2.0
12 mg Zn/l	0.057174 ± 0.001129	99.8 ± 2.0
50 mg Zn/l	0.057013 ± 0.000811	99.5 ± 1.4
100 mg Zn/l	0.053679 ± 0.001415	93.7 ± 2.5

CONCLUSION

Inhibitory effect of zinc on anaerobic stabilization of sewage sludge was studied using batch anaerobic fermenters at temperature 42 °C ± 1 ºC. Hydraulic retention time was 21 days. As toxic substance was used zinc chloride ($ZnCl_2$) in three different amounts: 75, 312 and 625 mg/l which represent 12, 50 and 100 mg/l Zn, respectively. Biogas and methane yield after 21 days hydraulic retention time were used as comparative parameters of inhibitory effect of zinc. There were no significant differences between biogas yields from all tested concentrations of zinc and blank. There was only one significant difference between methane yields from tested concentrations of zinc and blank. The reduction of 6.3 ± 2.5% in the cumulative methane production can be observed after addition of 100 mg Zn/l (625 mg/l $ZnCl_2$).

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ANALYSIS OF THE PHYSICO-CHEMICAL PROPERTIES OF THE HYDRAULIC FLUIDS IN ORDER TO MODIFY CHANGE INTERVALS

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Abstract: The present paper deals with laboratory studies of contamination of hydraulic oils with analysis of the physico-chemical properties, in order to determine and lengthen the change interval of hydraulic oils used in stationary pressing devices. The aim was to determine the degree of contamination of samples of hydraulic fluids, to prolong change intervals, to ensure the proper operation of hydraulic presses. Result of the analysis of hydraulic fluids was, that the physico-chemical properties of the used hydraulic fluids, after the two-year period of usage, preserve the properties, which determine the correct operation of the production of pressing equipment.

Key Words: hydraulic oil, hydraulic press, physico-chemical properties, contamination

INTRODUCTION

Today time offers us various kinds of industrial fluids. Hydraulics, which is used in transport and handling equipment, for its operation need the working medium - liquid in its hydrostatic systems (Kosiba 2013). Each liquid has its own characteristics that have different impact on the various elements of the hydraulic system of working equipment. Hydraulic fluid must meet all the conditions, which can occur during the operation of hydraulic systems.

Production of hydraulic oil in the required quality, with minimal environmental pressures and acceptable cost will become more complex, and thus creating a space for the use of specific hydraulic oils according to strict performance requirements.

Industrial oils are most often characterized and described by the device according to ISO and DIN standards. An important part, in some cases, is also the classification or possible approval by the standards of major equipment manufacturers.

Oil analysis can reveal the amount of wear metals, oil pollution, and the amount of additives and physico-chemical parameters of oil. Several authors, e.g. Vasishth et al. (2014), Wan Nik et al. (2005), Kumbár et al. (2012), deal with the similar issues. To determine the technical parameters of oil filling is necessary to use appropriate diagnostic methods.

MATERIAL AND METHODS

Hydraulic fluids in the hydraulic system have multiple functions. Besides energy transfer, universal oil must lubricate, dissipate the heat and be compatible with seal materials and metal materials of the components of the system (Majdan et al. 2014). The basic prerequisite for the proper functioning and effective care of hydraulic fluids is well chosen methodology of monitoring impurities in liquid and continuous comparison of the level of contamination with the behaviour of the machine (Orlík and Tkáč 2011). Contamination of the liquid can be divided into two categories, namely contamination by particles and contamination by fluids. Contamination by particles includes organic, inorganic and metal particles. Air, water and other foreign fluids are a group contamination of the fluids by other fluids (Tkáč et al. 2010).

The aim of the paper is analyses of the physico-chemical properties of hydraulic fluids. In case of the new hydraulic oil, as indicators were selected the basic parameters specified by the manufacturer, and subsequently evaluated changes in the physico-chemical properties of hydraulic fluids. Hydraulic oils sampled from running tests were subjected to the following laboratory analysis:

1) **Ferrographic analysis -** using mentioned analysis with magnetic separation of particles, which are separated during wear of friction pairs in the lubrication system, we investigated the size and the morphology of the particulates of wear.
2) **Temperature dependence of viscosity -** viscosity as one of the most important rheological parameters is defined as the resistance of a fluid to flow. Viscosity of most of the liquids decreases with increasing temperature according to Arrhenius equation (Figura and Teixeira 2007):

$$\eta = \eta_0 e^{\frac{E_A}{RT}}, \quad (1)$$

where η is, dynamic viscosity (Pa/s), η_0 is reference value of dynamic viscosity (Pa/s), E_A is activation energy (J/mol), R is gas constant (J/K/mol) and T is absolute temperature (K). Present data have been obtained from measurements performed on laboratory viscometer DV2T fy Brookfield. The experiments have been performed with use of ULA (0) spindle.

3) **Measurement of acid value -** the total acid number (TAN) is an important indicator of the quality of the used oil, and indicates the quantity of such acid in the oil, to determine the degree of degradation of the oil.
4) **Measurement of the content of water -** the water in oil is undesirable factor which arises during operation of the machine and causes unfavourable degradation processes which can result in various degrees of failure of the device. For this measurement, we used devices from HYDAC.
5) **Monitoring of pour point by differential scanning calorimetry (DSC) -** differential scanning calorimetry or DSC is a thermo-analytical technique which monitors heat effects associated with phase transitions and chemical reactions as a function of temperature, at pre-defined speed of heating (cooling), with assuming that both materials – sample and reference are under the same conditions (Haines 1995).

Description of the production plant

DS Smith Worldwide Dispenser ™ is a part of the division DS Smith Plastics a world leader in the production of plastic dispenser. DS Smith Plastics Division Worldwide Dispensers ™ operates a manufacturing facility in Nitra since 2008, focusing in the production of packaging materials, food industry, which are produced by compressing hydraulic and electric presses.

Description of handling equipment

In the manufacturing plant is located 53 press lines ARBURG, NETSAL, DEMAG, KRAUSS MAFFEI, which are working on the hydraulic principle and two press lines are electric. Facility requirements were to find out how much is contaminated oil, by physico-chemical analysis, at press lines ARBURG number 40, 41 and 42, and to propose suitable oil change interval. In view of the utilization of hydraulic fluid in a machine, it is the most important to know the running properties of the fluid, i.e. to know the influence of the fluid on the technical state of the hydraulic system parts (Jablonický et al. 2007, Kosiba et al. 2013, Tkáč et al. 2008, Žikla and Jablonický 2006). The parameters of the individual lines are described in the Table 1.

Table 1 Parameters of production press lines in manufacture DS SMITH

ARBURG 470C allrounder 1500–350								
Line No.	**Oil**	**Date of filling**	**Date of sampling**	**Volume (L)**	**Pressure (10^5 Pa)**	**Flow (L/min)**	**Cycle (s)**	**Products/Cycle**
40	OSO S 46 AGIP	05.09.2014	01.03.2016	260	60.2	145	32.86	8
41	OSO S 46 AGIP	15.08.2014	01.03.2016	260	52	68.7	21.93	8
42	OSO S 46 AGIP	11.08.2014	01.03.2016	260	39.1	44.5	25.35	12

RESULTS AND DISCUSSION

Analysis of physico-chemical properties of hydraulic oils was conducted in the laboratories of the Department of Transport and Handling, and in the laboratories of the Department of Physics at the Slovak University of Agriculture in Nitra. The Department of Transportation and Handling carried out the analysis, which results are shown in Table 2.

Table 2 The results of chemical analyses

Line No.	**Ferrographic analysis**	**Acid value (mg KOH/g)**	**Content of water (%)**
40	clean	0.75	0
41	clean	0.95	0
42	clean	1.00	0

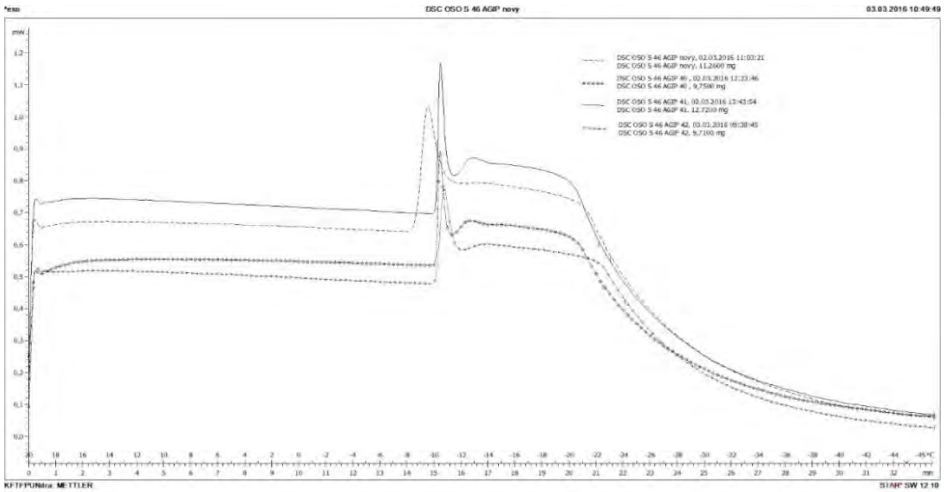
Ferrographic analysis – oil in hydraulic presses even after two years of use showed no metal particles. Samples of hydraulic oils were clean.

The acid number – the oil producers indicates use of oil up to 1.3 mg/g KOH because the hydraulic oil does not exceed this number, it is still suitable for use.

The content of water – the analysis of the water content of all the three samples was 0%.

For monitoring of pour point of oils (phase transition of oil components) by DSC method was used device DSC 1 (METTLER-TOLEDO). Samples of hydraulic oil OSO S 46 Agip with weight (9–13) mg were hermetically sealed in aluminium crucibles and thermally treated at the speed of heating (cooling) 2 K/min in the temperature range from 20 °C to the temperature of -45 °C. The measurement was carried out in an inert, dynamic atmosphere of N2. As a result we got a DSC thermogram, which was evaluated in STARe software (see Figure 1).

Figure 1 DSC measurement of different samples of OSO S 46 Agip hydraulic oil

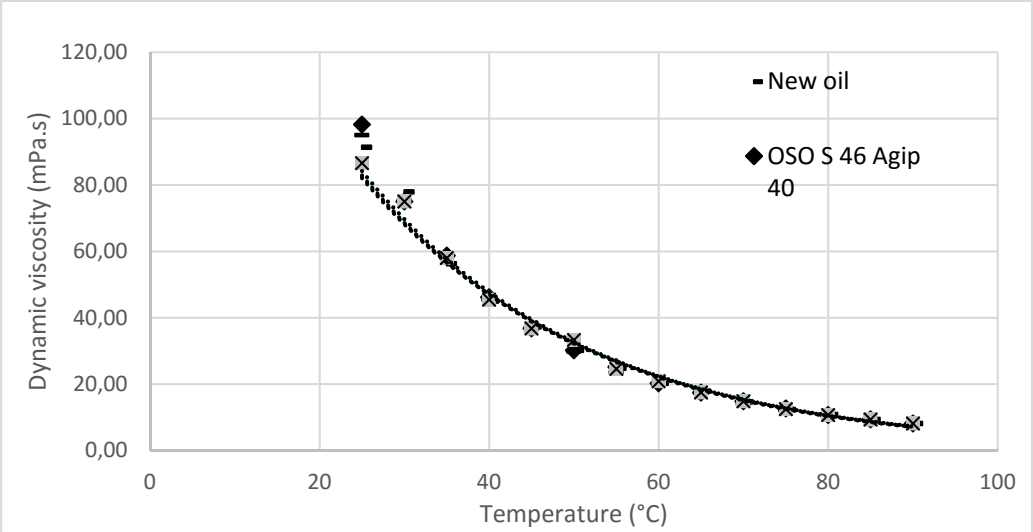


In the process of oil freezing and in the case of a new oil sample, we observed exothermal peak at the temperature -9.51 °C. This point is defined as a freezing point and temperature is nearly equal to the melting point (depending on the material purity). In the case of oil from press line No. 40, the temperature of peak was -10.41 °C. In the sample, where was used oil from press line No. 41, the temperature of exothermal peak was almost the same as previous -10.44 °C. The last sample was from press line No. 42 and its pour point was at -10.75 °C.

The graph indicates that peaks for used samples are almost identical, so we can assume that the difference between them is not significant in terms of pour point, but the difference between new and used sample is distinct. The experiment shows that the new sample has higher temperature of pour point.

Dynamic viscosity, as a function of temperature of four samples of hydraulic oil OSO S 46 Agip 46, has been considered. First sample was new. The others were used oils in three different press lines. The procedure of sample preparation for viscosity measurements corresponded to a typical sampling procedure. The adequate volume (20 ml) of oil was put into the apparatus cuvette. The viscosity data were obtained in temperature range from 25 °C to 90 °C. All samples were measured in approximately equal conditions. More precisely, at about the same torque (50%) and shear stress (0.36 N/m^2).

Figure 2 Temperature dependent viscosity of one unused and three samples of used hydraulic oil



As the samples of the oil were taken from the press lines where the operating temperature is 50 °C, most attention has been paid what happens with the viscosity at that temperature. It is possible to observe from Figure 2 that dynamic viscosity of hydraulic oils is decreasing exponentially with increasing of temperature, what was expected and corresponds with conclusions reported in literature (Hlaváč and Božiková 2014, Hlaváč et al. 2014, Severa et al. 2012, Trávníček et al. 2013, Valach et al. 2015, Vozárová et al. 2015). Regression equations and determination coefficients for individual samples are in the Table 3. As it can be seen from the results, the determination coefficients for all the samples are very high, which also confirms strong exponentially decreasing dependence.

Table 3 Determination coefficients and regression equations

Sample	Regression equation	Determination coefficient R^2
New oil	$\eta = 210.6e^{-0.038t}$	0.9910
OSO S 46 AGIP 40	$\eta = 216.84e^{-0.038t}$	0.9899
OSO S 46 AGIP 41	$\eta = 213.77e^{-0.038t}$	0.9887
OSO S 46 AGIP 42	$\eta = 207.72e^{-0.037t}$	0.9935

CONCLUSION

Hydraulic equipment are widely used in executive mechanisms of agricultural and forestry machinery, as well as in many other areas. Development of advanced hydraulic components is aimed at

increasing the transmitted power, reducing energy severity, minimize environmental pollution and improve the technical life and reliability of the machine (Tkáč et al. 2007).

Hydraulic oil is often faces unforeseen operating conditions that have a significant impact on its life. The reason for the exchange of the hydraulic oil is degradation, loss of additives and impurities in the oil. Kumbár (2014) states that the suitability rises when the samples of used oil are compared with the sample of new (unused) oil with same specification.

Our analysis consists of comparison physico-chemical properties of samples of new and used hydraulic oil OSO S 46 Agip. Results of ferrographic analysis show that oil in hydraulic presses even after two years of use showed no metal particles. Samples of hydraulic oils were clean. Values of the acid number of the used hydraulic oils does not exceed number which the oil producers indicates (up to 1.3 mg/g KOH), oils are still suitable for use. The water content of all the three examined samples of used oils was 0%.

In the case of pour point of oils the experiment shows that the new sample has higher temperature of pour point. However, it is important to indicate, that pour point specified by hydraulic oil producer is -27 °C. This difference in temperature between the information from the producer and the measured values is not so significant in the case of using oil in hydraulic systems, because press lines are located in halls with temperatures above zero. But it is considerable when it comes to the storage of oil, because the warehouse is not heated, practically oils are exposed to the outside temperature. If the winter temperatures are below -10 °C, it may change the characteristics of the aforementioned material.

It can be concluded that knowledge of viscosity behaviour of a hydraulic oil as a function of temperature is of great importance, especially when considering running efficiency and performance of press line. Thus, its function can be sensitive to the viscosity characteristics of the oil. Viscosity influences the oil's ability to flow through the hydraulic system, therefore affects the pressure required to push the oil sufficiently to develop the necessary flow. The rate of oil flow is important to the life of the hydraulic system. Our measurements of viscosity as a function of temperature did not show significant differences between new and used hydraulic oils. Dynamic viscosity of hydraulic oils is decreasing exponentially with increasing of temperature in accordance with theory (Arrhenius equation) and other authors (Hlaváč and Božiková 2014, Hlaváč et al. 2014, Severa et al. 2012, Valach et al. 2015, Vozárová et al. 2015). That results are also in compliance with results of ferrographic analysis that showed no metal particles. Samples of hydraulic oils were clean, they probably content no particles (neither non-metal), which should change viscosity of used oils.

Result of the analysis of hydraulic fluids was, that the physico-chemical properties of the used hydraulic fluids, after the two-year period of usage, preserve the properties, which determine the correct operation of the production of pressing equipment.

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PROPOSAL METHODOLOGY OF DIESEL ENGINE EMISSION MEASUREMENT BY MODIFIED METHOD OF FREE ACCELERATION

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Abstract: The article illustrates the problems related to the production of emissions of diesel engine, while the negative influence of the exhaust gases emitted is undesirable to humans, for ecosystems and for the actual working environment of agricultural machinery. In this time, methods of measurement of exhaust gas of diesel engines equipped with an exhaust system with exhaust gas filtration are considered inadequate. The purpose of the allowance therefore consists of establishing the methodology of measuring the exhaust gas NO_x on the selected reference engine. Established methodology consists of loading the engine modified with free acceleration from idle to a maximum speed and scanning the emission of NO_x at introduced current speed and time monitoring the regeneration of the NO_x emissions at idle speed. For an objective assessment of mentioned parameter, the required number of repetitions is set with the help of statistical methods. At the beginning of the methodical process of measuring emissions, it is necessary to warm up the engine to operating temperature, which is performed by running the engine at higher speed and subsequent five rinsing accelerations from idle speed to a maximum. Motor load was formed by inertial forces of dynamic changes during the acceleration of the engine rotating parts. When measuring with free acceleration, results were evaluated based on the recovery time to baseline NO_x max. 25 s while we were waiting for stability NO_x emissions to baseline with a tolerance of -1% ppm of baseline. The results obtained from experimental work serve as a proposal for the further development of science in the area of transport and diesel engine. Based on a sample of measured data, it is possible to set the limit values of NO_X emissions for the reference engine. These were important for determination of the NO_x emissions curve depending on speed, whereby the course we have expressed mathematically by power function.

Key Words: engine emission measuring, limit values of exhaust gases, emissions curve

INTRODUCTION

Presently, diesel oil and petroleum products belong to the most utilized fuels. Because of its irretrievability the crude oil is often called ‘the black gold’. Unfortunately, fossil fuels are not renewable or inexhaustible sources of energy. On the other side, ecology and environmental protection are the world´s global interests. There are a lot of negatives on fossil fuels, on which our society is depending to a high degree. One of the most important disadvantages is fouling the air and causing the greenhouse effect, which affects weather in a matter of temperature (Angelovič 2013).

Exhaust gases, i.e. products of combustion, are one of the most serious shortcomings of internal combustion engine (Lenďák et al. 2014, Szabó et al. 2013, Vitázek 2014b).

The emitted exhaust gases of the diesel internal combustion engine have a negative impact not only on human beings but also on the environment, thus on the air, agricultural land and water. To avoid the major impact of emissions of internal combustion engines, not only on the environment but also on humans, it is necessary to establish a regular inspection of exhaust gases of diesel engines (Vitázek et al. 2014a, Janoško 2014).

With regular measurement of exhaust emissions for example during regular maintenance, periodic emission controls it would be possible to avoid increasing emissions by certain components of exhaust gases of combustion engines. Suitable methods should provide sufficient information about the engine, not only about its technical and emission status but also about hidden damages, which may not be manifested during the normal operation (Lenďák et al. 2013, Vitázek et al. 2015).

Engine wear is closely related to the quality of fuel combustion and also with the amount of produced harmful emissions. That is why it is necessary to look particularly at choosing the right method that will be financially advantageous and time-saving, but especially versatile and precise enough to assess a wide range of used engines (Jukl et al. 2014, Polonec and Janoško 2014).

For this reason we proposed new methodology of measuring emissions of diesel engine, which is not depend from smoke quantity.

MATERIAL AND METHODS

For measurement, we have used a diesel engine LOMBARDINI LDW 502 M3, with an emission standard EURO 2, because of the widely used engines with a similar speed characteristic of agricultural practice. A condition for meeting the emission standard EURO 2 is the engine equipped with venting the crankcase. Selected engine is water-cooled two cylinder in-line four strokes with indirect injection of fuel and the injection system pump – nozzle. The valve timing is OHV, with two valves per cylinder. The basic technical parameters of the engine are shown in Table 1.

Table 1 Technical parameters of the engine LOMBARDINI LDW 502

Basic technical parameters of LOMBARDINI LDW 502 M3	
Number of cylinders, pc	2
Engine capacity, cm^3	505
Compression ratio, -	22.8 : 1
Engine output (according to 80/1269/CEE), ISO 1585, kW / HP	9.3 / 12.6
Max. torque, Nm	26
RPM at max. torque, min^{-1}	2,200
Idle, min^{-1}	1,100
Rated speed, min^{-1}	3,750
Maximum speed, min^{-1}	3,820

Measuring device for emission measuring

Diesel emissions will be measured with five gas analyzers MAHA MGT 5. The analyzer measures (CO, CO_2 and HC) on the principle of non-dispersive infrared absorption, and (O_2 and NO_x) are measured by electrochemical cells. The analyzed gas is taken with a probe from the car exhaust. Subsequently, the measured gas separates the water and flue gases flow into the measuring chamber. Gas molecules with the same atomic number (like $H_2/N_2/O_2$) do not cause absorption in the infrared spectrum. Molecules with different atomic numbers are in the infrared absorption spectrum of various absorption scales. The stronger the absorption the higher the gas concentration. Electrical signal produced by the chemical sensor is proportional to the percentage of oxygen or NO_x.

Speed sensor

To ensure the accuracy of measurements, two speed sensors will be used. The main speed sensor will be a device MAGTROL TMB 310 and the second, additional, will be AVL DiSpeed 492. The scanning of speed is based on sensing the signal of vibrations of the engine, or recording the sound of vibrations that are detected by the sensor which is attached to the engine of inspected vehicle with a permanent magnet. In terms of the correctness of the measurement, it must be ensured that the magnetic sensor is placed in the movement direction of the inspected engine piston.

The results of measuring NOx emissions

Before the measurement, it was necessary to warm up the engine to its operating temperature with the aid of increasing engine speed, and then we performed 5 rinse accelerations from idle to the maximum engine speed. We performed 50 free accelerations of engine, as listed in Table 2 to Table 4. Idle speed at 1,100 min^{-1} indicated by manufacturer was adjusted by adding and subtracting the value of 50 min^{-1}, and maximum speed values were modified by adding 150 min^{-1} to the maximum speed. The accelerator pedal was pressed during the measurements for one second, to be inferred the greatest possible load on the engine as well as to ensure the maximum dose of fuel. Engine load comprised of inertia forces of dynamic changes during the acceleration of engine rotating parts. The minimum number of measurements has been determined using mathematical and statistical methods based on the 50 measured samples in Table 2 for idle speed and in Table 3 for the maximum engine speed. At idle speed (Table 2), the minimum number of repetitions 1 was determined by mathematical and statistical analysis, and at the maximum speed (Table 3), the number of repetitions was 2. Based on experience and existing methodological guidelines for measuring the values of smoke, the minimum number of repetitions of NO_x emissions was determined to 3.

Table 2 Determine the minimum required number of repetitions – idle speed

Maximum value			***225***
Minimum value			***205***
Arithmetic average			***214.36***
Critical value of the normal distribution, t_α		90%	***1.645***
Critical value of the normal distribution, t_β		90%	***1.282***
Required accuracy Δ, m^{-1}			***0.1***
Maximum permissible error δ, %			***10***
Standard deviation, s			***4.797***
Coefficient of variation, v_k, %			***2.24***
Determination of minimum number of repeated measurements			
The number of repetitions of the known coefficient of variation for t_α			***1***
The number of repetitions of the known coefficient of variation for t_β			***1***
The number of repetitions at δ/v_k	90%	4,469	**-**

Table 3 Determine the minimum required number of repetitions – maximum speed

Maximum value			***76***
Minimum value			***52***
Arithmetic average			***63.56***
Critical value of the normal distribution, t_α		90%	***1.645***
Critical value of the normal distribution, t_β		90%	***1.282***
Required accuracy Δ, m^{-1}			***0.1***
Maximum permissible error δ, %			***10***
Standard deviation, s			***5.44***
Coefficient of variation, v_k, %			***8.56***
Determination of minimum number of repeated measurements			
The number of repetitions of the known coefficient of variation for t_α			***2***
The number of repetitions of the known coefficient of variation for t_β			***2***
The number of repetitions at δ/v_k	90%	1.168	**-**

In the design of methodology for measurement of NO_x emissions, we consider the notion of regeneration of NO_x emissions by engine load free acceleration. Regeneration will be given by the time it takes for emissions of NO_x to get after loading to the initially measured value before loading. In Table 4 we introduced a suitable time range, the value of which we received by individual measurements. Its value ranged between 17.08 and 23.01 seconds.

The minimum number of repetitions was determined from the set of measured values as above. Mathematic and statistical analysis indicates that the minimum number of repetitions is 2.

Whereas in previous cases we determined the number of repetitions 3, also in this case we determine the number of repetitions 3.

Table 4 Determine the minimum required number of repetitions – time of regeneration

Maximum value		***23.01***
Minimum value		***17.08***
Arithmetic average		***20.305***
Critical value of the normal distribution, t_α	90%	***1.645***
Critical value of the normal distribution, t_β	90%	***1.282***
Required accuracy Δ, m^{-1}		***0.1***
Maximum permissible error δ, %		***10***
Standard deviation, s		***1.73***
Coefficient of variation, v_k, %		***8.52***

Determination of minimum number of repeated measurements

The number of repetitions of the known coefficient of variation for t_α			***2***
The number of repetitions of the known coefficient of variation for t_β			***2***
The number of repetitions at δ/v_k	90%	4.469	**-**

To confirm the correctness of our proposed NO_x emission tolerance and time range of regeneration, were carried out on the reference engine experimental measurements, with results reported in Table 5. The total number of measured data was 15, divided into five measurements in three repetitions. The experiment will be valid if the time range of regeneration and tolerance based on the input measured value of NO_x emissions is not exceeded. Table 5 indicates that the proposed tolerance and time ranges are correct.

Table 5 Measured and evaluated values of NO_x emissions during the regeneration test

Measurement	Repetitions	Value NOx measured before measurement, ppm	Stable value NOx after measurement ppm	Allowed tolerance	Regeneration time, t, s	
				-1%	max	25s
	1	210	209	-0.48%	20.48	in the range
1	2	216	216	0.00%	20.63	in the range
	3	217	217	0.00%	18.60	in the range
	4	210	209	-0.48%	24.92	in the range
2	5	210	210	0.00%	15.00	in the range
	6	216	214	-0.93%	22.99	in the range
	7	214	214	0.00%	15.20	in the range
3	8	214	213	-0.47%	22.14	in the range
	9	211	210	-0.47%	20.48	in the range
	10	214	212	-0.93%	25.00	in the range
4	11	213	212	-0.47%	17.39	in the range
	12	215	213	-0.93%	21.00	in the range
	13	205	203	-0.98%	24.85	in the range
5	14	214	213	-0.47%	20.61	in the range
	15	211	210	-0.47%	21.48	in the range

RESULTS AND DISCUSSION

In this article we were looking for an alternative, fast, time and financially efficient method of assessing the emission state of the diesel engine, because it is not possible to assess the emission state of the engine equipped with an exhaust filtration with the method of smoke measurement.

Our suggested method of measuring nitrogen oxides (NO_x) replaced the method of measurement of smoke. During the measurements, 150 samples of nitrogen oxides NO_x were taken from the reference engine. The method is defined by the principle of loading the engine by its own inertia and its consequent unloading. When unloading, NO_x recovery time to baseline recorded before acceleration is monitored. The results were evaluated in terms of recovery time of NO_x to baseline, and the maximum permitted value was 25 seconds while we were waiting for stabilization of NO_x emissions to baseline with a tolerance of -1% ppm of baseline. For the correctness of the measurement, a temperature stabilization of the engine is needed, in our case at 70 °C. Results achieved to this date can contribute to the search for optimization measurements of emissions of nitrogen oxides NO_x so as to fulfill the conditions of simplicity, repeatability and user-friendly instrumentation and preparation of the inspected vehicle, in order to state an objective assessment of the emission of inspected vehicle.

The advantage of the proposed method is time and financial efficiency. Despite their simplicity, there may be deficiencies that could cause bias to influence of measured values and therefore cause wrong conclusions. It will be necessary to continue to test the proposed method of measuring the engine emission state by measuring NO_x, to verify and eliminate deficiencies that may arise at different ways of forming, burning the mixture, and modification of exhaust gases.

To avoid the bias of results by the human factor, it is needed to optimize the software and hardware. After each stage of verification of methodological guidelines and proposals for measurement devices, the method of measuring NO_x emissions should be implemented in the international directives of the European Community dealing with the assessment of vehicle emission state (Tkáč et al. 2014, Králik et al. 2015).

CONCLUSIONS

Our suggested method, unlike the measurement method of smoke, evaluated also other approval emissions, based on which it is possible to determine the state of the engine exhaust system. The article focuses on the measurement of nitrogen oxides NO_x for the reference engine in order to propose such a measurement method which would be able to objectively assess the emission state of diesel engines. Part of the article is proposals for evaluating the measured emissions under free acceleration. For the measured engine, we set the limit conditions when the engine suits the emission test. I tis when the time of regeneration of NO_x emissions is below 25 seconds and the stable value of NO_x after measurement is in 1% ppm tolerance of baseline. To verify our proposed methods of measurements and their potential applications in a wide range of diesel engines and their exhaust systems, it is necessary to carry out further measurements and evaluating the results with other combustion engines that can form the basis for development of database systems.

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MODERNIZATION OF LEARNING FACILITIES ON EVALUATION OF COMBUSTION PROCESSES

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Abstract: The paper focuses on modernization of learning facilities on evaluation of combustion processes, parameters and graphic indication of limiting states. Primal part of device was older type of dynamometer used for measuring power of tractors, which was redesign and modernized for measuring the power about 140 kW and torque about 450 Nm of small vehicle's engines. For data acquisition and calculations was designed new software in programming language. The control of hydrodynamic brake was automatised and all important parameters of learning facilities and engine was recorded in PC and visualized on the main monitor. Designed and modernized learning facilities can measure basic parameters: engine torque, engine power, RPM, fuel consumption with gravimetric method and other numerous internal engine parameters, that can be set "on line" through a special engine control unit. The whole modernized learning facilities are designed on open platform with free access for administrator.

Key Words: combustion processes, modernization, engine parameters, learning facilities

INTRODUCTION

Monitoring of performance and technical parameters of internal combustion engines is an important part of the scientific research process. It is mainly used to verify the theoretical knowledge and practical assumptions (Janoško et al. 2013).

In order to reliably and securely verify the effects of different technical solutions, settings or operating conditions on the performance parameters of the engine, the most suitable location of engine is on a test device that allows to simulate different operating conditions and to read the greatest number of motor parameters in real-time (Chrastina et al. 2014).

The market offers number of more or less complex devices, which allowing monitoring various parameters of internal combustion engines. Their complexity is reflected in their price (Uhrinová et al. 2013).

By utilizing existing facilities, their modernization and expansion of the new features can save a substantial part of the funds (Jablonický et al. 2015).

We have therefore decided to modify and modernize existing equipment to measure performance of tractors through the P.T.O. shaft whereby can be monitored and simultaneously modify selected parameters of conventional internal combustion engines for passenger cars. Designed device combines the feature of a chassis hydrodynamic brake with the possibility not only of monitoring engine parameters but it also allows control and modify of the activity of the internal combustion engine in real-time.

MATERIAL AND METHODS

Hydrodynamic brake was constructed by rebuilding of older type of dynamometer that are used to measure the performance of the tractor through P.T.O. shaft. From the initial dynamometer was used water brake with the driven and a reaction turbine without reduction gearbox (Figure 1), allowing to measure high torque at low speed through the P.T.O. shaft. Its technical parameters are shown in Table 1.

Figure 1 Hydrodynamic brake with mounted an internal combustion engine

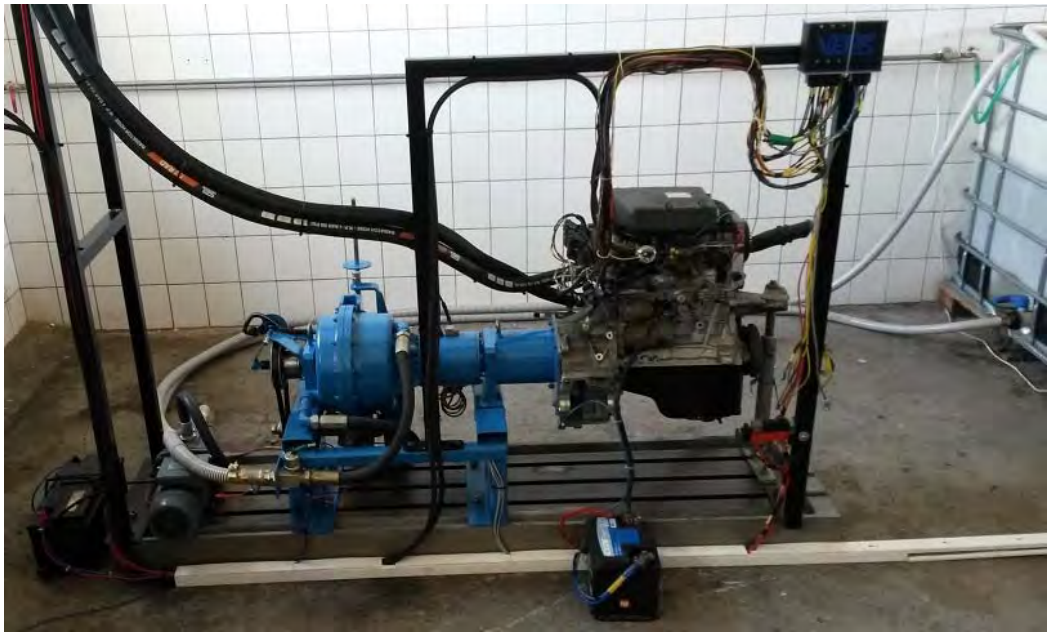


Table 1 Technical parameters of the original dynamometer

Maximum measurable power (kW)	140
Maximum rpm of P.T.O shaft (min^{-1})	2,000
The maximum measurable torque (Nm)	1,500
The gear ratio between the input shaft and turbine (-)	0.3036
The maximum allowable torque of input shaft at maximum power and maximum rpm (Nm)	668

Basic parameters of hydrodynamic brakes

Before starting conversions were carried out basic calculations to determine the suitability of using a dynamometer for the purpose of measuring power and torque of conventional combustion engines. Calculation of the maximum permissible rpm of the turbine $n_{t\ max}$ based on the maximum permissible rpm of the drive shaft of dynamometer and from gear ratio of reduction gearbox of initial dynamometer. From equation (1) results, that the maximum permissible rpm of the drive turbine of dynamometer are about 6,600 min^{-1}. This limit of the maximum permissible rpm is fully sufficient for diesel and most common petrol engines for passenger cars.

$$n_{t\ max} = \frac{n_{h\ max}}{i}, \quad (1)$$

Where:

$n_{t\ max}$ – the maximum permissible rpm of turbine (min^{-1}),

$n_{h\ max}$ – the maximum permissible rpm of the drive shaft (min^{-1}),

i – Gear ratio of reduction gearbox (-).

According to equation (2) we can determine the maximum measurable brake torque of turbine M_{kt}, which represents the value in the level $M_{kt\ max}$ = 455 nm, which is sufficient for most conventional diesel and petrol engines.

$$M_{kt\ max} = i.M_{kh}, \quad (2)$$

Where:

$M_{kt\ max}$ – torque on the turbine (Nm),

M_{kh} – torque of input shaft of the dynamometer (Nm),

i – Gear ratio (-).

The maximum permissible power of measured engine may be determined by the equation (3) to the level of $P_{t\ max}$ = 139.6 kW (at 6600 min^{-1}), which is sufficient for most existing, common, especially small-volume petrol and diesel engines of passenger cars.

$$P_{t\ max} = \frac{2\pi . M_{kt\ max} . n_{t\ max}}{60}, \quad (3)$$

Where:

$P_{t\ max}$ – the maximum allowable braking performance of dynamometer (W),

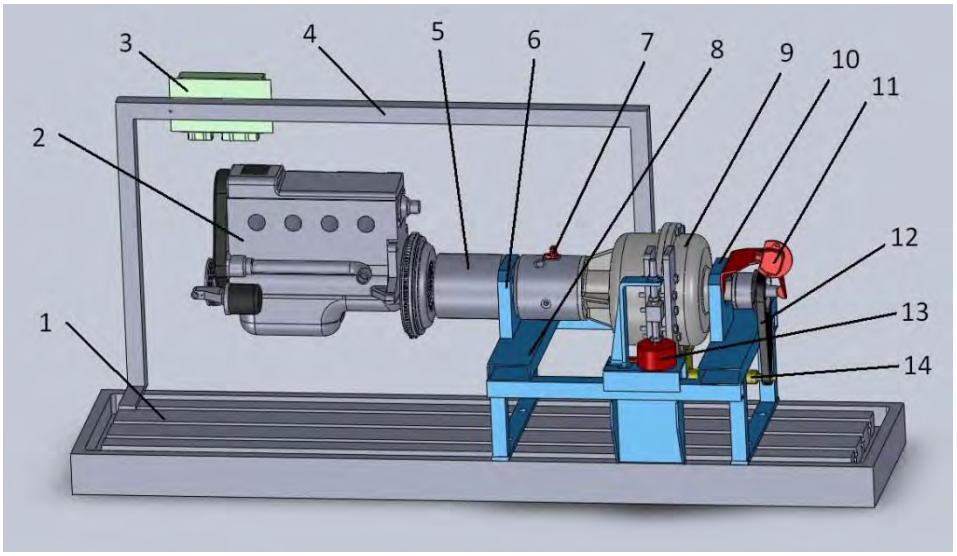
$M_{kt\ max}$ – maximum permissible safe torque of turbine (Nm),

$n_{t\ max}$ – the maximum permissible rpm of turbine (min^{-1}), (Janoško et al. 2014).

Mechanical construction of equipment

Proposal of test device for braking of engines (Figure 2) consists of a base plate, frame and body of hydrodynamic brake with turbines, reaction and calibration arm, main mounting flange, mechanism of adjustment load, water management and accessories with other components.

Figure 2 Assembly scheme of hydrodynamic brake - major parts



Legend: 1 - base plate, 2 - combustion engine, 3 - control unit of engine, 4 - auxiliary frame, 5 - flange for mounting the internal combustion engine, 6 - front pivoting of body brakes, 7 - rpm sensor, 8 - frame of dynamometer, 9 - hydrodynamic brake, 10 - rear pivoting of body dynamometer, 11 - sensor of eject position reaction turbine, 12 - belt gear, 13 - force sensor, 14 - electromotor at adjusting the position of the reaction turbine

The electronic part of the device

The electronic part of the proposed facility consists of two main parts, sensors and actuators. The sensing part through input-output card LabJack converts signals from the sensors to the computer. The reading of the other parameters is using control unit VEMS, which sends the measured values via the RS232 interface to the PC. The control part using the appropriate action and switching actuators executes commands sent from the computer. Using the designed software, it is possible fully automated to control the load of the hydrodynamic brake.

By using a sensing electronics of hydrodynamic brake it is now possible read the following parameters:

- Rpm of engine,
- Torque,
- The air temperature in the room,
- Air pressure in the room,
- Position of eject reaction turbine of hydrodynamic brake,
- Fuel consumption, mass method.

By using the control unit of engine can measure and record the following parameters:

- The oil temperature of engine,
- The temperature of the coolant,

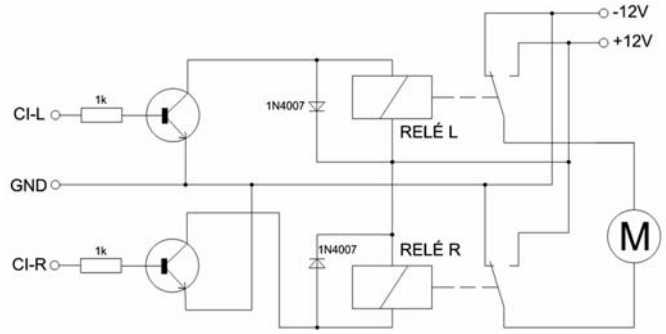
- The temperature of exhaust gases in two places,
- The air temperature in the intake manifold,
- Air pressure in the intake manifold,
- The position of the throttle,
- The richness of the fuel mixture, (Janoško and Chrastina 2014).

Modernization of equipment consisted inter alia in modification of mechanism adjustment of burden. Originally hand-operated mechanism was replaced by electromechanical system with automatic regulation. Electromotor by using worm gear and by belt gear (Figure 3) changes the position of driven and driving turbines of hydrodynamic brake.

Figure 3 Load setting mechanism of the hydrodynamic brake with the feedback

Operating of the electromotor is solved by using electronic switching unit (Figure 4) controlled by hydrodynamic brake software. Load adjustment can be performed in a manual or automatic mode.

Figure 4 Schematic diagram of the control electronics load setting of the hydrodynamic brake



Control and measurement software

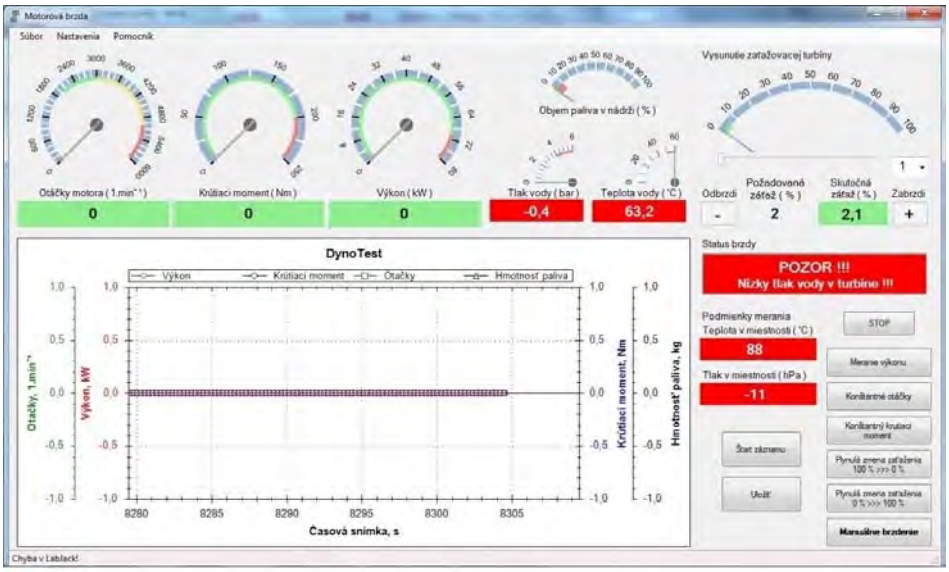
For operating of the hydrodynamic brake, data collection and data visualization was designed and created personal computer software.

Following of monitored parameters provides the user overview of actual measured values of performance, rpm, torque and fuel consumption in numeric and graphic form (Szabó et al. 2013).

The measured values can be saved and exported to a file in *.csv. format. The software enables step, continuous and automatic control of braking performance of hydrodynamic brake with the feedback sized load of internal combustion engine. Software has except operating and display functions also protective functions. In the event that any value exceeds the permitted limit value, the program immediately notifies the operator, respectively depending on the significance of safety occurs interruption of a measurement. The software was developed in C# on the platform of Microsoft® Visual Studio 2013 by using some freely available dynamically linked library. After starting the program, user

can immediately see the value of each measured variables and control settings of regulation in the graphics, respectively alphanumeric form (Figure 5).

Figure 5 *The main screen of the hydrodynamic brake software*

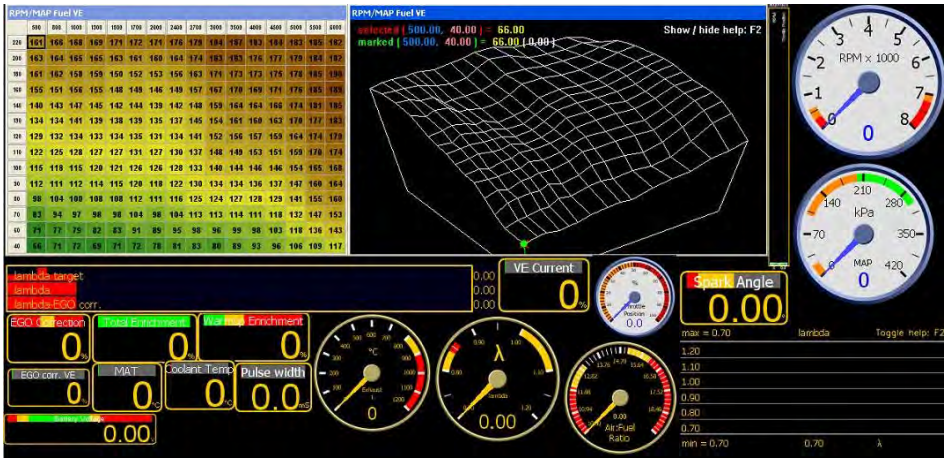


The software allows you to view in the chart a time frame of power, torque, rpm and volume of the fuel at tank. Furthermore, it is possible to record and portray also the rpm curves of engine.

Optimization of engine parameters

By use the fully configurable universal control unit VEMS, integrated into the system of the hydrodynamic brake is possible except the recording parameters of the internal combustion engine also manage his operations. The unit is useful for any petrol engine with a maximum of eight cylinders. By using VEMS control unit we can change the parameters of the combustion process, such as the richness of the fuel mixture, ignition advance, or turbo boost pressure. This change is possible due a rewritable memory of control unit transferred in real time and on the fly of the internal combustion engine. For monitoring and adjustment of all key parameters of the internal combustion engine is used software VemsTune (Figure 6).

Figure 6 Program for the monitoring and management of data in the control unit



RESULTS AND DISCUSSION

The operation of hydrodynamic brake is also on base an open platform of setting the parameters required not only for spot cycles but also in automatic mode according to a programmed load. The learning facility was designed as an open system, which allowing complements various modules for measuring other parameters. The device can be easily extended at the measurement of emissions or

thermo vision testing. This characteristic is specific to the hydrodynamic brake, which is different from commercial facilities.

CONCLUSION

Despite the fact that designed the test equipment is still in development, already now can be a good instrument in the education process, or may be use for scientific experiments in short-term or long-term measurement of internal combustion engines of cars. By using the integrated, fully configurable control unit it can manage the operation of a petrol combustion engine and change the combustion parameters in real time.

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MODELLING OF PHOTOVOLTAIC MODULE CONVECTIVE HEAT TRANSFER COEFFICIENT

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Abstract: In this article are presented facts from photovoltaic theory and practise. The amount of energy produced by PV system is influenced by many internal and external factors. One of the most important factors is temperature which has significant influence on PV system energy production. The temperature of PV module is affected by emissivity, absorptivity of cell surface and convective heat transfer coefficient. In the text are presented parameters of real PV system installed on RES laboratory roof in Slovak University of Agriculture in Nitra. Measured parameters by PV systems were used for verification of mathematical model for time temperature relation. The main aim was modelling of convective heat transfer coefficient dependence on temperature and wind speed. There were compared time relations of measured and modelled convective heat transfer coefficient.

Key Words: photovoltaic module, mathematical model, temperature, convective heat transfer coefficient, relation

INTRODUCTION

The PV system is one of the most important ways for conversion of solar energy into electric energy. The photovoltaic system consists from photovoltaic modules or panels. Basic design component of photovoltaic panel is photovoltaic cell. From physical point of view the operation principle of photovoltaic cell is based on photovoltaic effect. The usage of PV system depends on many technical and physical factors such as: solar irradiation, azimuth angle of sunlit surfaces orientation, the angle of surface inclination which is dazzled, local climate and temperature conditions. All mentioned conditions have significant influence on PV cell energy production, but one of the most important factors is temperature. Temperature of PV cells is important parameter for assessing the long term performance of PV module systems and their annual amounts of electrical energy production. This temperature depends on many parameters such as the thermal properties of materials used in PV module encapsulation, types of PV cells, configuration of PV modules installation and climatic conditions of the locality. Typically, PV module efficiency strongly depends on its cells operating temperature. Increasing cell temperatures during operation generally deteriorates the performance of the PV module in electricity generation. The analysis of photovoltaic panel temperature and power output was presented in literature (Marc et al. 2012).They created simulation of electric and thermal model of PV modules and they verify theoretical results in real operation conditions.

The thermal model of photovoltaic system and combined model of energy transfer processes was presented by authors (Jones and Underwood 2001). They showed that the response of the module temperature is dynamic with changes in irradiance and the accurate module temperature, particularly during periods of fluctuating irradiance. Based on the previous facts were made experiments in real climatic conditions with localization in central Europe region.

MATERIALS AND METHODS

The photovoltaic system is located on the RES laboratory roof in Nitra in the campus of the Slovak University of Agriculture. The PV panels were installed fixed PV system which consists from 6 photovoltaic modules made from monocrystalline silicon. Every photovoltaic module contains six photovoltaic cells STP040S – 12/Rb developed by SUNTECH. The PV cells are used in the combined serial-parallel connection. The total efficiency of photovoltaic modules is 12.6%. Optimal operating voltage is 17.6 V and optimal current is 2.56 A. Maximum power is 45 Wp (1000 W/m^2) and operating temperature is from -40 °C to +85 °C. Active surface of measured PV cells is 1.95 m^2. Block scheme of PV systems is shown on Figure 1.

The technical and physical parameters were monitored for 24 hours a day and time interval between measurements was 10 s. Monitored parameters and their measurement uncertainties were: ambient temperature ($\pm$0.5 K), module temperature ($\pm$0.5 K), solar irradiance ($\pm$ 2 W/m^2), electric current ($\pm$10 mA), electric voltage ($\pm$0.0075 V). As the main aim of the research were observed temperature characteristics of photovoltaic module and relation between temperature and power output of PV system in real climatic conditions located in central Europe region.

The temperatures were measured by calibrated digital temperature sensors DS18B20. Communication between control microprocessor and sensors was realized by 1-wire protocol. Standard accuracy is ± 0.5 °C in temperature range from -10 °C to + 85 °C. Accuracy is better than 0.25 °C in temperature range from –10 °C to 100 °C. The temperature sensors were additionally calibrated for this range.

For battery charging was used TriStar TM controller TS-45. The controller operates in one mode at the time. Rated solar current of the controller was up to 45 A and system voltage was set to 24 V in our case. Accuracy of the voltage measurement was lower than 0.1% (± 50 mV). Modbus communication protocol was used. Communication was realized on the RS-232C physical layer.

Figure 1 Block scheme of PV system

System was loaded by bulbs, which were switched by the module Load Control. Output current of system and battery voltage was measured by this module. Converter resolution was 12-bit. The sine wave inverter AJ1300 from producer Studer was used. Maximum output apparent power was 1300 VA and efficiency was up to 9%. Input voltage was optimally 24 V. Inverter output voltage is sine waveform with effective value 230 V/50 Hz, it is generated by the PWM principle with passive filtration.

The measurement system was controlled by the single-chip microcontroller modules. Data were loaded by the program in Labview via USB port and measurement results were saved in Matlab.

Temperature model of photovoltaic module

From the literature are known two types of photovoltaic module temperature models. First type of model was described by Schott (1985) for steady state conditions, but for real climatic conditions is better non-steady state temperature model, which was presented by Jones and Underwood (2000). We assume that the thermal energy exchange in PV module with its environment is realised by three heat transfer ways – conduction, convection and radiation. The most significant are two ways of heat transfer – convection and conduction which are applied on the front and back surfaces of photovoltaic module.

The resulting rate of time-temperature changes can be expressed by equation:

$$C_{PV}\frac{dT_{\mathrm{mod}}}{d\tau}=q_{LW}+q_{SW}+q_{conv}-P \qquad (1)$$

where C_{PV} - is heat capacity of photovoltaic module, q_{LW} - is heat flux per unit area of PV module surface which characterizes long wave electromagnetic energy exchange, q_{SW} - is heat flux per unit area of PV module surface which characterizes short wave electromagnetic energy exchange, q_{conv} - is the total convective energy exchange from photovoltaic module to surface, P - is DC electrical power generated by PV modules. The solution of differential equation (1) could be obtained by Euler method of integration and temperature of PV module at time step $(\tau+1)$ could be described by equation (2) (Jones and Underwood 2001)

$$T_{\mathrm{mod}}(\tau+1)=T_{\mathrm{mod}}(\tau)+step\frac{dT_{\mathrm{mod}}}{d\tau}. \qquad (2)$$

The temperature of PV module was obtained as solution of differential equation (1) and could be described by next formula (3):

$$T_{\mathrm{mod}}(\tau)=\int_{\tau_0}^{\tau}C_{\mathrm{mod}}\left[\sigma\varepsilon T_{\mathrm{mod}}^4+\alpha\Phi S_{\mathrm{mod}}-h_c S_{\mathrm{mod}}(T_{\mathrm{mod}}-T_{amb})-P\right]d\tau \qquad (3)$$

where σ - is Stefan-Boltzman constant and ε - is emissivity, α - is the absorptivity of cell surface, Φ - is the total incident irradiance on module surface and S_{mod} – is the area of surface, h_{c} – is heat transfer coefficient, T_{mod} – is temperature of PV module, T_{amb} – is ambient temperature (Jones and Underwood 2001).

RESULTS AND DISCUSSION

Collected energy from PV system was approximately equal to supplied energy during the measurement. Therefore, battery voltage was regulated to 26 V. Constant battery voltage is controlled by the program in Labview. The basic role of load control module is regulation output power. System is based on the industrial single-chip microcontroller C8051F340, which is manufactured by Silicon Laboratories. All-important parameters was monitored and saved to the file. Namely they are: cells output voltage, cells output current, battery voltage and load current. These data are very important for energy relationship evaluation.

Days without wind were selected for measurements. Selected value of global solar radiation intensity was 760 W/m^2. Ambient air temperatures were selected in range from 5 °C to 18 °C during the measurement and the temperature of PV module was from 6 °C to 33.5 °C. Selected day for measurement of PV system parameters was 19.03.2015.

The temperature of PV module is influenced by many factors. Very important factor is emissivity ε. Normal range of emissivity for objects goes from 0.1 to 0.95 and typical value of glass emissivity is 0.85. The total emissivity depends on emissivity of surface of ground, emissivity of PV module and emissivity of the sky but in our case was used emissivity of the glass because it represents the frontal surface of PV panel (Green 1995). In generally the emissivity range for PV module is (0.85–0.91) according to the external conditions.

The next important factor that has an effect on the PV module temperature is absorptivity of cell surface α which is defined by formula (4) (Santbergen and van Zolingen 2007). The absorptivity of cell surface depends on the extinction coefficient *k* and wavelength λ.

$$\alpha = \frac{4\pi k}{\lambda} \tag{4}$$

The values of extinction coefficient *k* are presented in literature (Green 1995). The absorption coefficient depends on the material and also on the wavelength of light which is being absorbed. Semiconductor materials have a sharp edge in their absorption coefficient, since light which has energy below the band gap does not have sufficient energy to excite an electron into the conduction band from the valence band. Consequently this light is not absorbed. The relations of cell surface absorptivity α for crystalline silicon and wavelength is known from literature (Santbergen and van Zolingen 2007). In our case was used constant absorptivity which is simplification for the calculation of values during the day. For silicone modules with antireflection coating it is 0.7 (Jones and Underwood 2001).

Figure 2 Relation between coefficient of convective heat transfer, temperature and wind speed

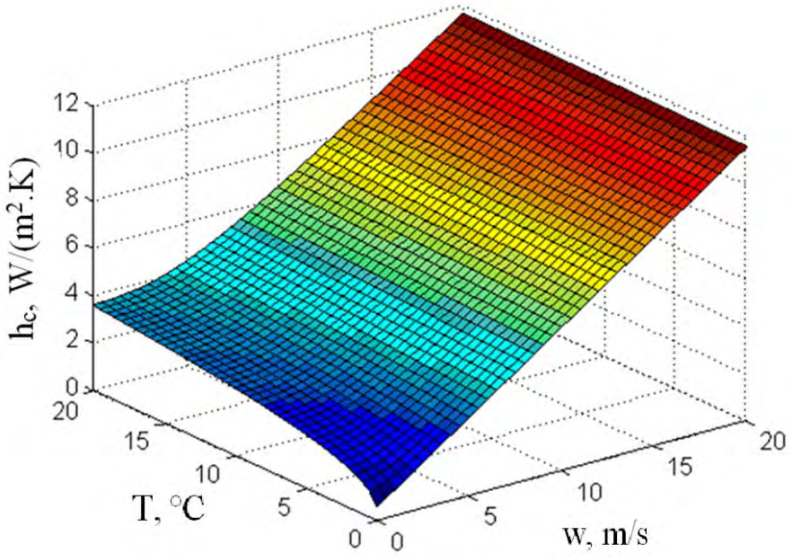


Figure 3 The time dependence of convective heat transfer coefficient obtained from measured parameters of PV system

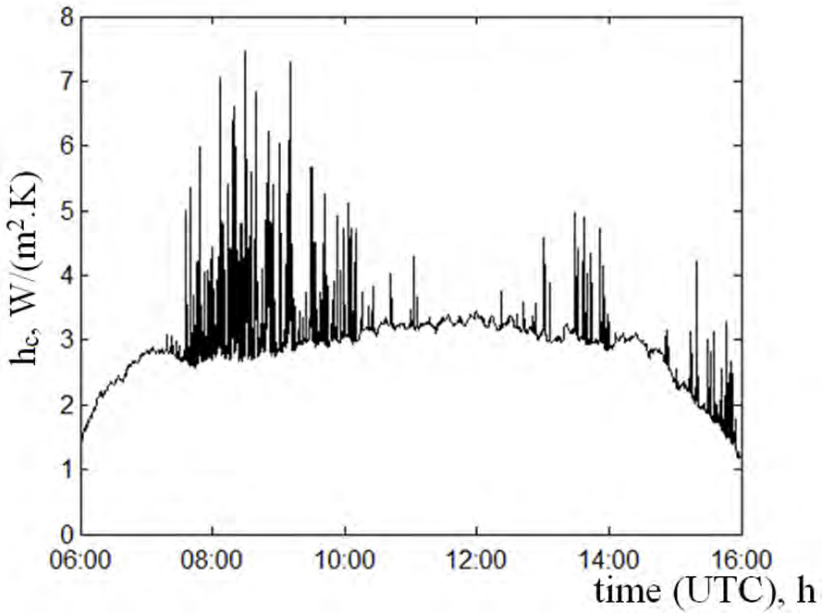
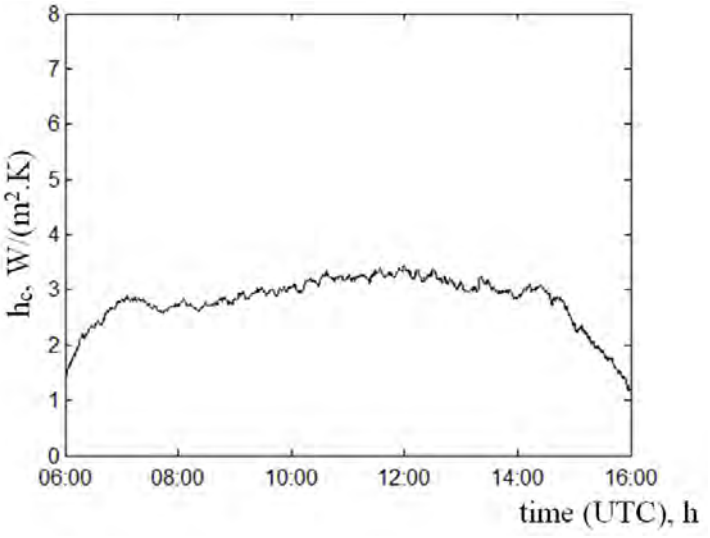


Figure 4 The time dependence of convective heat transfer coefficient obtained from mathematical model



The last factor which has effect on PV module temperature is coefficient of convective heat transfer h_c, which was examined. It depends on the physical situation for example it contains wind conditions, free convection and forced convection. The values of heat transfer coefficients for different wind speed are presented in literature (Schott 1985). In generally it can be approximated as a linear function of wind speed. In our case was modelled relation between coefficients of convective heat transfer h_c, temperature of PV module in the temperature range from 0 °C to 20 °C and wind speed in the range 0 m/s to 20 m/s. Final relation is showed on Figure 2. From Figure 2 is evident that temperature dependence of convective heat transfer coefficient h_c has nonlinear shape. The time dependence obtained from measured values is shown on Figure 3 and the same relation obtained by mathematical model application is presented on Figure 4. In the measured relation – Figure 3 are some variations from mathematical model, mainly in time range from 7:30 h to 10 h and in (13–14) h and also in (15–16) h.

The variations of convective heat transfer coefficient were caused mainly by temporary cloudiness and also by chemtrials smog which had significant influence on the solar radiation intensity. In the statistical evaluation were not included extreme measured values in mentioned time ranges. The statistical evaluation of the results showed, that measured values of convective heat transfer coefficient co-vary with modelled values with correlation coefficient of 0.9862.

CONCLUSION

Solar energy is one of the most popular types from group of renewable energy sources. The PV systems usage depends on many factors. From the technical and physical point of view most relevant factors are: solar irradiation, azimuth angle of sunlit surfaces orientation, the angle of surface inclination which is dazzled, local climate and temperature conditions. All mentioned factors were examined on photovoltaic solar system installed on the roof RES laboratory of SUA in Nitra. Presented article is focused on mathematical model which describes dependence between temperature of photovoltaic module and factors as: emissivity, absorptivity of cell surface and convective heat transfer coefficient. Main attention was oriented on modelling of convective heat transfer coefficient dependence on temperature and wind speed. In the second part of results were compared time relations of measured and modelled convective heat transfer coefficient. Both results were in good agreement which was proved by correlation coefficient. Obtained results showed that temperature is one of the most influencing factors.

ACKNOWLEDGEMENT

The impact of external factors on the photovoltaic cell efficiency in real conditions of micro-region Nitra, VEGA1/0696/11.

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EVALUATION OF GRAPE SEED OILS USING COLOUR SYSTEM METHOD (CIELAB)

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Abstract: Customers are still unfamiliar in the areas of quality, varietal purity, and keeping quality of grape seed oils. These parameters can influence consumers in their purchase decisions. An important indicator for determining the variety's purity, stability, and oil degradation due to storage is their colour intensity. A variety of methods can be used for evaluation of colour intensity of oils. For example CIELAB method. Utilization of the obtained results, particularly the hue (h_{ab}) and chroma (C^*_{ab}) factors, facilitates the use of CIELAB method to verify the authenticity of grape seed oils and exclusion of impurities, especially of cheaper oils.

Key Words: colour system, CIELAB, grape seed, oil

INTRODUCTION

Oil from grape seeds is an interesting raw material in the food industry for its dietary value. It has a high content of essential fatty acids and tetraphenols (Anastasiadis et al. 2010). The production of these oils at home and abroad has been growing significantly and it opens up new possibilities for vineyard and winery operations in the application of the residual primary products (Dědina et al. 2013).

Customers are still unfamiliar in the areas of quality, varietal purity, and keeping quality of grape seed oils. These parameters can influence consumers in their purchase decisions (Ranalli et al. 2005). An important indicator for determining the variety's purity, stability, and oil degradation due to storage is their colour intensity (Criado et al. 2008). Moyano et al. (2008) state that the colour intensity of oils is among others related to the content of healthy substances.

A variety of methods can be used for evaluation of colour intensity of oils. For example Moyano et al. (2008) state that the best method for the assessment of colour intensity of olive oil is the CIELAB method, which is fast, inexpensive, and objective. Also Meléndez-Martínez et al. (2007) state that this method is suitable for long-term examinations of a large number of analysed oil samples. It can be used as selective for more comprehensive and precise analysis even for samples of grape seed oils. For the target consumers, the colour intensity of oils is the only evaluable parameter that can be biased by partially subjective approach.

The aim of this paper is to verify the possibility of using the colour system method (CIELAB) to identify colour differences in grape seed oils obtained from chosen varieties. Another objective is to determine the effect of storage duration on their colour changes.

MATERIAL AND METHODS

Samples

For the purpose of this paper, grape seed oils of six grape varieties were used. The varieties included Dornfelder (DR), Blaufränkisch (BF), Pálava (PA), Riesling (RR), Pinot Gris (PG), and Zweigelt (ZW). The oil was pressed on the UNO FM 3F press. This press model is designed for cold pressing of all oily seeds at 80 rpm. After pressing, the oils were settled by gravity, then filtered, and poured into glass jars (volume 500 ml). Oils were not technologically treated or stabilized in any way.

The samples were then evaluated using the CIELAB method. Another evaluation was carried out after six months of storage (estimated minimum durability) in conditions of absence of light at 12 °C.

CIELAB measurements

Colour changes in the oils were monitored by determining transmittance on the Lovibond RT850i device by X-rite Incorporated, USA. The resulting colour was defined as a colour space L*a*b*. Oil samples were measured in plastic cuvettes with 10-mm optical path length. The evaluation was carried out by OnColor™ Premium software application by Lovibond. The differences between the colour changes of the individual samples were expressed through the colour difference ΔE*ab in colour space L*a*b*, which indicates the size of the difference but not its direction. It is defined by the following equation:

$$\Delta E^{*}ab = \sqrt{(\Delta L^{*})^2 + (\Delta a^{*})^2 + (\Delta b^{*})^2} \quad (1)$$

Where: ΔL*, Δa*, and Δb* are the differences of these values between the samples just after pressing and after six months of storage.

Statistics

A statistical analysis was carried out using the software package 'Statistica 12.0' (StatSoft Inc., USA).

RESULTS AND DISCUSSION

These are the initial results of measurements that aim to verify the possibility of using the CIELAB method for quick evaluation of grape seed oils.

Objective colour assessment - CIELAB colour space

Measurements have shown that the values of hue (h_{ab}) of the oil ranged between 87.15° (DR) and 94.26° (RR) and averaged 91.48°. This is a narrower range when compared with the results by Moyano et al. (2008) and their virgin olive oil samples with values between 84.63° and 100.84° and a comparable average of 91.37°. Thus grape seed oils show greater shade balance.

The chroma (C^*_{ab}) ranged between 63.70 (RR) and 111.81 (DR) and averaged 80.73. The results have again a significantly narrower range compared with Moyano et al. (2008) (between 20.12 and 134.66 CIELAB units, averaging 85.42 units).

The lightness (L*) ranged between 77.64 (DR) and 90.52 (ZW) units, averaging 86.87 units. Moyano et al. (2008) reported similar results between 49.88 and 99.28, averaging 85.42 units. Grape seed oils are thus similar in lightness to virgin olive oils.

The a* values ranged between -4.74 and 5.57 units, averaging -1.53 units. The b* values ranged between 63.52 and 111.67 units, averaging 80.65 units. The results represent a markedly yellow colour of oils (b* values).

The factors hue (h_{ab}) and chroma (C^*_{ab}) of the DR and RR varieties were both at the ends of the spectrum. In the lightness factor (L*) it was again the DR and ZW varieties. However, within the set, the order of varieties alternated. Table 1 shows the average measured values of the six monitored varieties, in 3 repeated measurements for one variety. The measurements did not show significant differences between varieties or between white and red groups of varieties. However, the obtained results confirm a relatively wide range of hue values (h_{ab}) and chroma (C^*_{ab}), which is typical for these oils. Based on the results of this work, it is not possible to positively confirm the usability of CIELAB to assess the varietal purity of grape seed oils. It is possible to continue research and focus on groups of varieties, such as Traminer, Riesling, or Muscat, which have possibility for positive results. Another realistic possibility is using the CIELAB method and its results, especially the factors of hue (h_{ab}) and chroma (C^*_{ab}), for verifying authenticity of grape seed oils and exclusion of oils with admixture of other oils such as virgin olive oil, sunflower oil and others.

Table 1 Average values of measurements of grape seed oils using the CIELAB method (n = 3)

First measurement

Var.	L*	L* STD	a*	a* STD	b*	b* STD	C*	C* STD	h°	h° STD	ΔE*ab
DR	77.65	0.06	5.55	0.02	111.67	0.00	111.81	0.00	87.15	0.02	–
PA	89.87	0.02	-2.34	0.03	81.96	0.00	82.00	0.00	91.64	0.02	–
BF	86.52	0.02	-3.09	0.01	71.56	0.00	71.63	0.00	92.47	0.01	–
RR	89.77	0.01	-4.75	0.02	63.52	0.00	63.69	0.00	94.26	0.01	–
PG	86.87	0.01	-2.75	0.00	79.36	0.00	79.40	0.00	91.99	0.02	–
ZW	90.53	0.02	-1.83	0.00	75.81	0.00	75.83	0.00	91.39	0.01	–

Measured after 6 months

Var.	L*	L* STD	a*	a* STD	b*	b* STD	C*	C* STD	h°	h° STD	ΔE*ab
DR	44.40	0.01	13.02	0.01	75.60	0.17	76.71	0.17	80.23	0.03	49.61
PA	63.62	0.02	10.36	0.01	104.62	0.18	105.13	0.17	84.35	0.02	43.09
BF	71.13	0.00	8.11	0.02	113.15	0.02	113.44	0.02	85.90	0.01	42.41
RR	65.89	0.05	10.42	0.02	108.63	0.11	109.13	0.11	84.52	0.01	54.55
PG	77.29	0.02	10.94	0.02	124.86	0.22	125.34	0.22	84.99	0.02	38.36
ZW	73.15	0.02	10.71	0.01	119.46	0.14	119.94	0.14	84.88	0.01	52.39

Ripening stage

Table 1 shows the average values of the first measurement after pressing and the second measurement after six months of storage. When comparing the values of the two measurements for each variety, the overall darkening of oils, or the lower L* values become obvious. In the DR variety, the value decreased from 77.64 to 44.40, which represents the most significant difference of 33.24 units. The smallest decrease was observed in the ZW variety which represents a difference of 13.23 units. On average, the value decreased by 20.96 units.

Differences were also found in the values of a* and b*. There was an overall expansion of the range of a* values, but to a lesser extent. Moyano et al. (2008) and Criado et al. (2007) presented similar results. Significant changes of value were recorded in the factor b*, which represents intensifying of yellow colour. This change corresponds with a decrease in lightness (L*). The DR variety was the only one, where there was a drop in the b* value from the original 111.67 to 75.60. In combination with the most significant decrease of lightness (L*), after six months of storage, this variety was the darkest. The measure of colour difference, defined as deviation delta E (ΔE) for each oil stored for six months reached relatively high levels. Vik (1995) reported that values greater than three units express colour difference that can be perceived visually by the human eye. The results in Table 1 show that the smallest influence of the storage on the colour was found in the oil from the seeds of PG variety, while the largest changes were observed in the RR variety.

Comparison with the results by Moyano et al. (2008) and Criado et al. (2007 and 2008) reveals that the change in the colour of grape seed oils is more pronounced than in virgin olive oils. In formulating conclusions, it is necessary to keep in mind that the oils in the experiment, compared to commercial products, underwent only minimal technological modification.

Using the CIELAB method can be recommended for testing of larger sample sizes for future use in the evaluation of the authenticity and quality of grape seed oils and their shelf lives.

CONCLUSION

Utilization of the obtained results, particularly the hue (h_{ab}) and chroma (C^*_{ab}) factors, facilitates the use of CIELAB method to verify the authenticity of grape seed oils and exclusion of impurities, especially of cheaper oils. The values of hue (h_{ab}) ranged between 87.15° (DR) and 94.26° (RR) and averaged 91.48°. The chroma (C^*_{ab}) ranged between 63.70 (RR) and 111.81 (DR) and averaged 80.73.

The six-month storage generally resulted in darkening of oils, or lower L* values. In the DR variety, the value decreased from 77.64 to 44.40, which represents the most significant difference of 33.24 units. On average, the value decreased by 20.96 units.

The results of the work demonstrate a limited shelf life of oils and suggest that their storage should not exceed one year. Assumptions that grape seed oils have a relatively short shelf life without changing their sensory characteristics (colour) were confirmed. In this experiment they were also multiplied by the minimum technological treatment provided.

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DETERMINATION OF PRODUCTIVE PERFORMANCE OF LAWN MOWERS ON SLOPES

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Abstract: The paper evaluates the effects of the inclination factor which determines the performance of deployed lawn mowers. The identified coefficients facilitate the assessment of the impact of inclination on the really attained performance of most used machines on a real territory. The results show a significant impact on the slope above 9.0°. The impact of 12.0° slope, which is the limit value for conventional techniques, showed a reduction of performance coefficient to be 0.76. The effect of gentle slopes up to 8.5° (15.0%) showed a reduction of performance coefficient to be 0.93.

Key Words: park areas, machinery performance, maintenance factors, lawn mower

INTRODUCTION

The economic situation of many local governments requires a constant search for new ways and procedures to streamline the maintenance of public spaces. Technical means used in greenery maintenance are deployed in different conditions that affect the difficulty of maintenance and thus cost.

Syrový (2008) assert in the computing of the areal performance of machines in maintenance of permanent grasslands as the most important parameters to be the working width and working speed. They also consider the slope to be of considerable influence, similar to Altmann et al. (2007) and Mimra et al. (2007).

Zemánek and Burg (2008) evaluated the factors that affect the maintenance of permanent grassland and using a numerical scale, they quantified their impact. They evaluated the site conditions, area size, shape and roughness of terrain, obstacles, terrain conditions, and accessibility for machinery. Based on measurements, Celjak (2012 and 2014) indicated that the actual performance of machines, compared with theory can differ by up to 50%, which is significantly influenced by the nature of the site.

Stonawská (2010) added to the previously mentioned factors in maintaining grasslands also the inclination factor of the terrain and evaluated it by the coefficient of performance reduction. Most of green spaces in communal areas are located on level surfaces or gentle slopes, and only rarely on slopes above 8.6° (15%). Despite the lower incidence of inclination surfaces, this factor is according to the author not insignificant.

The objective of this paper is to quantify the impact of the terrain inclination factor on the performance of lawn mowers.

MATERIAL AND METHODS

Monitored machines

The monitored lawn mowers included both professional self-propelled and manually operated machines under normal operating conditions. The evaluation of exploitative indicators used the Methodology of Time Measurement Log (ČSN 47 0120 standard). The measured values were used to calculate the achieved productive performance W_{04} (ha/h) of individual machines at different terrain inclination.

Terrain inclination

The evaluation was based on the hypothesis that any deterioration of the machine performance due to the influence of terrain inclination will be reflected by an increased consumption of time to carry out the same amount of work compared to optimal conditions, namely mowing on flat area. Another assumption was that different terrain inclinations have different effects on different machines. To quantify the influence of terrain inclination, a method for the coefficient (K_i) calculation for each reference slope was chosen (degrees 1 to 5). Each degree was verified in triplicate. The individual degrees were chosen with respect to the uniformity of their distribution and range within the standard use of monitored machines (Table 1). Terrain inclination (slope of worked terrain) was measured using two spirit levels. The first one was 1.0 m long and was used to measure the plane, while the other one (vertical) was used to measure the altitude difference of the terrain. The altitude differences of the terrain were measured at least three times and after averaging, the inclination was determined.

Table 1 Distribution of ground inclination

Degree	Slope (°)	Slope (%)	Performance ${}_xW_{04}$	Coefficient of the inclination impact – K_i (–)
1	0.0–5.7	0.0–10.0	W_{04}	1.00
2	5.8–8.5	10.1–15.0	${}_2W_{04}$	
3	8.6–11.4	15.1–20.0	${}_3W_{04}$	
4	11.5–14.4	20.1–25.0	${}_4W_{04}$	
5	from 14.5	from 25.1	${}_5W_{04}$	

Generally, the inclination coefficient (Ki) is determined as a fraction of performance affected by the terrain inclination (degrees 2 to 5), and the performance under ideal conditions (degree 1 - plain) by the relationship:

$$K_i = \frac{{}_xW_{04}}{W_{04}} \quad (-) \qquad (1)$$

Where:

${}_xW_{04}$ - Actual performance of the machine in a particular terrain inclination (ha/h)

W_{04} - Productive performance of the same machine under ideal conditions (ha/h)

Statistical analyses

T-test was used to compare the measured values. To determine the influence of a factor in different degrees, correlation analyses with a significance level of 0.05 and obtained linear dependences were used for determining the coefficients. The final coefficient is the average of the obtained values. The statistical analysis was carried out using the software package 'Statistica 12.0' (StatSoft Inc., USA).

RESULTS AND DISCUSSION

Figure 1A–F illustrate the values of detected productive performance (W_{04}) of lawn mowers used for maintenance of grasslands, with the addition of linear correlation with interval of confidence 0.95.

Regression analyses allow evaluating the impact of slope on the change in performance of machines of different constructions. Figure 1A–D show that the regression coefficient values vary in lawn tractors ranging from 0.0066 to 0.0083. While, on the other hand, at the manually operated machines seen on Figure 1E–F the regression coefficient values stay approximately the same, which is around 0.002. Therefore, one can conclude that the slope of the land at a greater extent reflects in the performance of lawn with lawn tractors and not so much with manually operated machines.

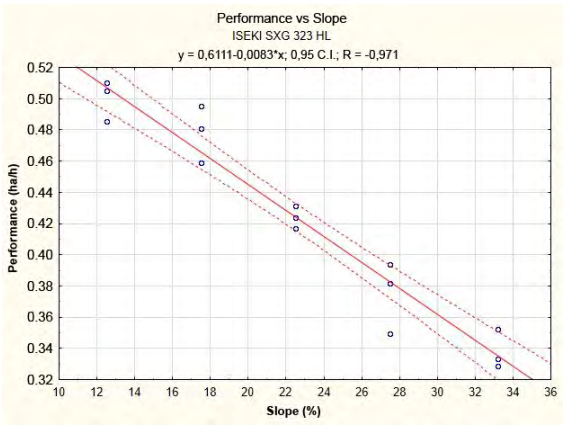
By further analysis of the data in Figure 1C there is seen less tightness of measured values of the performance to a regression line. This might be due to the worse terrain conditions at the time of measurement. In contrast, on Figure 1A–B and Figure 1D there is a greater tightness no matter what the construction level of the machine is.

On the Figure 1E, which describes the impact of slope on the performance of professional machines, tightness is higher than that one of semi-professional machine on the Figure 1F.

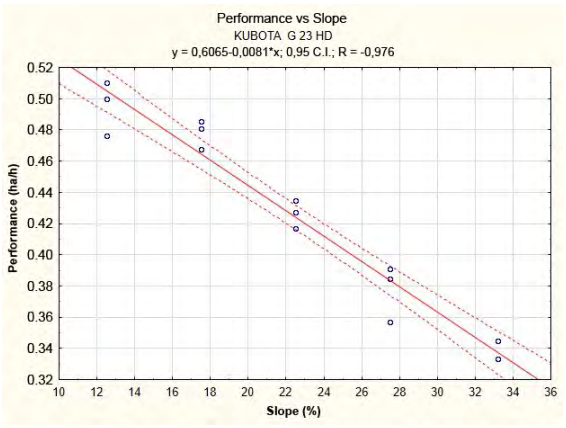
The comparison of Figure 1E (HONDA HRX 537) and Figure 1F (VIKING MB 448 T) with other graphs shows that in manually operated machines, there was no significant reduction in performance with increased terrain inclination. This is due to the lower weight and better possibility to operate the machine. Facts mentioned above confirm that correlation coefficient which values range from -0.921 to -0.978, tells about a very high impact and dependence of the slope on the machine performance. Table 2 shows K_i values for individual machines and terrain inclination.

Figure 1 Performance of individual lawn mowers on a particular terrain inclination

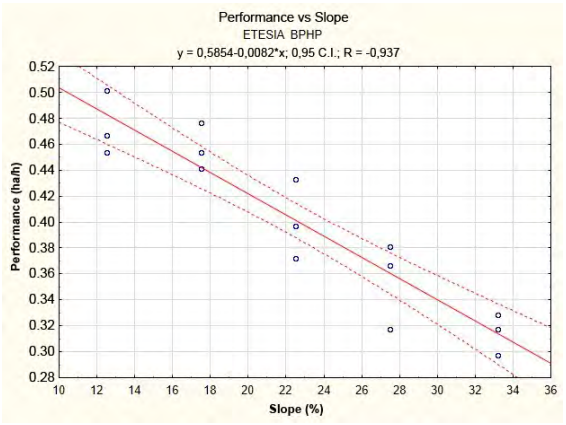
A) ISEKI SXG 323 HL



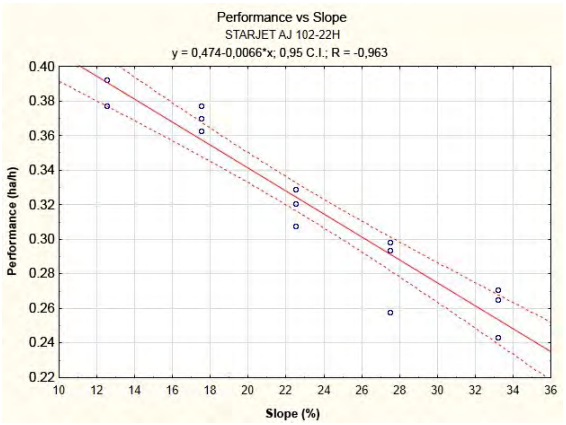
B) KUBOTA G 23 HD



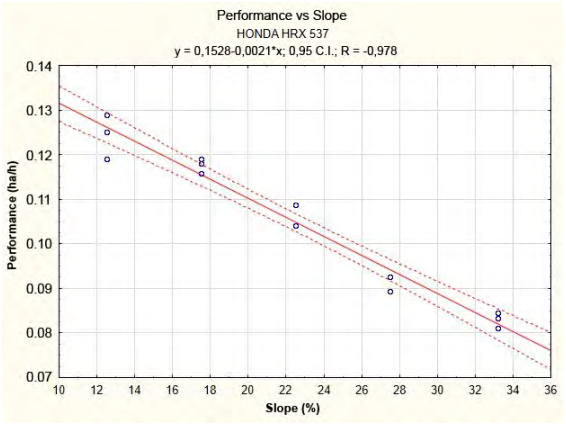
C) ETESIA BPHP



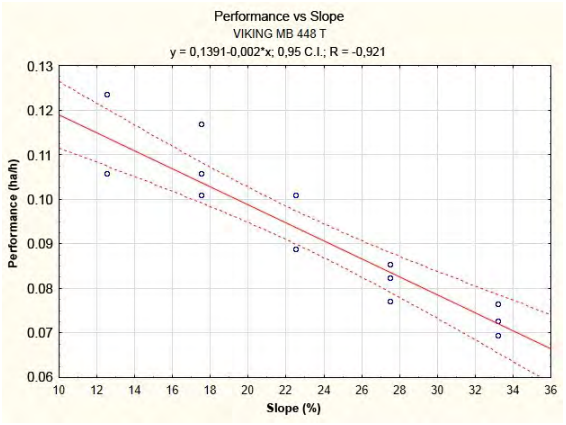
D) STARJET AJ 102-22H



E) HONDA HRX 537



F) VIKING MB 448 T



The results of the generated coefficients can be compared with the results of other authors. Syrový (2008) stated that the effect of terrain inclination on exploitation, energy, and economic indicators occurred especially on slopes greater than 10.0°. The results show a significant impact on the slope above 9.0°. The impact of 12.0° slope, which is the limit value for conventional techniques, showed a reduction of performance coefficient to be 0.76. The effect of gentle slopes up to 8.5° (15.0%) showed a reduction of performance coefficient to be 0.93. Syrový (2008) quoted the terrain inclination coefficient to range between 0.98 and 0.75.

Stonawská (2010) evaluated the terrain inclination up to 25% by a coefficient of 0.20 compared to the coefficient shown in this paper, which is 0.66. This is probably due to the operating conditions of machines where the author evaluated the machines at meadow stands with presumably poorer state of the terrain, taller and denser growth of the vegetation, and higher humidity.

Table 2 Values of coefficients of monitored lawn mowers according to the degree of terrain inclination

Degree	Slope (°)	Slope (%)	Monitored machines						Average
			KUBOTA G 23 H	ISEKI SXG 323HL	HONDA HRX 537	ETESIA H100 DBPHP	STARJET AJ102-22H	VIKING MB 448 T	
			Coefficient of the inclination impact – K_i (–)						
1	0.0–5.7	0.0–10.0	1.00	1.00	1.00	1.00	1.00	1.00	1.00
2	5.8–8.5	10.1–15.0	0.94	0.92	0.94	0.92	0.91	0.93	0.93
3	8.6–11.4	15.1–20.0	0.84	0.84	0.84	0.84	0.86	0.85	0.85
4	11.5–14.4	20.1–25.0	0.76	0.76	0.76	0.76	0.75	0.75	0.76
5	from 14.5	from 25.1	0.66	0.66	0.66	0.66	0.65	0.65	0.66

For lawn mowers, the impact of terrain inclination on efficiency is still considered to be the most important. The results of the work indicate that the values of the coefficient of terrain inclination in the degrees 1 to 4 were evaluated at 1.00 to 0.76. The obtained coefficients can be used to provide more efficient and realistic performance of other lawn mowers.

CONCLUSION

Using coefficients for efficiency decrease of mowing machinery based on the conditions at a given site as presented in this paper can facilitate projects for maintenance of greenery and more accurately determine the time required for mowing. To that extent and to the required extent, we have not addressed the effects of slope on the ornamental grassland. The goal is to derive the costs for the performance of mowing operations based on time requirements, and thus specify the costs for ensuring quality maintenance of the greenery. The data obtained can be used for creating the formula, which, unlike the price list takes into account the real operating conditions of machines. Respecting and considering slope coefficients and their inclusion in the cost calculations can be used in subjects which do the lawn maintenance as well as determining the cost of operation performed as a service. This obtained data show that the performance of mowers might be reduced, based on the slope of the land, on average by 34%. This directly reflects in an increment of operating costs. Only quality greenery, including grass areas, can adequately fulfil its function and delay the need for revitalisation and the significant costs associated with it.

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PROPORTION OF VOLATILE MATTER IN SELECTED BIOFUELS

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Abstract: Biomass provides a great diversity of input materials and universal use, not only for heat production but also for electricity production in modern combustion devices. The quality of biofuels depends on the total content of combustibles. Course of combustion is affected by the levels of volatile and solid combustibles. The aim of this research was to determine the amount and release course of combustibles in selected biofuels depending on temperature by the means of gravimetric method. For this purpose, we used a gravimetric furnace Nabertherm L9/11/SW/P330. The results are processed in tabular and graphic form and enable to characterize the release course of combustibles in the tested fuels. The highest content of combustibles was observed in samples of softwood pellets, in the amount of 99.64%. The highest proportion of volatile matter from the total amount of combustibles was present in cherry wood, in the amount of 74.4%.

Key Words: gravimetry, ash, biomass, combustible, dry matter

INTRODUCTION

Renewable energy has become more important globally especially with the current fuel and economic crisis (Bernama 2008). Solid biofuels in particular will be increasingly used as a source of thermal energy. Biomass refers to all organic matter that arose through photosynthesis, or to the material of animal origin. This term often represents plant biomass usable for energy purposes as a renewable energy source (Maga et al. 2010). Biomass provides a basis for renewable energy sources, without any doubts. It accounts for 75% of renewable energy sources such as wind, water, sun etc. Biofuel is the fuel derived from biomass. According to the chemical composition, the biofuels can be divided into solid, liquid and gas. Quality of solid biofuels as an energy source depends on the content of moisture, ash and combustibles (Vitazek et al. 2014). Chemical energy is released mainly from biofuel combustion process. The combustion process is considered as oxidation process, where the combustible components of the fuel are oxidized by atmospheric oxygen, while the energy content of the fuel is transformed into heat (Jandacka et al. 2011, Pitel et al. 2013).

Biofuels as an energy source depend on the quality of combustibles and the content of ballast - moisture and ash. Compared to solid fossil fuels, biomass has significantly higher proportion of volatile matter, which is essentially due to its origin. Biomass combustion does not pollute the environment by excessive production of CO_2 (Holubcik and Jandacka 2016). Another advantage of the biomass combustion is that the ash as a by-product of combustion can be used as a high-quality fertilizer. Biomass offers a great variety of raw materials and becomes universally used in the energetics. It is used for production of heat, as well as electricity in modern combustion plants (Misakova 2014, Trenciansky et al. 2007). Original composition of solid biofuels (wood, straw, corn) in terms of combustion is as follows (Piszczalka 2010): volatile matter (wood gas) 60–70%; non-volatile solid combustible (wood charcoal in the case of wood) up to 20%; ballast - water (up to cca. 14%), and ash from the burning of charcoal 0.5–4%.

This paper presents a method of determining the proportion of the biofuel components by gravimetric method. The release course of combustibles at a selected time interval and determined proportion of volatile matter is shown.

MATERIAL AND METHODS

In terms of the combustion of biofuels, the release course of combustibles and proportions of volatile and solid matter is important. The residue after combustion is ash. Tested sample consisted of pellets from spruce wood, spruce and fir wood (90% and 10%), unspecified softwood (labelled as Baumax), sunflower pressing and waste from post-harvest processing of grain. In addition, the wood chips from cherry wood with bark and charcoal sample were included in the experiment. To measure the proportion of components in solid biofuels, gravimetric method was used. For this purpose, a furnace Nabertherm L9/11/SW/P330 was used. Input power of the heater is 3.0 kW. The control unit P330 enables to program selected courses of the heating and endurance using the computer. Heating of tested samples is possible up to 1 100 °C, while our experiments were terminated at 815 °C. Digital scales Kern are also a part of the equipment. The device is connected to a computer and records the temperature and weight courses at the selected time intervals. Such device enables to determine the proportion of moisture, combustibles and ash in the tested solid biofuels. From the change in weight of the sample after the removal of moisture it is possible to obtain the release profile of combustibles and determine its individual components.

Figure 1 Gravimetric furnace Nabertherm L9/11/SW/P330

Proportions of particular components are calculated according to the following relations
Moisture content wet basis w:

$$w = \frac{m_1 - m_2}{m_1} \quad (1)$$

Ash content

- in original sample A´:

$$A' = \frac{m_3}{m_1}$$

- in dry matter p_{ps}:

$$p_{ps} = \frac{m_3}{m_2} \quad (2, 3)$$

Combustible content

- in original sample h´:

$$h' = \frac{m_4}{m_1}$$

- in dry matter p_{hs}:

$$p_{hs} = \frac{m_4}{m_2} \quad (4, 5)$$

where:

m_1 – original weight of sample, g
m_2 – weight of dry matter, g
m_3 – weight of ash, g
m_4 – weight of combustible, g

The identification of the proportion of volatile matter follows the norm STN EN15148. Ash content is determined in accordance with the norm STN EN 14775. In accordance with the standards we have carried out experiments. Using a computer, we have programmed the required temperatures and impact periods. Table 1 shows the parameters of gravimetric measurements.

The analysed sample is first heated to 105 °C ± 2 °C and then dried for 120 minutes in accordance with

the norm STN EN 14774–2. Weight loss in the interval of 0–180 minutes is accounted for the removed moisture. Mass residue at the end of the experiment is made up of ash.

Table 1 Parameters for gravimetric measurement procedure

Parameters	Time interval					
Impact period	1	2	3	4	5	6
minute	60	120	60	60	60	60
Temperature, °C	20–105	105	105–500	500	500–815	815

RESULTS AND DISCUSSION

Temperatures above 100 °C lead to the removal of moisture – drying of the fuel. At temperatures above 150 °C, volatile matter begins to release. After exceeding temperatures from 260 °C to 410 °C, the release of volatile matter is significantly accelerated. The solid portion of combustible begins to oxidise at a temperature of about 500 °C. Temperature 815 °C leads to a perfect oxidation of solid residue.

The results of gravimetric measurements of analysed samples are shown in Table 2. Proportions of moisture, ash and combustibles are calculated according to the relations 1 to 5. Heating with biomass is increasingly used as an alternative to natural gas. The manufacturers of boilers for biomass heating began to react to this trend. A high proportion of volatile matter affects the supply of the secondary or even tertiary air. The aim is to provide enough air supply for complete combustion of the fuel and reduce the production of solid and gaseous emissions. Even with the gradual arrival of new innovations of biomass boilers, high ash content and inadequate temperature can lead to ash sintering. This can cause temporary interruption of combustion and lead to partial or permanent damage to the boiler. This is one of the reasons why it is necessary to know the information about the following parameters: composition of fuel, proportions of different types of combustibles and their release course in solid biofuel.

Table 2 Processed results of gravimetric measurements of analysed samples

BIOFUELS	PARAMETER, %				
	w	A'	h'	p_{ps}	p_{hs}
spruce wood pellets	7.0359	0.3434	92.6208	0.3639	99.6307
waste pellets	7.3492	5.1206	87.5302	5.5267	94.4733
spruce-fir pellets	10.3288	0.5485	89.1228	0.6116	99.3884
Baumax pellets	8.5671	0.3322	91.1007	0.3633	99.6367
sunflower pellets	9.8347	3.5885	86.5768	3.9799	96.0201
cherry wood with bark	13.5512	0.4254	86.0235	0.4920	99.5080
charcoal	4.4884	2.3522	72.7785	3.1309	96.8691

Figure 2 shows the course of the experiment of the selected fuels during the whole time interval. Figure 3 depicts course of the experiment from the 180. minute to 300. minute, when the release of volatile matter occurs. The graphic course shows the mass calculated to 1 gram of dry matter. From the obtained data were calculated proportions of volatile matter shown in Table 3. These are the proportions of the total combustibles p_{hs} (Table 2).

Figure 4 shows the course of the experiment with biofuel with the highest proportion of volatile matter (cherry wood). The course for charcoal is given for comparison.

The overall proportion of combustibles in the tested samples is presented in Table 2. Proportion of the amount of volatile matter in dry matter at the interval from 180. minute to 240. minute is shown in Table 3, column 2. Values are quite balanced again and indicate high proportions of volatile matter in solid biofuels. In addition, the proportions to the period of 300 minutes, i.e. to the end of heating endurance at 500 °C, are listed as well. It is no longer regarded as volatile matter, but as a proportion of total oxidized combustibles. Course of the experiment for charcoal, where the proportion of volatile matter is very low, provides evidence of this.

Table 3 Proportions of combustibles at given time intervals

BIOFUELS	Volatile matter (180–240 min), %	Proportion of combustibles (180–300 min), %
spruce wood pellets	71.18	86.21
waste pellets	74.23	88.09
spruce-fir pellets	73.08	86.78
Baumax pellets	70.70	83.37
sunflower pellets	70.11	83.14
cherry wood with bark	74.40	88.32
charcoal	57.6	90.40

The highest proportion of combustibles in dry matter was present in the sample of Baumax pellets (99.64%). On the other hand, the lowest was found in the waste pellets (94.47%), i.e. that they had the highest ash content (5.53%). The data in Table 3 shows that the highest proportion of volatile matter was present in the cherry wood with bark sample (74.4%). The case of waste sample is particularly interesting, because it indicated the highest ash content and the second highest proportion of volatile matter (74.23%).

Figure 2 Course of the experiment of selected biofuels during the whole time interval

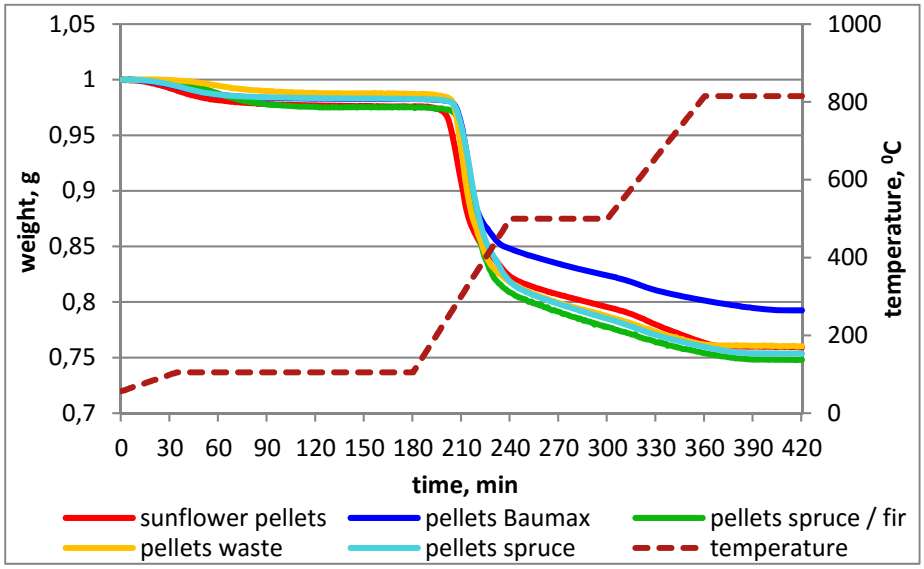


Figure 3 Course of the experiment of selected biofuels in the interval from 180. to 300. minutes

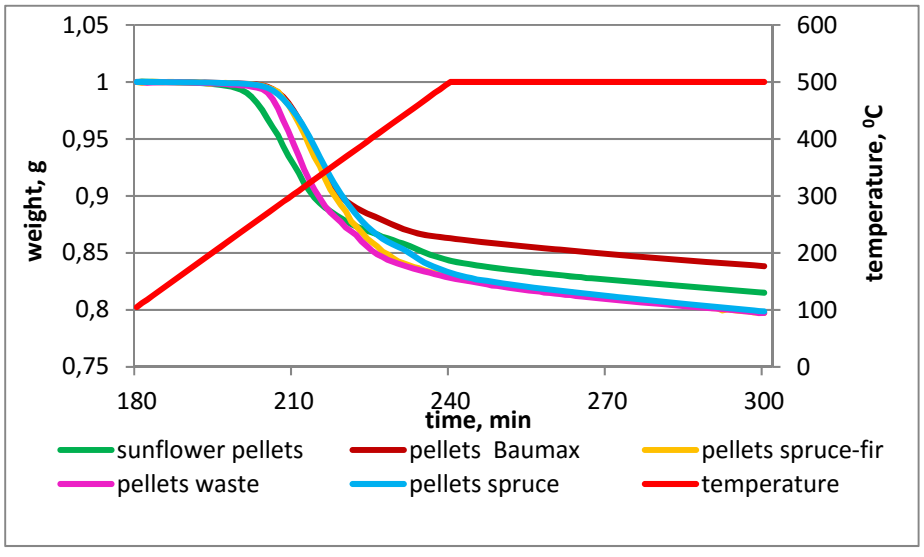
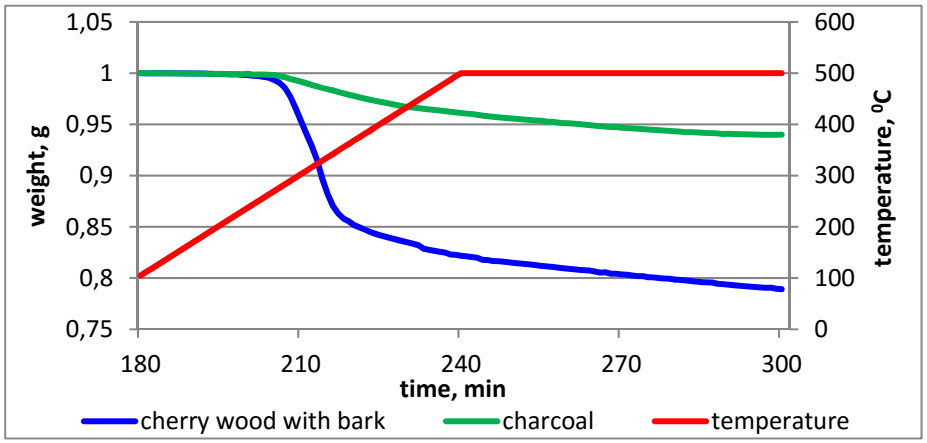


Figure 4 Course of the experiment of biofuels with the highest and the lowest proportion of volatile mater



Since we dealt with the waste from post-harvest processing, dust particles significantly account for high ash content. At the same time, husk and straw particles contain a high proportion of combustibles.

At low moisture, the wood burns virtually without smoke, easily ignites and produces low ash content of about 1% to 1.5% of the original weight (Hutla et al. 2005). The paper (Kazimirova et al. 2013) shows the results of gravimetric measurements. The research has shown that dry matter of the seeds of oil-seed rape of Catania variety contains, compared to cereal grain, higher ash content - namely 4.73% (hence the content of combustibles was 95.27%). Further findings indicate that the average ash content in the dry matter of tested cereal straw was 6.14% (hence the average content of combustibles was 94.92%). The dry matter of oil-seed rape Catania straw, compared to cereal straw, contained higher ash content of 9.20% (hence the content of combustibles was 90.80%). Similar results were obtained and described also in the works (Chrastina et al. 2015, Branca and Di Blasi 2015) – distillery waste from corn distiller dried grain with solubles contains 4.64% of ash. The paper of (Vitazek and Vitazkova 2012) presents the processed gravimetric measurements of selected types of biofuels. The obtained values of the ash content are as follows: pellets from rapeseed waste plus cereals: 7.98%, corn stalks pellets: 5.19%, burnt corn pellets: 4.70%, corn spindle pellets: 4.37%, softwood pellets: 1.02% and softwood without bark pellets: 0.46%. The paper of (Mikulova et al. 2014) shows that the fastest decline in weight loss and thereby the fastest release of volatile matter is indicated in the pellets composed of 10% brown coal, 90% sawdust (hardwood), while the lowest decline in weight loss is present in crushed brown coal briquette. Briquette composed purely of brown coal has ash content of 3.77%. Pellets composed of 10% brown coal, 90% sawdust have a slightly lower ash content, namely 3.48%.

Results presented in the paper contribute to the previously obtained knowledge about the proportion of moisture, ash and combustibles in solid biofuels and broaden it by the new findings about the release course of combustibles at selected time intervals. The decrease in weight from 180. minute to 240. minute (interval 3 in Table 1) can be accounted for the volatile matter.

CONCLUSION

In the agricultural sector, various types of biomass are suitable as a secondary raw material for the combustion process. Tested samples were made from different materials. These materials have different physical properties which determine their further processing and the choice of combustion device. Gravimetric method described above is highly applicable for examination of release course of combustibles in selected solid biofuels. Graphical representation of the weight loss course in the selected interval of time or temperature enables to observe the rate of volatile matter release, or more precisely, the rate of its oxidation. It affects the speed of combustion, consequently the construction of the boiler. The suitability of the examined fuel (or suggestions for its replacement with other biofuel) can be assessed for a particular boiler. Pellets from the mixture of crushed brown coal and biofuel in various ratios are already available on the market. They were also included in the experiments. Even in the case of new boilers with innovative technology, ash sintering caused by the high content of combustibles in

solid biofuels and inadequate temperature in the combustion chamber may occur. It may lead to permanent damage of the combustion devices. This threat only underlines the importance of sufficient information about the proportion of combustibles in the used solid biofuel, their release course and other thermophysical properties.

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OPTIMIZING COLLECTION ROUTES OF COLLECTION PLACES

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Abstract: This thesis attends to the optimization of separated waste collection routes, the waste is being collected by the company Technické služby VM s.r.o. The solution is carried out according to the salesman's methods with farthest insertion's algorithm. This algorithm provides possible solutions in an interface of two algebraic systems Maple and Bjornson's application. The routes for separated waste collection, which are compared, constitute an output.

Key Words: collection route, algorithm, waste, separated waste, optimisation

INTRODUCTION

Waste production has become part of everyday life. With the increasing number of inhabitants of our planet is increasing the amount of waste produced. Nowadays, it is therefore necessary to ensure the minimization of waste and the efficient management of waste. There are many effective methods of waste recovery or removal. In most cases, however, necessary to move the waste from the place of origin to the place of its processing. For effectively loading with waste, must also be provided efficient methods of collecting, gathering, treatment and transportation of waste. This work is primarily devoted to the issue of waste collection routes from sites, where waste is collected, to the sites, where waste is inspected, used and maintained.

To tackle the issue of efficient waste collection method is used Salesman (Cook 2012). The traveling salesman problem belongs to the category of optimization problems where the objective is to find the shortest route between points so that the optimized route passing through each point exactly once and returning back to the starting point. Using optimization methods for collection and transportation of waste, the loading of waste can significantly streamline and accelerate (Burdová 2011). Many information and logistics systems are able to streamline and automate a large portion of processes. This leads to an increase in the quality of services, centralization of data, easier administration tasks and possibilities of evaluation and planning activities.

This work focuses on optimizing the routes of collection points. Salesman methods are therefore applied to the selected collection area and an optimized path is created between the villages.

MATERIAL AND METHODS

The aim was to create optimized routes for the collection of plastic, paper, colored glass, white glass and biodegradable waste. Collecting area, which is administered by Technical Services Velké Meziříčí (TSVM), representing 54 municipalities, including local areas around the Velké Meziříčí. For each type of waste was made a list of municipalities where there is a collection of the waste (Běloch 2014). As input data were used maps that are available from the server www.mapy.cz/, and GPS position coordinates villages (www.mapy.cz).

Creating of collection routes was made in the program Bjornson's application available online from websites and http://bjornson.inhb.de (Bjornson 2008) and in program Maple. Both of these software creates optimal a collecting route by using the algorithm farthest insertion. Editing and route optimization was performed manually and by using Maple (Bartoň 1999).

Algorithm of farthest insertion belongs to the methods of insertion algorithms. The principle of the method of inserting cities into partial paths is to start building a partial path with several points and in further steps this way adjusted to the final stage include all the points. These techniques are generally referred to as the insertion algorithms. Currently, there are several variants of these algorithms, which differ by the selection of the next point. In all variants of these algorithms have the total length of the partial route increase minimally. Variant of farthest insertion selects one next point of partial route, which is farthest from one of the points in already built partial route.

Algorithm of farthest insertion in Bjornson's application does not allow you to insert your own data, it was necessary to all municipalities in which it is carried out of the collection of separated waste, assign coordinates [X, Y] that determine their position and mutual distance on the axes. Instead villages that appear normally on the map portals, has therefore created a set of points, supplemented by one point [0], which represents the starting position of each vehicle, and ending with the highest numerical value that represents the last visited site on the route (in this case, always local landfill where there is a weighing of all vehicles on vehicle weight) before the vehicle returns to the starting point [0], where there is a emptying of a collection vehicle, the sanitation, maintenance and necessary administrative tasks. These two points are always part of optimized routes for each separated waste separately.

In the program Maple, to optimize data was used the Petřík's algorithm of farthest insertion. This algorithm is fully described in the collection 11th Summer School of Applied Informatics and for his extent is described here only briefly. (Hřebíček et al. 2014). This algorithm has been after professional consulting adapted and adjusted to optimally processed input data. The first step of Petřík's algorithm in the program Maple was retrieved the data. From the assigned GPS coordinates were calculated coordinates of the center collecting areas and take into account the curvature of the earth. The middle of the collecting area is always assigned to coordinate [0,0] on the chart (Hřebíček et al. 2014). Points in the argument then was assigned serial numbers in alphabetical list of municipalities and new coordinates [X, Y]. Furthermore, the determined number of processed points, which varies depending on the type of separated waste. The smallest number of points includes races for collection of biodegradable waste. Most points within the route for collection and transport of plastic. It was subsequently determined the distance between any two points and then was created a matrix clearance distances of individual points. It was now possible to create an algorithm farthest insertion.

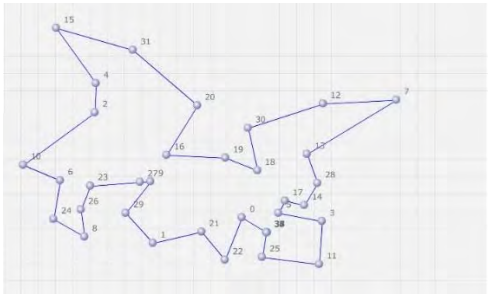
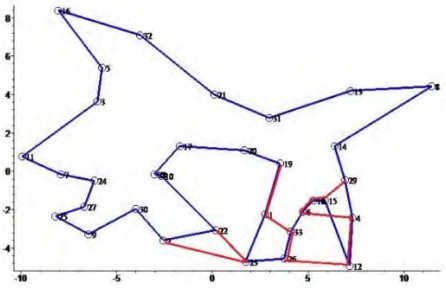
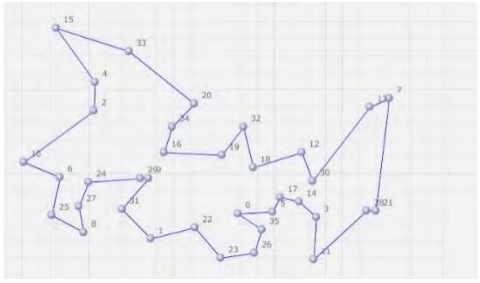
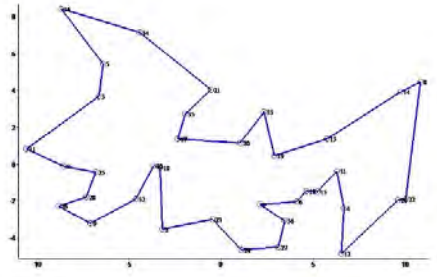
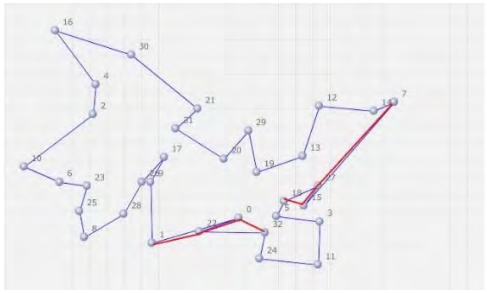
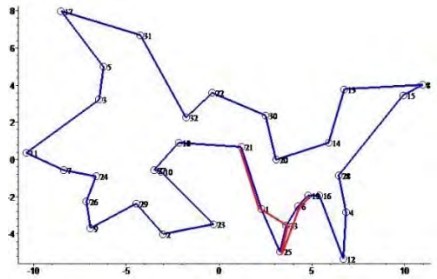
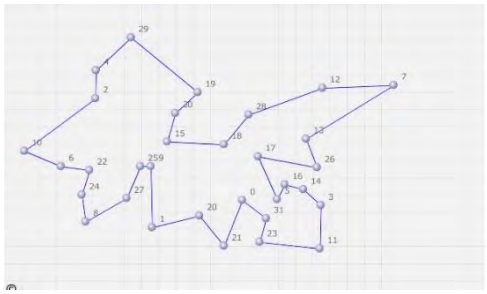
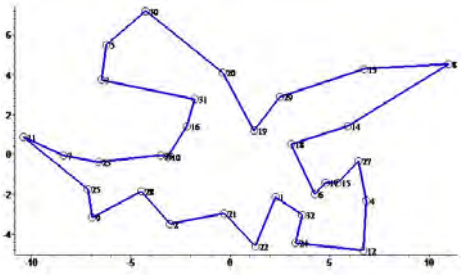
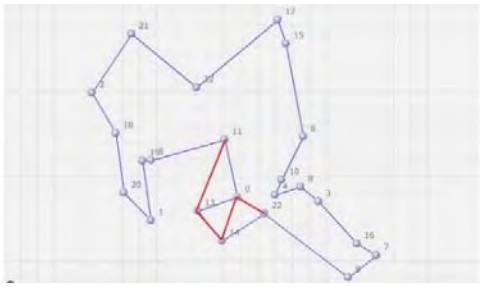
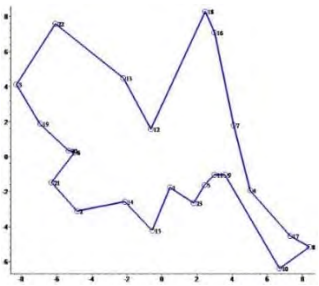
At the beginning the loop has been created, which gradually passed through all the possible pairs of the initial points. Among the selected pair was created segment and these two items were removed from the matrix. It was determined the remaining number of points in the array. It was subsequently determined distance remaining points from the resulting lines and was selected point, which was located furthest from the segment. With this item was created the triangle that formed the basis of partial routes. Point was eliminated from a matrix and there was a re-conversion points from the remaining distance lines. Again, it was chosen farthest point. Into partial routes has been incorporated so that there is a disconnection of the existing lines, and created two new line segments with a given point. The procedure of finding and subsequent inclusion in the farthest point to an existing partial route was repeated until the matrix was left in no point. If using a loop attempting to find a better pair of starting points, the result was a shorter route than in the previous selection, initial route was replaced by a new shorter route. The last step listed the course of the route point by point by the assigned serial numbers and calculated the total distance of the route. Optimal route created be using Maple were graphically displayed and for individual folders sorted waste are presented with descriptions for the following part of this work.

RESULTS AND DISCUSSION

Initial analysis of the data held in the program Bjornson's application, which is available online (Bjorson 2008). This application offers to solve the traveling salesman problem using four optimization methods. The disadvantage is that the points to be placed manually on a preformed grid so it is necessary to take into account human error factor. Bjornson's application may well serve for an initial analysis of data, based on which it is possible to opt for a more appropriate selection algorithm or choose a different approach. Thanks to the preliminary data analysis for the next steps of this work chosen algorithm of farthest insertion. The algorithm of farthest insertion showed the best results in comparison with the

other three algorithms (next neighbour, nearest insert and cheapest insert) from Bjornson's blog (Novotná 2016). Also Cook (2012) reported that algorithm of farthest insertion is best out of these four.

Table 1 All of optimized routes for separated waste with manual adjustment

Waste	Bjornson's application	Petřík's algorithm in Maple
Paper		
Plastic		
Colored glass		
White glass		
Biodegradable waste		

For collecting paper occurs in 31 municipalities, plastic is collected from 34 municipalities, colored glass from 31 municipalities, white glass from the 30 municipalities and biodegradable waste from 21 municipalities (Novotná 2016).

In the Maple system was created five optimized routes using the Petřík's algorithm of farthest insertion. Some of the routes were manually adjusted. Adjustment was necessary to satisfy the conditions set TSVM. This condition is weighing of a collection vehicle on vehicle weight and his move to the headquarters TSVM after the vehicle clears all the collection container of optimized routes. For these adjustments were not used special methods. But as Cook (2012) says in his publication, can be used for further modification n-opt algorithms. The optimized routes were created in Bjornson's application too. All of these routes are included in the Table no. 1. Manual adjustments are displayed by red lines.

The outputs of the two applications cannot be combined. Therefore, there was a measuring lengths of optimized routes by independent tool. It was made by tools "Measuring lengths", which is available online on server www.mapy.cz/. The measurement results are shown in Table no. 2 and individual data are compared with each other. Header table presents the types of separated waste for which routes has been optimized. The first column indicates the type of the optimization and final difference in length. All figures are shown in meters.

The output of Bjornson's applications were routes for collection of paper, plastic and white glass, which were in line with the condition imposed by TSVM and these routes did not need to be changed. These routes have been measured airline ideal distance. The optimized route for the transport of paper contained 33 points, where cartage vehicle must pass in order to empty all containers collecting this kind of separated waste. This route with an accuracy of tens of meters has a length of 86,640 meters. The route for the transport of plastic is 88,310 meters and the car must pass 36 points. For the collection of white glass collecting vehicle must pass 32 points and the length of the optimized route is 81,160 meters. Routes for collection of colored glass and biodegradable wastes that were created using Bjornson's application had to be manually adjusted. Modifications were carried out with respect to all field conditions. Flying route for collection of of colored glass, after all editing has been reduced by 7,960 meters, its total length is 85,520 m. Pick-up truck has to pass 33 points. Air route length for collection of biodegradable waste is 68,570 meters, and the route was shortened adjustments of 770 m. For this kind of collection of separated waste collecting vehicle must pass 23 points.

Adjusted Petrik's algorithm in Maple created optimized routes for collection of plastic, white glass and biodegradable waste, which no longer need to be modified. The length of the route optimized for the collection of plastic is 88,470 meters. In comparison with the final route of Bjornson's application, the route created in Maple was extended by 160 m. This distance is negligible with respect to the total distance of the route. The route for the transport of white glass, which was created in Maple, is long 83,350 meters. Compared with optimized route of Bjornson's application is extended by 2,190 meters. The Maple created an optimized route for collection of biodegradable waste, which was long 65,020 m. This route compared with Bjornson's route is shorter by about 3,550 meters. Routes for the collection of paper and colored glass that were created in Maple, had to be manually adjusted. There was the condition set by TSVM. Both routes lead to modifications extension of these routes. At routes for collection of paper was an extension of the original route of 1580 meters. The final route has a length of 88,950 meters. It is about 2,310 meters longer than the route created by Bjornson's application. The route for the transport of of colored glass has been extended by 490 m. The final length of the routes is 87,440 meters, and the route is about 1,920 meters longer than the route created by Bjornson's application. After comparing the lengths of the final routes by using the system Maple was created a single route whose length is shorter than the route optimized through Bjornson's application. This route is designed for the collection of biodegradable waste. Another route for the collection of other types of separated waste were compared with Bjornson's applications longer. The smallest difference in comparison final routes was 160 m and the biggest difference 3,550 meters. These routes are of course ideal air optimization and they do not coincide with the real used routes. Solution does not include the municipality in which only leads one way or does not reflect the actual profile of the terrain and the condition of roads.

Author of Bjornson's application sees as a disadvantage that it is not possible to insert your own GPS coordinates to the application (Bjornson 2008). Points representing municipalities must be manually placed on a display grid. This can cause deviations in the route optimization and better results compared to the system Maple. The advantage of using the Maple program is that it enables complex processing of the problem. It allows input your own coordinates, transformations of coordinates,

calculations of the lengths of the routes and processing of already transformed data. Maple enables gradual simplification of the problem by using command of library Graphtheory (Petřík and Bartoň 2016).

Current outputs of both systems offers further possibilities for reflection to optimize the routes. One possible suggestion is to create a command line that includes the condition set by TSVM directly into the algorithm. The next step could be to take account of municipalities, which leads to only one path, and also create arrays that would contain the actual distance between the villages.

Table 2 Length of optimized routes

Application	Kind of separated waste				
	Paper [m]	Plastic [m]	Colored glass [m]	White glass [m]	Biodegradable waste [m]
Bjornson‘s application	86 640	88 310	85 520	81 160	68 570
Petřík’s algorithm	88 950	88 470	87 440	83 350	65 020
Difference in length	2 310	160	1 920	2 190	3 550

CONCLUSION

This article shows how the Salesman problem may be used in the issue of waste management. Because there is currently no way to create the optimal route in a reasonable time, there are disclosed two methods, how the algorithm of farthest insertion can be used to optimize waste collection routes and how this algorithm behaves in two different programs. These programs are Bjornson’s application and algebraic system Maple. Both of these systems have been tested on five waste collection routes under management of TSVM.

System Maple exhibits a high accuracy because optimized routes are made with using the GPS coordinates of villages that can be loaded into the program. It is impossible to enter the same data to Bjornson’s application. Points representing municipalities must be manually placed on a display grid. This may cause deviations in the route optimization. But both of these methods can be used by the collecting companies for making waste collection more efficient processes while sparing the environment.

ACKNOWLEDGEMENT

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THE USAGE OF ALGAE IN BIOGAS TRANSFORMATION

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Abstract: Using of algae in a biogas transformation is still in the beginning. However, the microalgae have large potential from the perspective of growing demands on biogas quality and trend of using natural resources. First of all, it is their ability to fix carbon dioxide (CO_2) using photosynthesis and presumed ability of some algae to metabolize hydrogen sulphide (H_2S). Biogas contains not only required methane but also components causing its worse quality such as mentioned CO_2 and H_2S. Therefore, the algae are potential biological systems for biogas-conditioning. The microalga *Chlorella pyrenoidosa Chick (IPPAS C2)* was used for fixation of CO_2 and H_2S in our experiment. The microalgae were cultivated in a medium BG 11. The algal suspension was aerated with the biogas during two weeks. Different values of CO_2 concentration measured in the input and output confirmed decrease of CO_2 caused by intensive growth of algal culture. Decline of H_2S was not confirmed.

Key Words: biogas purification, carbon dioxide, methane, hydrogen sulphide, biological treatment

INTRODUCTION

The first references of using algae are dated back to several centuries BC. One of the first record which proves that algae were used as a food, feed and fertilizer in old China, is dated approx. 500 years BC (Barsanti and Gualtiery 2006). The scientists tried to research the morphology of algae and possibility of their cultivation in the 19th century. At the beginning, they tried to find suitable conditions for cultivation in the laboratory and to keep algae viable for some time. The first information about the isolation of pure alga culture comes from Dutch microbiologist Beijerinck. He managed to isolate wild algae of the genus *Chlorella* in 1890. Then he successfully cultivated genus *Scenedesmus* in 1893 (Andersen 2005). The controlled cultivation of algae has started in the 20th century. In 1960, the first experiments with usage of algae for pretreatment of the wastewater were started. Related these experiments, first integrated systems of producing microalgae in the open ponds were designed. Tests of anaerobic digestion of produced biomass were performed only in the nineties of 20th century. (Sialve et al. 2005). The dominant benefit of algae is fast growth in a short time period. Thanks to this ability, the algae might be suitable substrate for the biogas production. Especially if the algal biomass could be obtained during the cultivation in wastewater (Chantrasakdakul et al. 2015).

Production of biogas from maize or other agricultural waste substrates is in the spotlight in the present time. It reflects the worries about depletion of the natural fossil fuels. Biogas is a mixture of methane (CH_4), carbon dioxide (CO_2), hydrogen sulphide (H_2S) and other substances. We can classify it as the fuel for an electric energy production. After the suitable treatment, it might be possible to use biogas under the same conditions as the fuel from fossil sources.

For the higher quality of the biogas, it is suitable to eliminate the substances which have negative influence on it. This includes the elimination of H_2S and CO_2. High concentration of H_2S in the biogas causes the corrosion of engines, pipes and other apparatuses. For these reasons, the various chemical and biological methods are used for H_2S removal. Usage of algae may become one of the biological

methods to treat the biogas. Some species are able to metabolize these unfavorable substances occurring in the biogas. For example, high content of CO_2 radically reduces biogas calorific value and increases emission of carbon monoxide during combustion. Algae have high ability of CO_2 fixation. Thanks to this ability, there is formed valuable product, which might be utilized again in the biogas production (Miyawaki et al. 2013, Rameshprabu and Dussadee 2015).

MATERIAL AND METHODS

The strain *Chlorella pyrenoidosa Chick (IPPAS C2)* was used during experiments. Suspension of algae was mixed with sterile culture medium BG11 containing necessary macroelements and microelements for algal growth. The elemental composition of medium is described in Table 1. (Stanier et al. 1971). The algal suspension volume 400 ml was filled up to four sterile Erlenmeyer flasks. Each flask was closed with rubber stopper which had two vents. One of them served as the input of biogas which aerated algal culture in the Erlenmeyer flask. Second gap was outlet of biogas. This output was also used for quality measurement of outgoing biogas. Figure 1 presents the scheme of the experimental setup.

Algal culture in flasks was placed in the laboratory fume hood to avoid gas escape to the laboratory. The algal cultures aerated with biogas were continuously illuminated by fluorescent lamp (Osram Lumilux T8 58W/840) with light intensity 20 µE µmol (photons) $1/m^2/s1$ of cool white light. Temperature in the laboratory fume hood was stable (24 ± 1 °C).

Flow rate of inflowing biogas was set up to 100 ml/min.

Used biogas was produced during fermentation tests. Laboratory gasholder served for storage of biogas. Its volume was 1 m^3. Concentration of CO_2, H_2S, CH_4 and O_2 were analyzed daily by gas analyzer (COMBIMASS® GA-s, BINDER GmbH, Germany). Measurements were performed in the biogas input and output nine days at the same time of the day. Values of pH and temperature were observed in one flask which was allocated for this purpose. The optical density was also measured in the mentioned flask. It was measured three times during the experiment, at the beginning and twice during the experiment. Optical density was analyzed by AquaPen C AP-C 100 (Photon Systems Instruments, Czech Republic). Pictures of each flask were taken every day.

Figure 1 Scheme of experimental setup

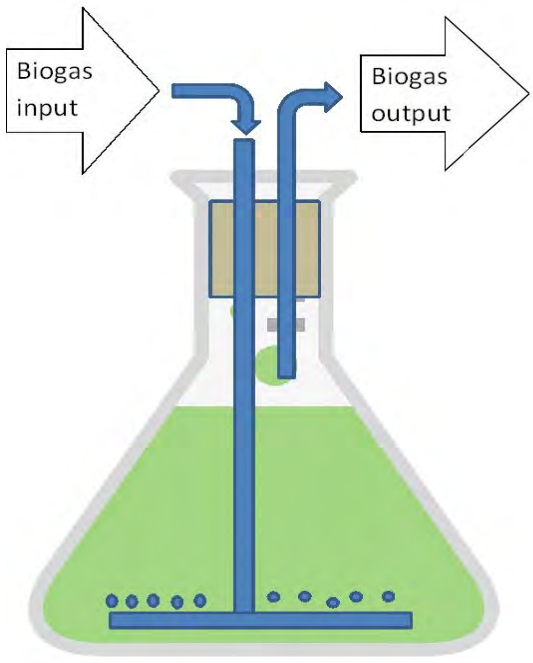


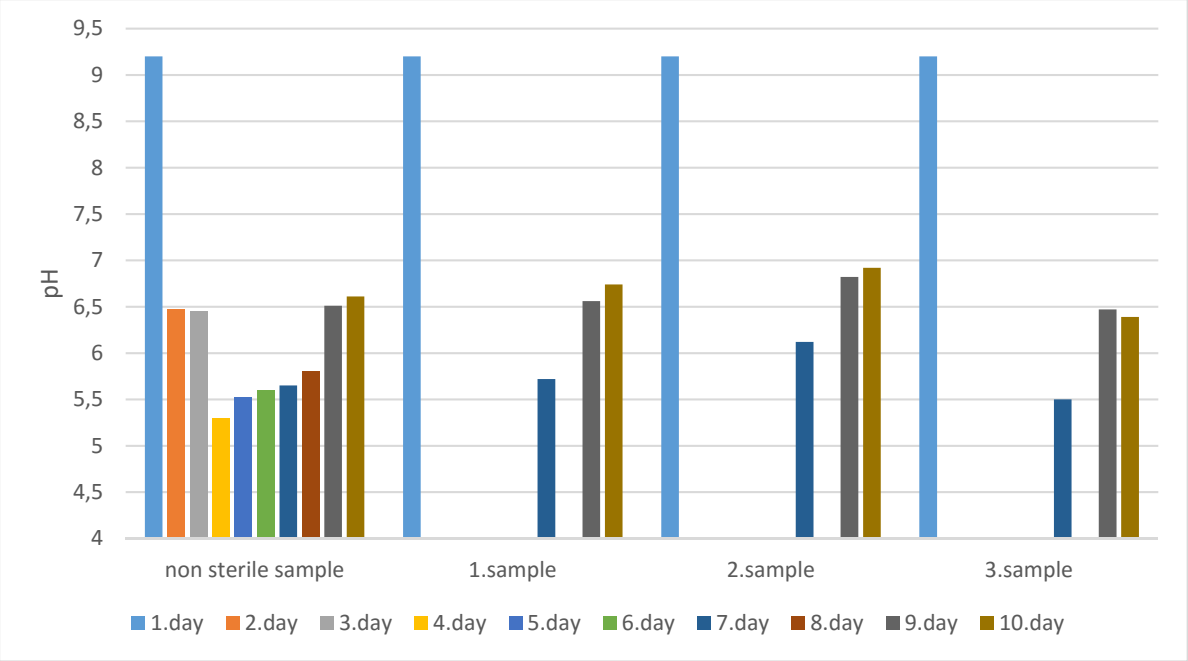
Table 1 Mineral composition of the culture medium for algal growth

Chemical substance	Concentration (g/L)
$NaNO_3$	149.6
$MgSO_4 \cdot 7H_2O$	7.48
$CaCl_2 \cdot 2H_2O$	3.6
Citric acid	0.6
Na_2-EDTA$\cdot 2H_2O$	0.123
Ferric ammonium citrate	6
Na_2CO_3	20
K_2HPO_4	30.5
H_3BO_3	2.86
$MnCl_2 \cdot 4H_2O$	1.81
$ZnSO_4 \cdot 7H_2O$	0.22
$Na_2MoO_4 \cdot 2H_2O$	0.39
$CuSO_4 \cdot 5H_2O$	0.079
$Co(NO_3)_2 \cdot 6H_2O$	0.049

RESULTS AND DISCUSSION

The pH value of the culture media for optimal growth of *Chlorella pyrenoidosa Chick (IPPAS C2)* was found 6.5 (unpublished results). At the first day of the experiment, the value of pH was 9.2. This alkaline environment was caused by transferring the culture into the fresh cultivate medium. Following days of cultivation, the medium showed rather acid reaction. CO_2 dissolving in solution was the reason for the pH value reduction. Measured values of pH ranged from 5.3 to 6.92 (Figure 2).

Figure 2 pH values during the time



Biogas composition changed during the test depending on the stage of the fermentation process. The composition was initially characterized by a low content of CH_4 (3.2%), 3.5% of CO_2, and high O_2 content of 18.6%. Concentration of H_2S was represented by the level of 10 ppm. During testing, CO_2 concentration gradually increased to a maximum of 47.1%. Later, when the process of methanogenesis fully started, CO_2 concentration dropped to 31% and concentration of CH_4 raised to the maximum value of 53%. Simultaneously O_2 concentration decreased to a minimum of 2.7%. The amount of H_2S increased from 10 ppm to 20 ppm in the second half of the test and stayed stable until the end of the test.

Measured concentrations of CO_2 and O_2 were verifiably different. Difference between the inlet and the outlet concentrations of monitored gases are shown in the Table 2. The change of CO_2 and O_2 concentration proved ongoing photosynthesis. Algae were consuming CO_2 (consumption is illustrated by negative values in the Table 2) and producing O_2.

The maximum decrease of CO_2 was 0.7%, the average decrease of CO_2 was 0.22% ± 0.026%.

The value for sample 1 of the 4th day of measurement was eliminated from calculations of the average CO_2 reduction. In this measurement an increase in CO_2 concentration has been recorded compared to the values of the incoming biogas. In the event of a leak, thanks to a lower concentration of CO_2 in atmospheric air, dilution of the mixture would occur and subsequently reduction of the CO_2 concentration in the Erlenmeyer flask. In samples 2 and 3, measured on the same day, a slight reduction in CO_2 versus entrant biogas was recorded. Based on these findings value was discarded as erroneous measurement.

The maximum production of O_2 was 0.4%, the average value of the O_2 production for the entire experiment was 0.15% ± 0.051%. Differences in H_2S are actually zero. Only in two cases, was recorded increase in the concentration of H_2S in an Erlenmeyer flask compared to entrant biogas. In the seventh day of the experiment in sample 1 and at the nineth day of experiment in the sample 2. In the fact, such

a phenomenon could not occur. This situation was caused by an error of measuring device COMBIMASS. In this experiment the ability of algae to metabolize H_2S was not proven.

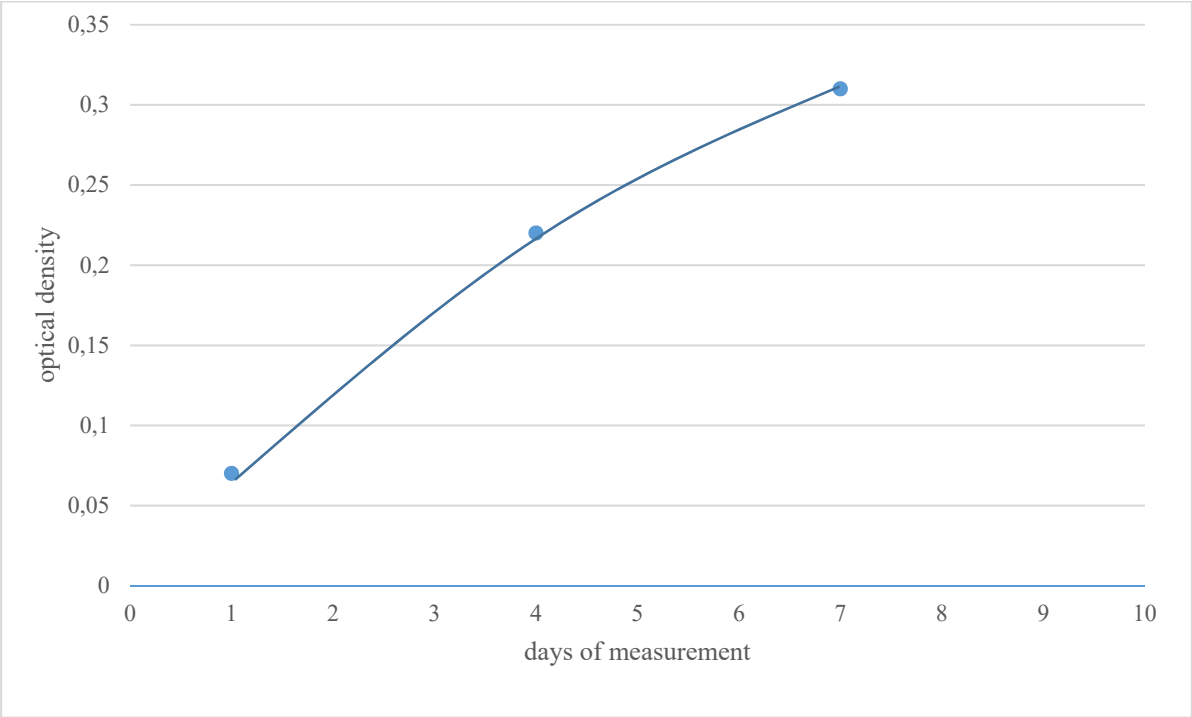
The measurement has shown that flowing biogas through the algal culture has insignificant influence to methane concentration in the outlet stream.

Table 2 Input and output concentration differences of monitored gases

	1.sample			2.sample			3.sample		
	CO_2 [%]	O_2 [%]	H_2S [ppm]	CO_2 [%]	O_2 [%]	H_2S [ppm]	CO_2 [%]	O_2 [%]	H_2S [ppm]
day 1	-0.2	0.1	0	-0.1	0.1	0	0	0.1	0
day 2	-0.2	0.3	0	-0.2	0.4	0	-0.1	0.2	0
day 3	-0.3	0.1	0	-0.3	0.1	0	-0.7	0.2	0
day 4	0.7	0	0	-0.1	0.3	0	-0.1	0.2	0
day 5	0	0	0	-0.1	0	0	-0.5	0.3	0
day 6	-0.2	0	0	-0.3	0.1	0	0	0.1	10
day 7	-0.1	0	10	-0.4	0.3	0	-0.3	0.1	0
day 8	-0.2	0.1	0	-0.3	0.4	0	-0.2	0.1	0
day 9	-0.4	0.2	0	-0.3	0.2	10	0.1	0	0

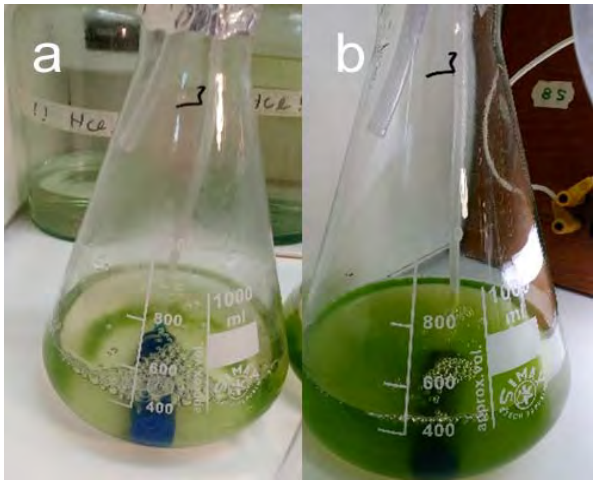
The growth of algae can be demonstrated also with growing optical density. It was increased approximately 4.5 times in comparison with an initial optical density observation. The growth of optical density is in the Figure 3.

Figure 3 Optical density development



Next algae growth proof is different color intensity of cultivated culture at the beginning and at the end of the experiment. Figure 4a illustrates the color of algal culture in the beginning of the experiment. Figure 4b shows the color in the end of the experiment.

Figure 4 Color differences



CONCLUSION

This experiment was carried out to find possibilities of using algae for biogas treatment. The experiment was focused to verify ability of tested algae *Chlorella pyrenoidosa Chick (IPPAS C2)* to fix CO_2 and to metabolise H_2S. Results of the experiment have shown that algae are able to fix CO_2 from biogas using the photosynthesis. Flowing biogas containing CO_2 caused growth of algal biomass which can be seen not only in measured optical density but also from taken photo documentation. There is visible gradual increase of colour intensity from beginning light to dark green. Further evidence of algae growth is a partial absorption of CO_2 from flowing biogas and higher content of O_2 from Erlenmeyer flasks. Unfortunately this experiment was not able to confirm hypothesis of chosen algae ability to metabolise H_2S. We did not observe statistically significant difference in concentration of H_2S between input and output of biogas from algae solution.

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DETECTION OF HARDENING PROCESS BY MEANS OF ACOUSTIC EMISSION

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Abstract: This article deals with the issue of martensitic transformation scanning using acoustic emission. The principle of which is the use of acoustic detectors, especially in extreme temperatures. Special waveguides were developed for these purposes, enabling this type of measurement. The maximum temperature achieved in the experiment was 850 °C. Samples of carbon steel were used for the measurement, due to their extensive use in the industry. The properties of carbon steel can be widely influenced by alloying carbon and a combination of thermal and thermomechanical processing. After a non-destructive measurement by acoustic emission, was used the university device XEDO with a piezoelectric sensor. The martensitic steel quenching was performed in water. After the samples were thermally treated, was determined their hardness in different locations on the sample. The samples were then subjected to metallographic abrasive cutting, and images of the resulting structure of the samples were taken with an electron microscope. The acoustic emission was used to create a record of the heating and cooling of samples martensitic ally quenched in water. The most important result was the creation of an acoustic record of the martensitic transformation itself.

Key Words: acoustic emission, austenite, martensite, hardening, heat treatment

INTRODUCTION

Martensitic transformation, more commonly known as quenching and tempering, is a hardening mechanism specific for steel. The steel must be heated to a temperature where the iron phase changes from ferrite into austenite, i.e. changes crystal structure from BCC (body-centered cubic) to FCC (face-centered cubic). In austenitic form, steel can dissolve a lot more carbon. Once the carbon has been dissolved, the material is then quenched. It is important to quench with a high cooling rate so that the carbon does not have time to form precipitates of carbides. When the temperature is low enough, the steel tries to return to the low temperature crystal structure BCC. This change is very quick since it does not rely on diffusion and is called a martensitic transformation. Because of the extreme supersaturation of solid solution carbon, the crystal lattice becomes BCT (body-centered tetragonal) instead. This phase is called martensite, and is extremely hard due to a combined effect of the distorted crystal structure and the extreme solid solution strengthening, both mechanisms of which resist slip dislocation (Alavudeen et al. 2006).

MATERIAL AND METHODS

The experiment examines the dependence of the change in the material's temperature on its acoustic manifestations. The austenitizing temperature reached 850 °C, the temperature of the quenching bath was 20 °C. The given temperatures were chosen pursuant to CSN 41 2050 1978.

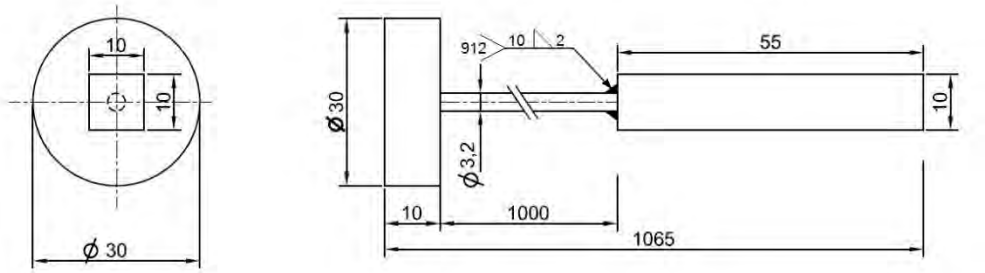
Generally, carbon is the most important commercial steel alloy. Increasing carbon content increases hardness and strength and improves hardenability. But carbon also increases brittleness and reduces weldability because of its tendency to form martensite. This means carbon content can be both a blessing and a curse when it comes to commercial steel (Capudean 2003).

For this reason was chosen material for the production of samples from carbon steel 1.1191 (C 45, 12 050) with a carbon content of 0.5%. Martensitic hardening was performed on ten specimens.

The samples were provided with a waveguide with a diameter of 3.2 mm and length of 1 m, which was pressed and soldered into the test sample. At the other end, a facet with a 20 mm diameter and a surface roughness of 3.2 Ra was attached for the placement of the acoustic emission sensor. This connection was also pressed with an overlap for better signal guidance, and a soldered joint was added. A diagram of the sample with a waveguide is in Figure 1.

The diameter of the waveguide was chosen with the purpose of minimizing the effect on the results of the acoustic emission measurement, as well as the possibility to handle the sample when inserting it into the furnace and quenching bath. The selected material for the waveguide and the facet was low-carbon steel, in order for it to coincide with the measured samples.

Figure 1 Diagram of the sample with a waveguide



Samples

The mentioned samples with the dimensions of 10 × 10 × 55 mm made of carbon steel were subjected to heating at a temperature of 850 °C in the school laboratory furnace MP 05–1.1.

Laboratory furnace MP 05 – 1.1

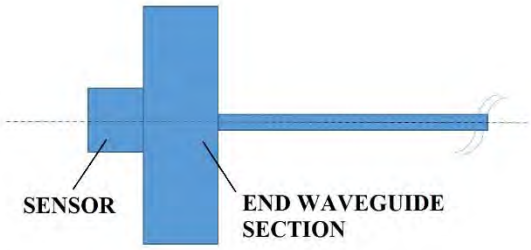
The furnace has a sheet metal casing. It consists of two parts joined with screws. The operation and control of the furnace is located at the bottom of the casing; a ceramic muffle with a heating coil and thermal insulation is located at the top of the furnace. The temperature in the furnace is sensed by thermocouple PtRh - Pt. The heating coil is prepared over the contactor contacts. The contactor coil is also controlled by the door switch and protective circuit of the controller.

The furnace had to be adjusted so that the material could be seamlessly inserted and removed. A chamotte block was inserted into the furnace, which reached the height of the opening in the back of the furnace. This adjustment was chosen in order to eliminate the stress on the waveguide and connections.

Measuring system

The measuring was conducted using the school device XEDO by Dakel with an IDK sensor. The sensor was attached to the prepared surface. (Figure 2).

Figure 2 Detail of sensor attachment



AE Diagnostic System The DAKEL – XEDO diagnostic system is an advanced device for capturing and recording of AE parameters, localization of AE sources, and signal sampling. Its main purpose is to monitor periodical pressure tests to detect any potential hidden defects in primary circuit

technology material and to identify locations that have the highest probability of material defect occurrence.

These locations can be then subject to more detailed examinations by other diagnostic methods. System sensors are permanently located on power plant primary circuit loops, in throat segment of reactor pressure vessel, volume compensator and pipeline network. Systems sensors can also act as electronic transmitters (pulsers) enabling the function check and calibration of sensors (Dostal et al. 2011).

The heating temperature of 850 °C was selected according to CSN 41 2050 1978 and can be confirmed on the basis of the phase diagram Fe-C found in the literature (Fiala et al. 2003). The precise hardening temperatures were set according to the diagram of an isothermal decomposition of the steel austenite from the article Totten and Howes (1997).

The heating temperature remained at the hardening temperature for 15 minutes, which is the optimum time for these samples, stated in the literature Llewellyn (2013) and Hosford (2010).

The cooling was conducted in a medium suitable for quenching - for each of the ten samples was used distilled water at a temperature of 20 °C. After the heat treatment, the hardness of the samples was measured according to Rockwell and the literature Herrmann (2011). The hardness was measured ten times at the edge of the material and at its center. The values measured by acoustic emission were set up in the Daemon system, and evaluated and visualized in the DaeShow program.

RESULTS AND DISCUSSION

The primary outcome was the finding that is possible capture an acoustic emission signal during martensitic quenching. As mentioned before, the testing was carried out with a cooling medium - distilled water at a temperature of 20 °C. A total of ten measurements were performed. After a cut was made in each sample, a Rockwell hardness test was performed. The hardness was measured at the center and at the edge of the material (See Table 1).

Table 1 Overview of fungicide treatments

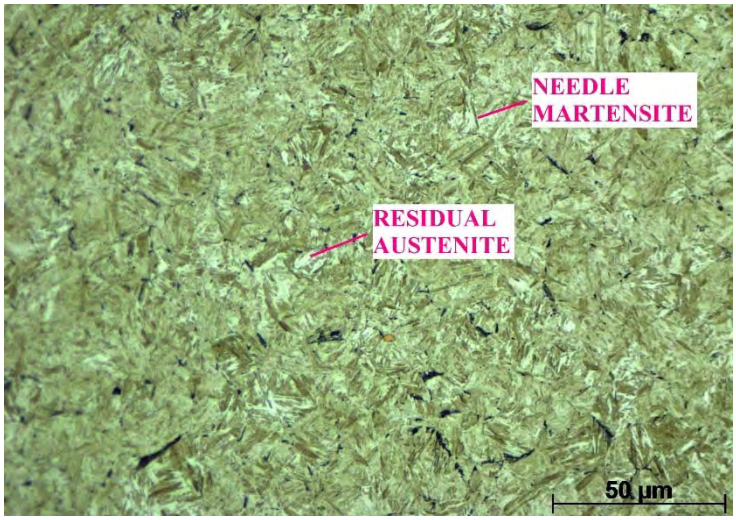
Hardness test HRC			
Number of tests	Center of material	Number of tests	Edge of material
1	59	1	60
2	59	2	59
3	60	3	60
4	59	4	60
5	60	5	59
6	60	6	59
7	59	7	60
8	59	8	59
9	60	9	60
10	60	10	59
Average	59.5	Average	59.5
Variance	0.25	Variance	0.25
Standard deviation	0.5	Standard deviation	0.5

The average hardness at the center of the material was 59.5 HRC. The same value was measured at the edge of the material. These high values can be explained by the fact published in the article (Votava et al. 2005). Carbon that is forcibly closed in an iron lattice induces high internal stresses, resulting in a high level of hardness.

Based on articles by (Ptacek et al. 2002) the measured value of hardness corresponds with the hardness of martensitic ally hardened steel containing 0.5% C, which ranges from 59–61 HRC.

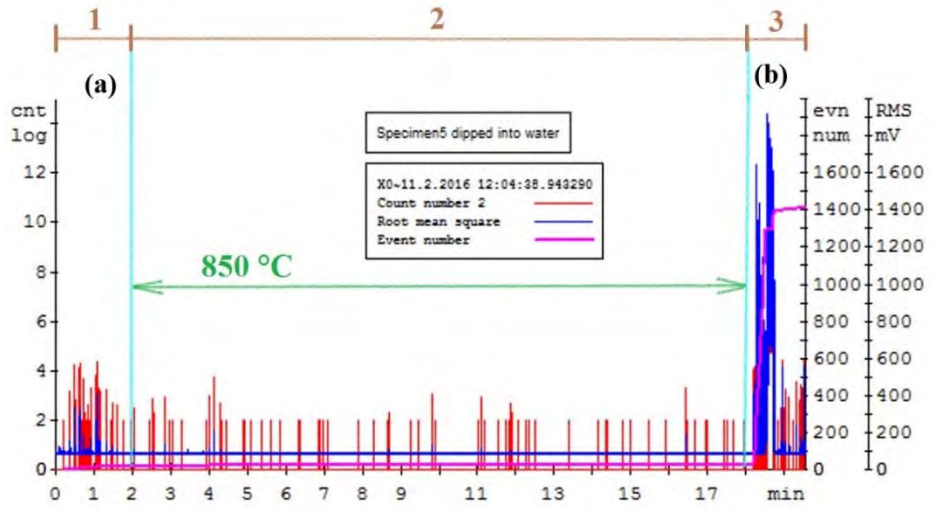
Another result includes images of a metallographic abrasive cut, shot with an electron microscope in collaboration with the Faculty of Mechanical Engineering at the Technical University. The metallographic images of samples quenched in water clearly demonstrate the complete transformation from austenite to martensite, which corresponds with the measured hardness value. The sample was hardened throughout the cross section, as the measured hardness values show. The structure of the steel after quenching in water is shown in Figure 3.

Figure 3 The structure of the steel after quenching in water



The austenite, which is transformed to martensite needles with rapid cooling, forms lenticular facets on the surface of the steel after metallographic treatment. The grain structure visible with corrosion is evidence of a diffusion less transformation of the original structure.

Figure 4 Record AE characterizing the water quenching with marked sections



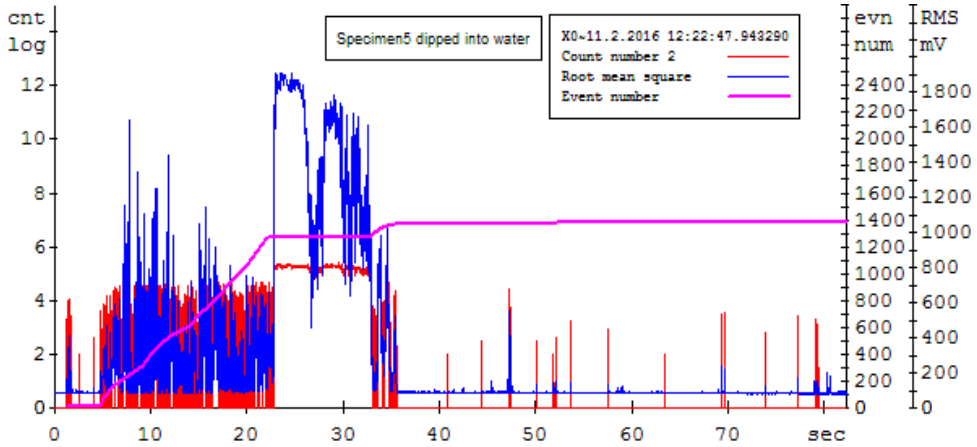
Legend: AE count number, root mean square and event number values versus time; (a) zone is AE parameter values at the beginning; (b) zone is AE parameter values at the end; (1) zone is heating; (2) zone is retention on the temperature; (3) zone is cooling

The resulting needles are caused by volumetric tension in the primary austenite grain, as a result of the transformation of the position of corner atoms in the fcc lattice model, from a face-centered cubic lattice to a body-centered tetragonal lattice.

The most important result of this experiment is the acoustic emission record, which captured the process of heating and cooling samples. It also captured the transformation from austenite to martensite. The hardening, i.e. the transformation of the austenite fcc lattice including the overall handling of the hardened sample, is shown in Figure 4.

A record of the transformation from austenite to martensite was created during the water cooling, which is shown in Figure 5. It is a more detailed visualization of the cooling section (b) from Figure 4.

Figure 5 Record of austenite to martensite transformation in an H_2O quenching medium



The record shows the removal of the sample from a prepared hole, the effect of the rapid oxidation, the transfer to a quenching medium (water), the formation of gas-saturated layers around the edges of the sample - water. The martensitic transformation itself begins approximately 23 seconds after the sample is removed from the furnace. In water quenching, the cooling, and therefore the transformation from austenite to martensite, is extensive. The AE record shows a secondary peak on the RMS curve in water quenching, which implies significant activity in terms of the energy of the signal, whereas events have a constant course and it is therefore another headlong structure change, a phase change such as the one recorded at the beginning of the hardening; it is most likely the additional hardening of the remaining residual austenite. The obtained results are compact and is possible therefore recommend a physical method of nondestructive AE testing for the evaluation of the entire phase transition. The acoustic emission records can be explained by the article Cerny et al. (2016).

It is evident that the correlation between material hardness and acoustic emission response is influence by the hardness and structure of tested material. The harder material the stronger, more readable a more regular is acoustic emission response. The main assessed and evaluated variables were the following: signal energy – RMS which was proportional to hardness i.e. the higher signal energy the higher hardness and another important indicator – the number of overshoots and total cumulative quantum of events. Together with increasing material hardness and thereby causing rise of indentation resistance, which results in increase of discontinuous signals of AE with very regular attenuation.

CONCLUSION

For the experiment, it was necessary to design a measurement system and develop appropriate waveguides for the transmission of the acoustic signal, which were designed and created specifically for the purposes of our measurements. The waveguides needed to have a sufficient length so as to protect the sensor, because the maximum temperature at which the sensor can be used in 60 °C. The test samples were sensed by acoustic emission during water quenching. The samples were subjected to hardness tests, which demonstrated an average hardness of 59.5 HRC.

This value of hardness is optimal for martensitic ally hardened steel, according to articles by Ptacek a kol. (2002). The samples were then subjected to a metallographic analysis, which shows us the resulting structure of the material after the heat treatment. When water quenching is used, is possible

see a complete transformation from austenite to martensite in the resulting structure. As all our measurements show, is possible consider the experiment demonstrable in terms of AE, because the results of the acoustic emission measurements are confirmed with the hardness tests and metallography. Is possible therefore recommend the method of nondestructive AE testing for verifying phase transitions. The content of this article, and especially the experimental measurements contained herein, can be used for further research in the area of nondestructive testing of materials in technical practice.

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DETERMINATION OF THE TRACTOR ENGINE POWER IN THE FIELD CONDITIONS

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Abstract: This article deals with the determination of the tractor engine power in the field condition. Effective engine power is the basic output parameter of the engine. At the evaluation of tractor output parameter, engine power is used for determination of drawbar pull. Engine power is product of torque and angular speed. CAN-Bus reading is widely spread technique in testing. Information about actually torque is also provided, but this information and its value is indicated in percent. For this reason, the direct calculation of engine power is not possible and it is necessary to proceed from other parameters. Application of the engine load and the engine speed for determination of the engine power is the objective of this article. In this paper, the individual steps of model creation including partial calculations mentioned above are described.

Key Words: least squares method, natural neighbor interpolation, CAN-Bus, engine power

INTRODUCTION

In the last years, progress of electronic control systems in vehicles is evident. The growing share of these element is not reflected only in engine control, transmission and other vehicles systems, but also in testing field (Štěrba et al. 2011). Installation of external sensors is not needed to use for measurement. Reading of relevant data can be realized with the use of CAN communication. Network parameters and data transfer are specified by standards, moreover the requirements crossing different categories of vehicles are strict based on line topology. Digital data acquiring brigs more benefits like time and cost savings, reduced complications in measuring chains adjustments etc. Fundamental evaluation of performance is based on engine power knowledge. Engine power can be calculated from torque and crankshaft angular speed. At analyzing the data from the vehicle network CAN-Bus, it is clear that information about engine torque is available only in percentage units and not in Nm. For this reason, this data cannot used for calculation the engine power. There is only one solution that offers. It is needed to find out relationship among actual torque, engine speed and engine power, for example in tractor laboratories by eddy current dynamometer. However, published study dealing with similar problems (Sedlák et al. 2010) shows that information about the actual torque corporates errors. For this reason, it is preferable to determine the engine power for example from engine load. The article deals with possibilities of using the engine load and engine speed to determine the power of tractor engine in field conditions. The paper also describes the various steps in the construction of a model showing the relationship among these parameters.

At the drawbar tests, calculation of the engine power is very important, because it can be used for the calculation of traction efficiency. Drawbar pull is calculated by dividing tractive power and efficient engine power. It expresses the efficiency of transfer engine power to pulling power (Bauer et al. 2013, Semetko et al. 1986).

MATERIAL AND METHODS

To determine the relationship among the engine load, engine speed and power, tractor Claas Arion 640 CMATIC was used. Technical specification of the tractor engine is given in the Table 1.

Table 1 Technical specification of the tractor Claas Arion 640 CMATIC

Manufacturer	DPS
Number of cylinders	6
Volume capacity [cm^3]	6788
Nominal engine speed [min^{-1}]	2200
Type approval value (97/68 EG)[1] [kW/k]	128/174
Output at nominal engine speed (ECE R 120) [kW/k]	124/169
Maximal output power (ECE R 120) [kW/k]	130/177
Maximal torque (ECE R 120) [Nm]	714
Engine speed at Mt_{max} [min^{-1}]	1200
Constant power output range [min^{-1}]	1800–2200

Laboratory measurement took place in the laboratories of the Department of Technology and Automobile Transport at Mendel University in Brno in accordance with OECD. In all tests have been complied with general condition and provision about allowed tolerances of the standard ČSN ISO 789-1. For data acquisition and saving information from bus CAN-Bus, proprietary software created by Department of Technology and Automobile transport was used. DLC (data link connector) was used for connection of the tractor to computer. CAN-Bus network of tested tractor was fully compatible with standard SAE J1939 in specification 2.0B. Communication speed was set to 250 kbps and appropriate messages related to engine power were monitored: actual torque, engine load, torque losses, fuel consumption, temperature of coolant, etc.

Figure 1 Tractor Claas Arion 640 CMATIC in Vehicle laboratories of the Department of Technology and Automobile Transport at Mendel University in Brno

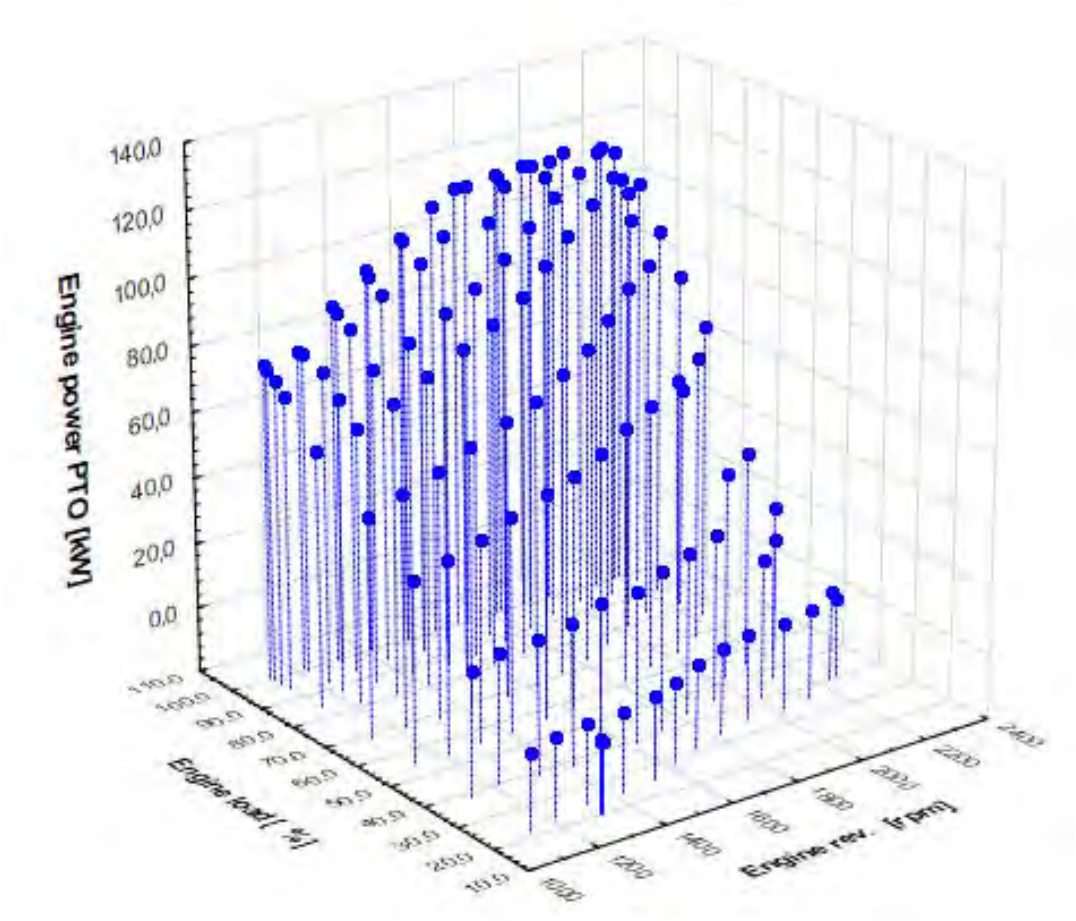
Sampling frequency of measuring chain was set to 20 S/s. For loading the engine and calculation of the engine power, eddy current dynamometer was used. Type of dynamometer: V500 (producer: VÚES Brno, speed [min^{-1}]: 150/1500/3000, power [kW]: 4/500/500, torque [Nm]: 254 / 3184/1592, cooling: water, load: permanent). The dynamometer was connected to the rear PTO of the tractor (see in Figure 1). Dynamometer regulation and saving of measured data was provided by the computer in laboratory.

To determine the relationship among the monitored parameters, measurement in full range of engine speed and engine load was conducted. It was done measuring one nominal and twelve partial characteristics with reduced fuel supply.

RESULTS AND DISCUSSION

Mentioned above, engine load, speed and engine power were major measured parameters. Engine power is labeled as the power on PTO. Since, the force (measured with the dynamometer) was not measured on engine crankshaft, but on the PTO shaft. PTO power and engine output is slightly different due to the mechanical losses. Figure 2 is 3D chart showing measured values.

Figure 2 Measured values in 3D chart

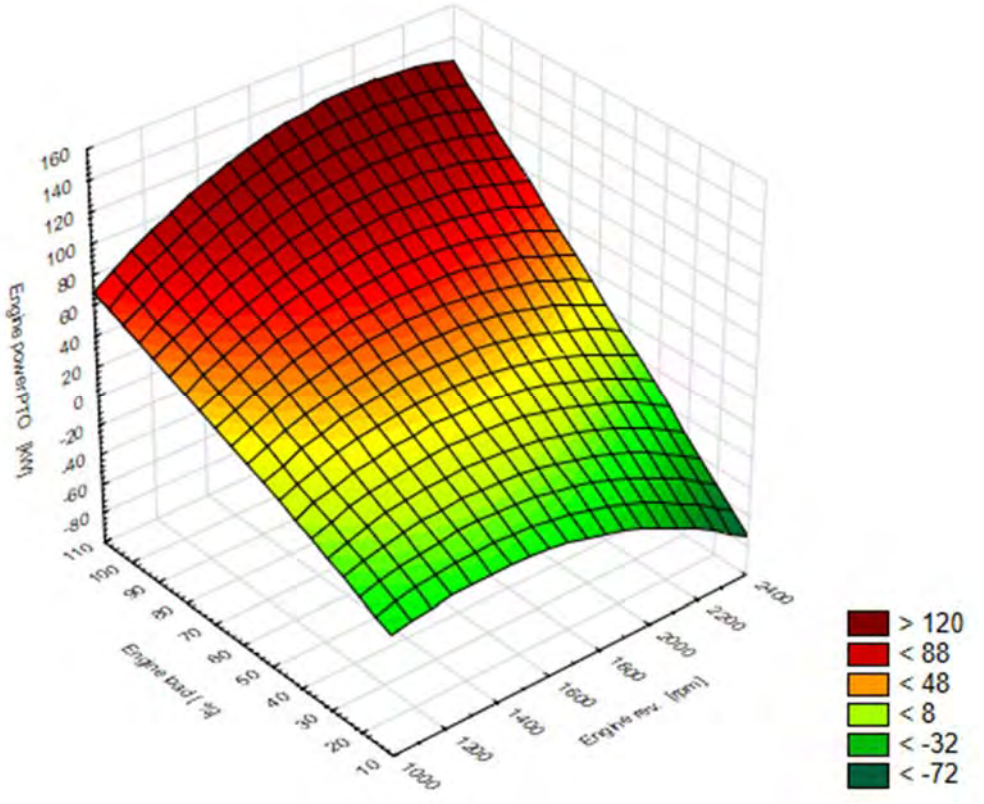


Data, in the chart (Figure 2), was used for intersperse of surface and for calculation surface equation. As independent a variables entering into the calculation, the load and engine speed were used. The dependent variable was PTO engine power. For the calculation of the equation, software Statistica 12 and Microsoft Excel 2013 (for checking of calculation) was used. For the calculation was used polynomial regression analysis using the least squares method. Calculated equation (2) has the form:

$$Engine\ power\ PTO = -127{,}9808 + 0{,}1571 \cdot Engine\ rev + 0{,}2311 \cdot Engine\ load - \\ -5{,}96 \cdot 10^{-5} \cdot Engine\ rev^2 + 0{,}0008 \cdot Engine\ rev \cdot Engine\ load - \\ -0{,}001 \cdot Engine\ load^2 \quad (2)$$

Determination index was calculated $R^2 = 0.99$. This value indicates that a high percentage of points corresponds with the calculated model. However, as absolute member show, the low engine speed and engine load will evince negative PTO power. This is also evident in the graphic expression of the equation (Figure 3).

Figure 3 Graphical representation of calculated model (Equation 2)



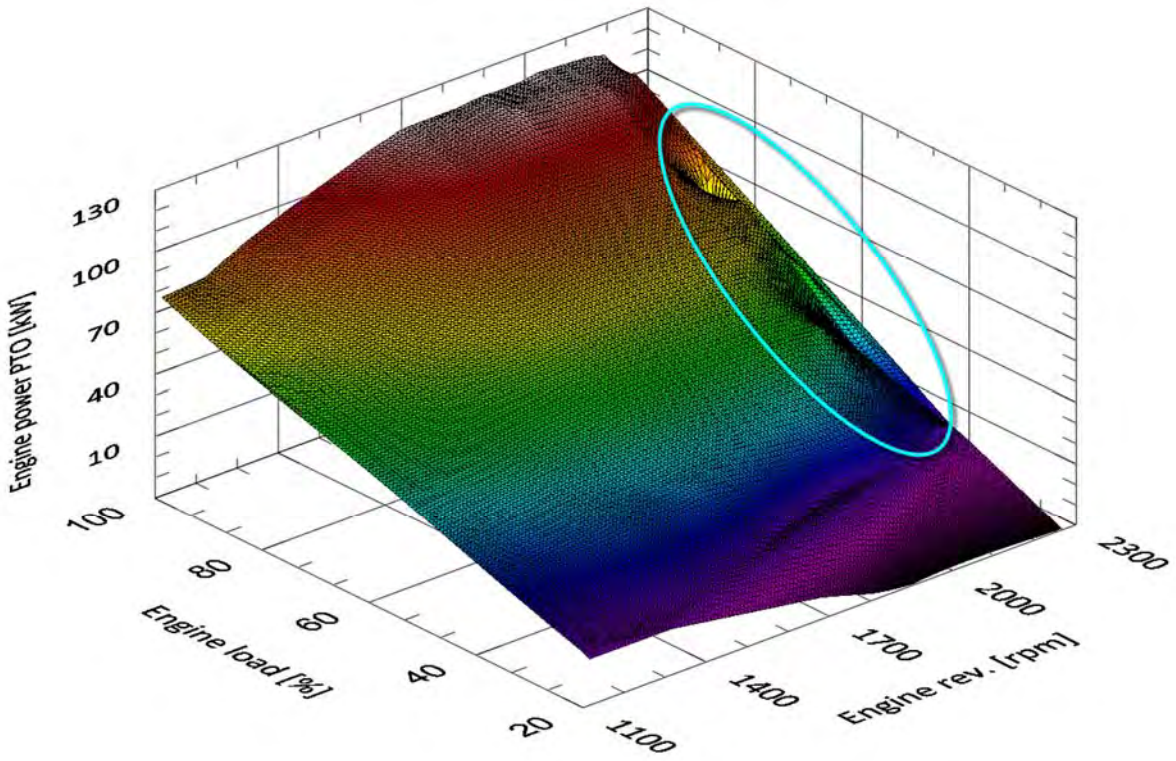
As is evident from Figure 3, not only low values of the engine speed, but also a high value of speed at low engine load indicate the negative PTO power. In this areas, negative power is realistically impossible to achieve. For this reason, using of this model for determining engine power would be very inaccurate. Hence, used polynomial regression analysis by the least squares method is inappropriate and it is needed to use another method. Another possible solution was to use the Natural Neighbor Interpolation using Watson algorithm.

Natural neighbor interpolation is a method of spatial interpolation. The natural neighbor algorithm uses a circular areal-based procedure for interpolation. It is the most general and robust method of interpolation available to date. The method is based on Voronoi tessellation of a discrete set of spatial points. It produces a conservative, artifice-free, result by finding weighted averages, at each interpolation point, of the functional values associated with that subset of data which are natural neighbors of each interpolation point (Watson 2002).

The natural neighbor relationships of data are specified by the shared natural neighbor circles. The Watson algorithm uses a simple weighted average of the *z values* of the natural neighbors of the interpolation point. This type of linear interpolation in natural neighbor coordinates is the equivalent of planar interpolation in rectangular coordinates. No gradient information is used for the non-tension interpolant. For the tension interpolant, natural neighbor gradients are computed using the natural neighbors of a data point, but not the data point itself. A compound exponential blending function is used to add the influence of the gradients and render the interpolant at the nodes. The blending function does not use the tension directly, modifying it in accord with an outlier or roughness index. For data fitted well by smooth functions, the highest tension setting is likely to produce the greatest accuracy.

From the description mentioned above, the result of interpolation is not one equations (as in the previous case), but the matrix points that will subsequently be used as an input matrix for calculation the PTO power from speed and engine load. The matrix used for description of relationship among the PTO power, load and engine speed contained 16 384 points. Graphical representation of this matrix is shown in Figure 4.

Figure 4 Graphical representation of matrix calculated Natural Neighbor Interpolation using Watson algorithm



As shown in Figure 4, data of matrix in areas with low engine load does not contain negative values of engine power and can be used for further processing. Subsequently, this data was transported into the software created for the purpose of reading relevant data from the CAN-Bus network and for determination PTO power. This program was created on the platform of LabVIEW.

As shown in Figure 4, it is possible to further notice of the slumps of engine power at high engine speeds (highlighted area). This decline is not caused by incorrect interpolation, but the regulator of the engine. This regulator intentionally reduced engine power. This effect is not seen in Figure 3 due to inaccurate calculated model.

Before the starting field measurements, it was also examined whether the PTO power determined by the calculated model exhibits deviations from the engine power measured in the laboratory. The errors in determining the performance fluctuated only in interval ± 0.25 kW. This is very small difference. Hence, this procedure is possible to use with high precision for calculation of the engine power of the tractor. As shown some studies (for example Čupera and Šmerda 2009), it is necessary to bear in mind, that before using the data from the CAN-Bus network, verify their accuracy is needed, or rather calibration of vehicle internal sensors is needed by accurately laboratory sensors.

CONCLUSION

The results of the paper show that the data from the network CAN-Bus is possible to use to determine engine power. Mentioned process is very accurate, fast and cheap, but at determining, the relationship among the individual parameters have to be sufficiently choose the appropriate calculating method.

The high level of vehicles electrification that take over control not only of engine combustion process, but also other vehicle systems. Topology of this systems allow their using at vehicle measurements. In recent years, reduction price and time-consuming arise in field measurements. Before each measurement, checking the accuracy of the sensors should be done. It is allowing to avoid introduce of needless errors in the vehicles system testing.

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THE INFLUENCE OF TRACTOR TYRES INFLATION ON PHYSICAL SOIL PROPERTIES

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Abstract: With increasing size and weight of machines using in agriculture is also increases concern of soil compaction. Soil compaction affects adversely the yield in turn. Basic connection between the soil and the machine are the tyres. Choosing tyres in agriculture affects not only the economy but also the effect of the machine to the soil. Manufactures of tyres and agricultural equipment develop new design solutions to smaller negative impact of driving the machine on the soil. It also chosen tyre pressure can cause the different effects on soil properties. In this work, we focused on the influence of tractor passes at three different inflation pressures – 1, 1.5 and 2 bars on physical soil properties - on bulk density and porosity. The results show that passing tractor through the field has significant influence on soil compaction. The change of these parameters is particularly evident in upper layers of soil (up to 0.15 m). Results also indicate that tyres inflation pressure has statistically significant influence on soil compaction in the upper layers of the soil profile.

Key Words: soil compaction, bulk density, porosity, tractor tyres, Phaeozem

INTRODUCTION

The soil compaction is a threat to the long-term productivity of soil (Brevik and Fenton 2012). The farm tractors and field equipment are becoming ever larger and heavier (Chamen et al. 2013). The compaction may occur within the tilled layer, frequently just below the zone of tillage, or even at greater depths and the subsoil compaction may persist for decades (Schjønning et al. 2015). The soil compaction affects nearly all soil physical, chemical, and biological properties and functions (Batey 2009, Hamza and Anderson 2004).

Soil compaction is the densification of soil by application of mechanical energy (Holtz et al. 2010), which can occur naturally or driven by anthropogenic activities. The result is an increase of bulk density and a reduction of pore space (Horn and Smucker 2005, Keller et al. 2013), affecting the percolation of soil water as well as gas exchange or production. It may increase the soil resistance to root penetration (da Veiga et al. 2007, Fasinmirin and Reicher 2011), water deficit during dry spells by the rooting system (Grzesiak et al. 2012, Reichert et al. 2009), saturated hydraulic conductivity, and water storage (Bhattacharyya et al. 2006, Cavalieri et al. 2009). Soil compaction has been strongly linked to the loss of nitrogen by the accelerated production of greenhouse gases (e.g. N_2O) through denitrification in anaerobic conditions (Keller et al. 2013).

DeJong-Hughes et al. (2001) reported that excessive compaction reduces crop roots grow and consequently also the overall contact with the soil. It causes the reduction in the ability of plants to take up nutrients and poor water management. The stunted plants due to reduced root activity can be in dry years. In contrast, the increased denitrification can be due to low breathability in wet years.

In our article we focused on the influence of tractor tyres inflation on physical properties of soil – bulk density and porosity. It has been applied 3 tyre inflation pressures: 1, 1.5 and 2 bars. We compared the soil properties changes in the track machine and off track machine (zero variant). The described depths were 0.05, 0.15, 0.25, 0.35 and 0.45 m.

MATERIAL AND METHODS

Characterization of locality

The field measurements were carried out at the autumn in the year 2015 of land near the village Otmarov located in the district Brno-country (49° 10′ N and 16° 67′ E), around 12 km S of Brno. The elevation of the land is 193 amsl.

Grain size composition of the land for each depth is presented in Table 1. The soil type is Phaeozem.

Table 1 The grain size composition of the land

Depth (m)	Faction	Value (wt %)	Moisture (%vol.)	Depth (m)	Faction	Value (wt %)	Moisture (%vol.)
0.05	Clay	29.30	30.59	0.35	Clay	30.98	29.40
	Silt	35.02			Silt	31.90	
	Sand	35.68			Sand	37.12	
0.15	Clay	31.24	31.61	0.45	Clay	34.34	31.81
	Silt	31.74			Silt	34.78	
	Sand	37.02			Sand	30.88	
0.25	Clay	34.36	30.29				
	Silt	33.22					
	Sand	32.42					

Legend: clay <2 μm, silt 50-2 μm, sand 2 000-50μm. Moisture – average of all samples from the place "in tractor rut" and "between the tractor ruts".

Experimental design and laboratory

It was chosen appropriate stretch of land on the locality in order to the load on the tractor´s wheels evenly distributed.

The tractor was fitted with a disc harrow. The disc harrow was divided into a working position for uniform distribution of load. Its height above the land remained in the maximum lift position of the rear three-point system of the tractor.

It was elected German production tractor Deutz-Fahr for measurement, specifically Agrotron X720 model. The tractor had at the time of measurement supplemented with all operating fluids. The carried disc harrow was clamped in the rear three-point hitch. The weight was used in the front three-point hitch for ballasting the tractor. The tractor parameters are given in Table 2.

Table 2 Selected parameters of the tractor

Parameter	Value
Tractor weight (kg)	10 160
Tractor front ballast weight (kg)	1 100
Specification of tractor front tyres	600/65R38
Specification of tractor rear tyres	710/70R42
Weight of disc harrow (kg)	3 490

The tractor tyres were inflated to 3 different pressures – 2 (A), 1.5 (B) and 1 (C) bar. The measurement section for each tyre inflation pressure was 10 m. The speed of the tractor at passing

through measurement section was 10 km/h. The inflation pressure was controlled using tyre filler with a pressure gauge manufacturer from Aerotec.

The soil samples were sampled in tractor rut (I) after crossing mechanization. As a zero variant were used samples collected between the tractor ruts (II). The zero variant was determined for each inflation pressure.

The core samples were sampled into the Kopecky rollers (volume 100 cm^3). The depths of samplings were 0.05, 0.15, 0.25, 0.35 and 0.45 m. Soil samples were taken in row spacing within 4 repetitions for each variant. Samples were processed according to the methodology of the Central Institute for Supervising and Testing in Agriculture (Zbíral 2002). The laboratory results identified values of bulk density and porosity. The bulk density and porosity are some of the physical properties, which are used to evaluate the physical condition of the soil in the Czech Republic. The limit for this soil type is for bulk density < 1 450 kg/m^3 and for porosity > 45%.

Statistical analysis

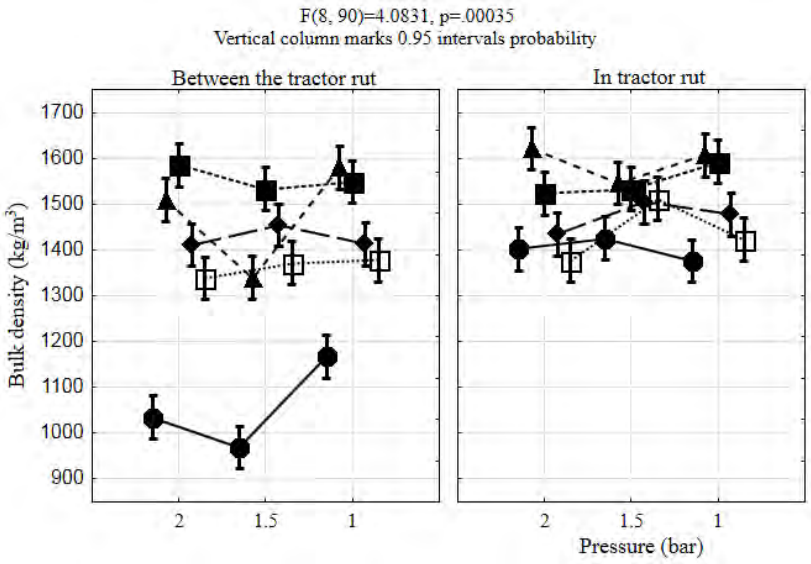
The data obtained were subjected to Grubbs test extreme deviations. Results of bulk density and porosity were statistically compared. The values were analyzed by ANOVA with interaction. Post-hoc tests were carried out by Tukey HSD test at the level $p < 0.05$. The statistical software was used Statistica 12 (StatSoft, USA).

RESULTS AND DISCUSSION

Bulk density

The graphical results from ANOVA are shown in Figure 1. The Figure 1 shows the significant difference between bulk density at the depth 0.05 m for sampling I and II. Value of II indicated significant statistical difference between the depths of 0.05 and 0.15 m. similar fact was found in I. There was the statistically significant difference between the depths of 0.05 and 0.15 m for all three variants of inflation pressure. While the bulk density at the depth 0.05 m had values with limit, the value at the depth 0.15 m was longer above the threshold. Other significant differences were apparent between the depths 0.25 and 0.45 m for all inflation pressures and especially by II.

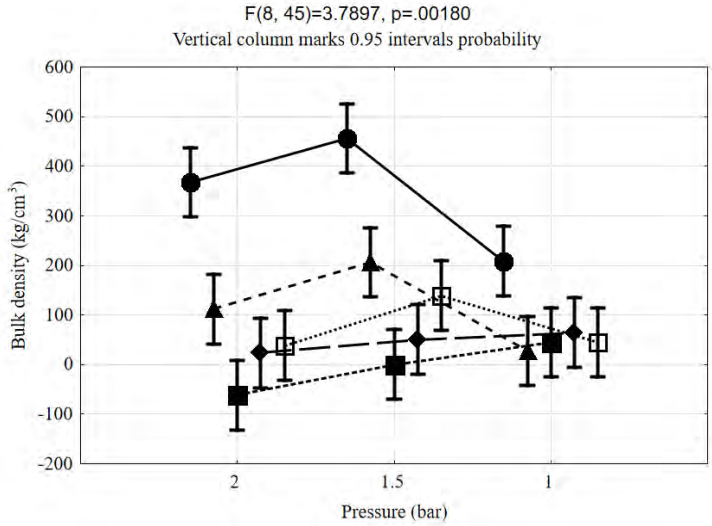
Figure 1 Dependence of bulk density on the depth and tyres inflation pressure



Legend: ● *0.05 m,* ▲ *0.15 m,* ■ *0.25 m,* ◆ *0.35 m,* ▯ *0.45 m*

Differences among bulk densities for sampling I and II are shown in Figure 2. Influence and statistically significant difference among tyre inflation pressure 1 bar, 1.5 bar and 2 bar is evident from the figure. Statistically significant differences were found out for 0.05 m and 0.15 m depth.

Figure 2 Differences among bulk densities for sampling I and II and different depths

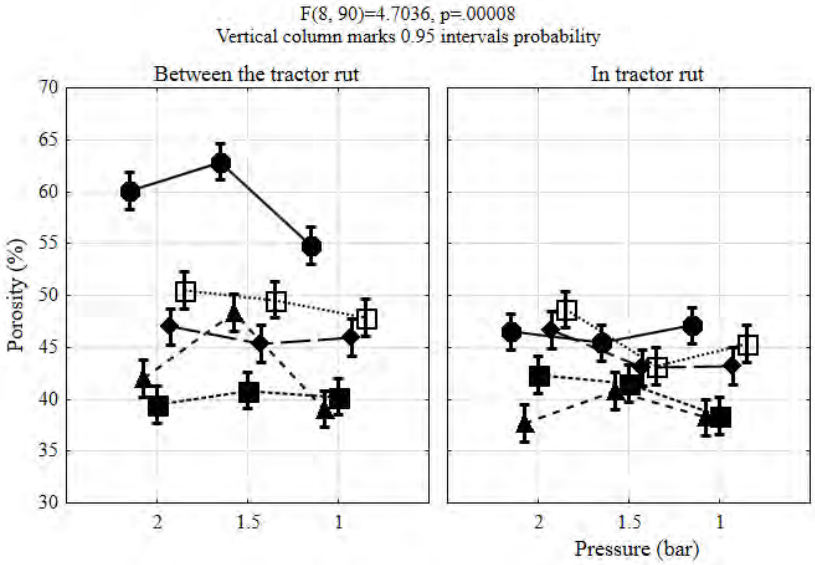


Legend: 0.05 m, 0.15 m, 0.25 m, 0.35 m, 0.45 m

Porosity

The Figure 3 shows the porosity for all tyres inflation pressures. As the bulk density and porosity values together correlate, can be observed the similar trend as in the previous graph. The relationship between porosity and bulk density has the character of an inverse. It might therefore be expected that the porosity will have the highest value by the depth 0.05 m. This trend was confirmed. The statistical significant difference was observed between the depths 0.05 and 0.15 m I and II by variants A, B and C. The statistical difference was observed also between the depths 0.25 and 0.45 m, similar to the bulk density. The difference was more pronounced by II. The statistical differences by I were only by variant A and C.

Figure 3 Dependence of porosity on the depth and tyres inflation pressure



Legend: 0.05 m, 0.15 m, 0.25 m, 0.35 m, 0.45 m

Due to the limited pages of this article, ANOVA graphical results from differences among porosities for sampling I and II are not shown, nevertheless the results are very similar as in the case of bulk density. It was found out statistically significant effect of tyre inflation pressure on porosity in depth 0.5 m and 0.15 m.

According Kostelanský (2004), aerating increases generally the porosity values from 50 to 60%. Our field was aerated twice to similar depths 0.10 and 0.12 m before sampled. The porosity value was

50–65% by our field (see Figure 3). Subsequent reduction of porosity sampled in tractor rut was thus demonstrably the cause of the passage tractor. Figure 3 also shows that the total porosity did not get worse with increasing depth. Some studies reported that the aeration of soil into more of the same or similar depths may be manifested by creating a so-called bottom plough. Bauder et al. (1981) detected the compacted layer just below the depth of tillage disc cultivator. Their experiment was carried out on clayey soils and it was ten years consecutively grown corn. The disc cultivator to the depth 0.08 m was the only represented the deeper aeration the soil processing for each year. Our field was aerated to limited depths of minimalization technologies in recent years. The allegations of creating bottom plough suggest mainly results in Figure 1 and Figure 3. The value of the bulk density and porosity in the depth 0.25m between the tractor ruts was exceeded to the limit value.

It was also apparent subsequent change both monitored physical soil properties of over limit at the depth 0.25 m to limit values at the depth 0.45 m. So, the state land before the crossing has a significant impact to the pressure spreading under the tyres and the subsequent compaction.

As already mentioned, the bulk density became the maximum just above the limit values by the depth 0.15 and 0.25 m (see Figure 1). The values reached between 1 500 and 1 650 kg/m^3. Svoboda and Červinka (2013) achieved the similar values of bulk density in their work.

CONCLUSION

Soil compaction is the problem of contemporary agriculture throughout the world. The causes of this condition are more. However, man and his intensive farming methods with the used technique have the great merit on it. The effect of a tractor on the soil significantly affects the choice of tyres – width, diameter, tread, but also tyre inflation pressure. It is also confirmed by results from measurements. Passing tractor through the field has significant influence on the parameters characterize soil compaction - bulk density and porosity. The change of these parameters is particularly evident in upper layers of soil (up to 0.15 m). The biggest changes of bulk density and porosity were occurred mainly in the lowest depth 0.05 m. In the deeper layers was not observed significant changes. However, primarily bulk density and porosity of the upper layer of soil are very important for the root systems of plants (particularly in the beginning of plant growth). One possible solution is minimization of number crossing on the field and also change tyres inflation pressure of tractor. Results from ANOVA show that tyres inflation pressure has statistically significant influence on soil compaction in the upper layers of the soil profile.

From this perspective, it is advantageous to use the modification of tyres inflation pressure not only for improvement traction of the tractors, but also to reduce the negative effects on the soil.

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WORKING LIFE OF PLOUGHSHARE RENOVATED BY HARD FACING

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Abstract: The aim of this paper is to emphasise the possibilities of lifetime improvement of working tools of agricultural machinery and especially to increase the abrasion resistance of the surface layer The life expectance was extended by welding of new cutting edge and hardfacings on functional areas of these new cutting edge (facet). I were used two technologies of surfacing by means of flame and an electric arc in the tests. The measurements were carried out over 40 ha and 80 ha of work tools in the soil.

Key Words: tool wear, renovation, weld deposit, metal powders, soil tillage tool

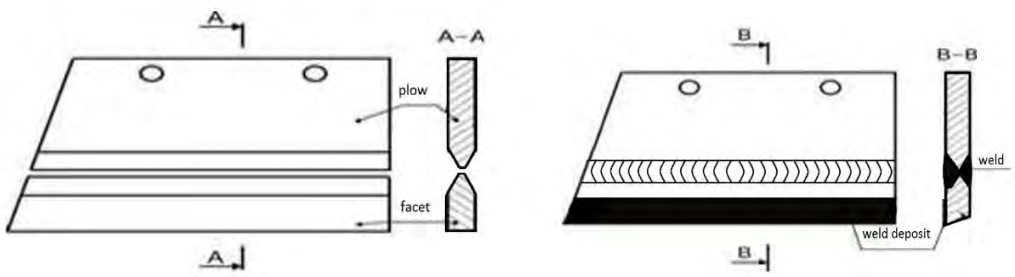
INTRODUCTION

The wear of cutting edges of agricultural tools in operating conditions significantly affects the quality of the work and energy demands of soil tillage processes. Traction force and fuel consumption increase significantly, while the work intensity and depth of tillage decrease with the greater thickness of the cutting edge of ploughshare. The quality of tillage decreases significantly with the greater thickness of the cutting edge of ploughshare. Therefore, when renovating particular tools, the possibilities of wear resistant cutting edge and self-sharpening effect achievement are researched. The latter can be reached by proper thickness of the coating and hardness ratio of the base and surfacing material. The size and intensity of wear can be measured using quantitative method, monitoring linear dimensions change per path unit in laboratory and operating conditions (Votava 2014, Paulíček et al. 2011, Kotus 2010, Kotus 2009, Pulíček et al. 2014). Agricultural users look for alternative methods to extend the lifetime of these tools. An application of hard layer to the tool' functional zone is one of the possibilities. Manual metal arc welding using hard facing electrodes is one of the most common ways of agricultural tool renovation. However, the flame oxi-acetylene technologies became an alternative for hard facing recently, with application of tube hard facing wires or metal powders. It's a low-tech method by which it can be achieved minimal mixing of base material with weld deposit and good wear resistant properties (Viňáš et al. 2013, Kolenič et al. 2007). High wear resistance and significant savings are achieved using this method, as evidenced by the work of authors (Daňko et al. 2011, Bujna et al. 2008). The structure of the weld deposit very significantly influences the weld deposit characteristics, weight losses and wear resistance ratio (Tolnai and Čičo 2001). The current trend of increasing operating speeds, reducing the consumption of materials and prolong service life is associated with the problem of wear resistance. They are parts which are important for quality in technological process (Votava and Ščerbejová 2007)

MATERIAL AND METHODS

An indexable seven ploughshares of plough Lemken EURODIAMANT was used in field tests. All the twelve worn ploughshares were renovated by cutting the worn ploughshare blades and replacing with the plates of size 490 mm × 50 mm × 8 mm welded to the ploughshare body to get the original shape and size (Figure 1a). The facets welded were made from STN 12050 steel. Than were made preventive hard weld deposit in the top of ploughshares (Figure 1b).

Figure 1 Modifications of ploughshare



Legend: a) preparation of facet and ploughshare; b) weld deposit and cutting edge modification

Completely were used three kinds of additional material. The results of their arithmetical mean of chemical composition were obtained by the SPECTROMAXx arc/spark OES (Optical emission spectroscopy) of weld deposits (Table 1) E 520.

Table 1 Chemical composition of additionals materials

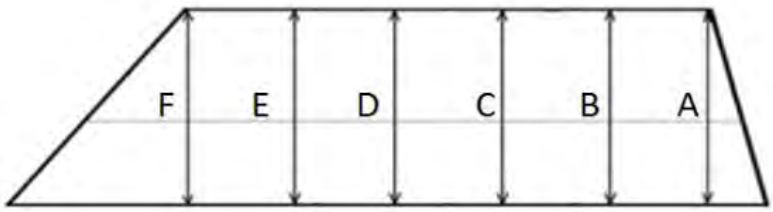
Additional materials	Chemical composition (wt %) made by optical emission spectroscopy									
	C	Si	Mn	Cr	B	Fe	Ni	W	P	S
E 520	2.48	0.81	0.7	22.05	72.26	-	0.11	-	<0.003	0.03
NP 60 WC20	0.77	3.98	0.017	18.13	4.1	7.37	65.6	0.01	<0.001	<0.001
NP 62	0.88	3.37	0.02	15.40	3.76	6.43	54.7	>15	0.004	<0.001

Every kind of additional material was used for four ploughshares including two left-site and two right-site ploughshares. Two kinds of additional materials are metal powders of the NiCrBSi base. The metal powders are very similar to the application from chemical aspect. However metal powder called NP60WC20 should contain 20% tungsten carbide as small grains. When is used surfacing technology by flame this small grains of tungsten carbide will not melted, they will remain in the matrix. They were plotted by using oxyacetylene burner called NPK-3. It was used neutral flame during the hard facing to did not chemically influenced the weld deposit. The last type of additional material is called ES20. It´s coated electrode for MMA. The hardfacing parameters with coated electrode: Diameter of electrode = 3.2 mm, electric current = 95 A, electrical voltage = 28 V. The last two ploughshares were used as etalons. The etalons were original and new ploughshares with the same geometrical dimensions and shapes which had renovated ploughshares.

RESULTS AND DISCUSSION

Field tests were realized in Kolíňany. The first measurement was done after ploughing of 40 ha and the final one after 80 ha. When measuring the wear, the ploughshares were removed and evaluated based on the loss of linear dimensions, since these describe the wear rate more reliably; the width of ploughshare plays an essential role in the quality of tillage in compare with the weight losses of ploughshares. The measurement locations on the ploughshares were strictly defined and marked. The measurement locations on the ploughshares were strictly identified, as shown in (Figure 2).

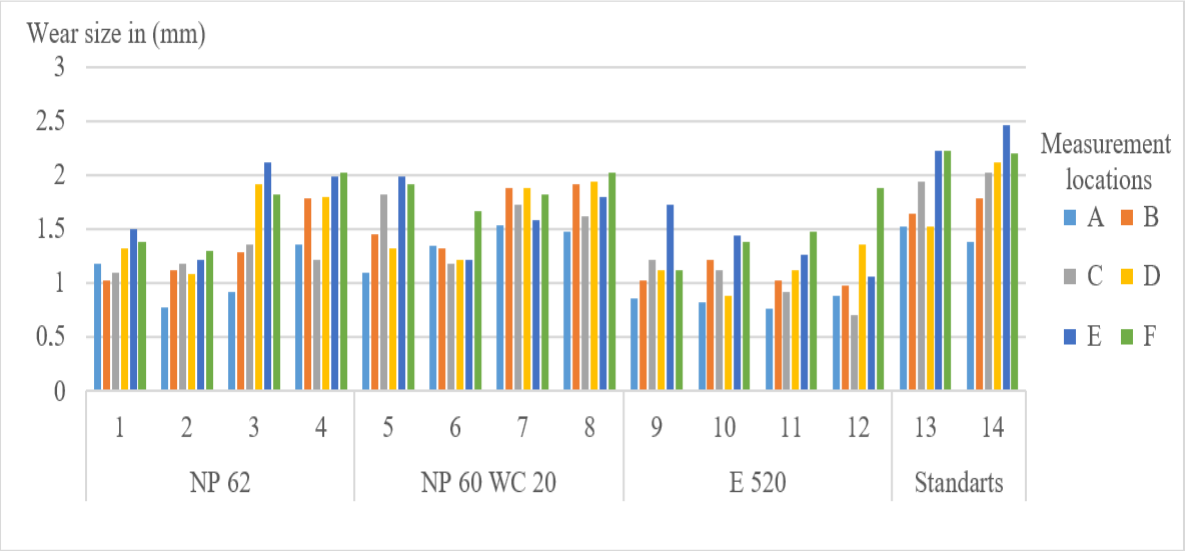
Figure 2 Positions of wear measurements



The ploughshares linear decrease after 40 ha of work is graphically illustrated in (Figure 3). The ploughshares linear decrease after 80 ha of work is graphically illustrated in (Figure 4) Field tests were

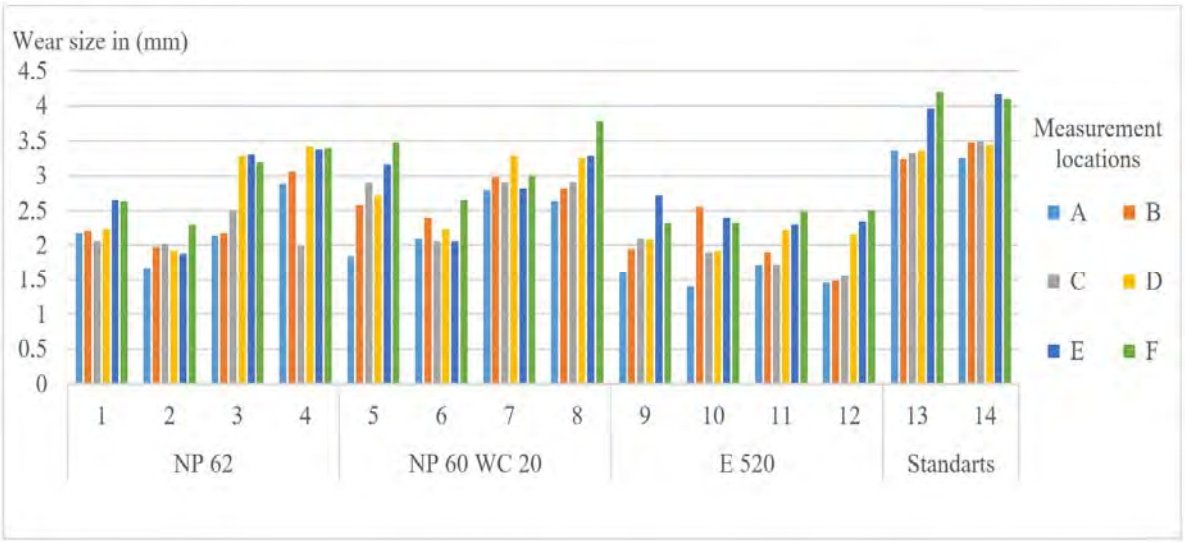
continuously evaluated. The land had 17.5% and 16.8% of moisture during the processing 40 and 80 ha of land by loughshares.

Figure 3 Changes in linear dimensions of ploughshares in particular zones after tilling 40 ha



Every renovated ploughshares had increase wear resistance. Graphical illustration shows that best results had additional material called E520. His average wear for one ploughshare was 6.8 mm. The results for NP 62 and NP 60WC20 were nearly same. Their average wear was 8.4 mm and 9.6 mm. Worst results had etalons. The average wear for one ploughshare was 11.51 mm. When compared etalons and renovated ploughshares with hardlayers made from E 520 it is 1.68 times higher wear after 40 ha.

Figure 4 Changes in linear dimensions of ploughshares in particular zones after tilling 80 ha



After 80 ha were results for renovated ploughshares still good. The worst results had etalons again whit arithmetic average of wear 21.7 mm. Results for weld deposit on the NiCrBSi base were nearly same again. Better result had metal powder NP 62. His average wear for one ploughshare was 15.12 mm. Metal powder called NP 60WC20 had 16.67 mm average wear. The best results had electrode called E520 with average wear 12.28 mm. The etalons have 1.76 times higher wear after 80 ha than electrode E520.The structure of hard facing material plays a very important role in material wear resistance determination. Structure of basic material STN 12050 shows lamellar perlite and ferritic net at grain boundaries (Figure 5).

Figure 5 Detailed structure of the basic material steel STN 12050

In this test the weld deposit with carbides in matrix NP62 (Figure 6) shows little bit worse wear resistance than the weld deposit on same based by NiCrBSi called NP60 WC20 (Figure 7).

Figure 6 Detailed structure of the NP62

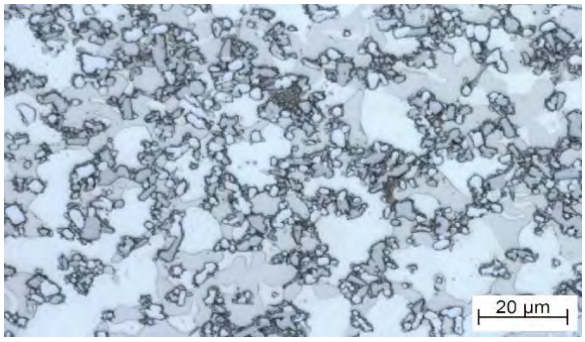
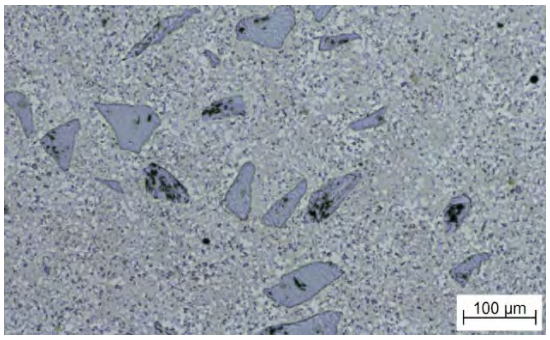


Figure 7 Detailed structure of the NP60 WC20



The matrix of weld deposit E520 is consist of ledeburit on the picture is shown also join weld on and basic material (Figure 8).

Figure 8 Detailed structure of the E520

CONCULSION

Ploughing is one of the most energy demanding operations in agricultural production; therefore, methods for energy demands reduction are searched. Worn ploughshares renovation is one of the methods. Hard facing is one of the most used one, applied to the new ploughshare in advance, or as a renovation method described in this paper. When surfacing, the majority of costs can be attributed to material, consisting of shielding gas and hard facing materials costs besides the labour costs. The hard facing renovation effect was technical life extension by 130% until 176% times if we notice even the renovation of the corrective dimension.

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COMPARISON OF METHODS FOR DETERMINING THE BIOLOGICAL OXYGEN DEMAND

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Abstract: Biochemical Oxygen Demand is one of the most commonly used group determination. The aim of work was to compare the selected methods used for determining the biological oxygen demand. For comparison respirometry and electrochemical methods for determining the biological oxygen demand were chosen. This article compares these methods when common matrices waste water and sludge from wastewater treatment plants were used. Furthermore, analysis duration, investment costs and operating requirements were evaluated for each method. The result is a comparison of the results of each method.

Key Words: biological oxygen demand, waste water, sludge, comparison of analytic methods

INTRODUCTION

Biological Oxygen Demand (BOD) is one of the parameter, which indicates the water quality. It is defined as a mass concentration of dissolved oxygen in solution, which is used during the biochemical oxidation of organic substances under specified conditions. Therefore it is an indirect indicator of the amount of biodegradable organic matter in the water. The standardized method in The Czech Republic is incubating of sample for five days, thus (BOD_5), when the BOD_5 is determined by dilution method, under aerobic conditions at 20 °C (Pitter 1999).

According to the Czech standards, it is possible to determine BOD_5 by various analytic methods, the result obtained should be comparable. Our objective was to test this hypothesis, therefore, to compare the analytic methods on various matrices processed and detect any statistically significant differences in the results. Selected methods of determining BOD_5 were compared in terms of analysis duration, investment costs, operating requirements and repeatability, therefore to show the similar results in the same matrices.

THE MATERIAL AND METHOD

For the determination of BOD_5 two different samples were taken. Waste water without inorganic particles was taken at the wastewater treatment plant (WWTP) after mechanical treatment and sewage sludge was taken from the activation tank. Individual samples were collected from two different wastewater treatment plants, WWTP Moravany, Czech Republic (5500 PE) and WWTP Brno, Czech Republic (513,000 PE). For the purpose of the research, the origin of the samples was not important because the main task was not to determine BOD_5 of the samples to find out an amount of biodegradable organic substances in it. The task of the measurement was to compare the methods of BOD determination.

After transport of the samples to the laboratory the samples were tempered at 20 °C and aerated so the initial oxygen concentration ranged between 8.5–9.0 [mg/l].

Measuring of the dissolved oxygen by the electrochemical method with a membrane probe

We followed the Czech standard CSN EN ISO 5814 which specifies the electrochemical method for assessment of the dissolved oxygen in water with an electrochemical cell. The electrochemical cell is separated from the sample by a membrane permeable for gas (CSN EN ISO 5814).

The measuring was done on multi-parameter portable meter ProfiLine Multi 3320 (Figure 1), Serial Number 15430032 manufacturer WTW, Germany in combination with a sensor CellOx® 325 which measures the concentration of the oxygen in milligrams per litres. It was necessary to dilute the samples before the measuring according to the Czech standard CSN EN 1899-1 and also aerate. Prepared samples were spilled into the Winkler flask. The first half of the samples was measured by the electrode, the second half of samples was put into the thermostat at 20 °C and measured after five days. The difference was calculated and expressed as BOD5 [mg/l].

Figure 1 Measuring device Multi 3320 and sensor 325 CellOx® in a calibration vessel OxiCal® SL

Respirometry determination

This method is based on the assumption that the microorganisms oxidising organic substrate, when use the oxygen dissolved in water and the oxygen in head space of the measuring bottle as oxygen source. Carbon dioxide (CO_2) generated during this process is absorbed (the absorbent is NaOH). Due to the decrease of the oxygen concentration and adsorption of CO_2 on NaOH the pressure in the bottle decreases. This change is detected by a pressure indicator (Figure 3) in the infrared head of a measuring bottle and saved into its memory. The data can be transferred to controller (Figure 2) which displays the exact BOD5 value. Thus, no other calculation is needed (Slevogt 2006).

Measuring bottles were filled according to the data visible at the display of controller (expected range of BOD and appropriate amount of sample). The device used in the laboratory is OxiTop® - the measuring head with inductive mixing system and OxiTop® OC 100 – a controller (Figure 2). Manufacturer WTW, Germany. No dilution of samples necessary (Slevogt 2006).

The bottles were cleaned and dried prior of the measurement. The bottles were filled with an exact volume of sample which represents the presuming BOD5 value (The controller allows display the table with an amount of sample for individual ranges of BOD5, so it is essential to know the presuming range so that the pressure sensor could work correctly).

After the bottle is filled up, the stirrer is inserted into the bottle. The rubber plug is filled with NaOH (1–2 capsules) and put inside the neck of the bottle. The measuring head is screwed on the bottle and the controller is switched on to the mode of communication with measuring heads. The presumed range of BOD and corresponding volume should be chosen and then confirmed. The measurement starts after the temperature of sample reach 20 °C. The head detects the pressure every 20 minutes and converts it to BOD5. The result is a chart of oxygen consumption development (Slevogt 2006).

Figure 2 OxiTop® set of 6 bottles with a controller

Figure 3 Bottom view of the sensor head and integrated in the pressure-sensitive membrane

RESULTS AND DISCUSSION

As is evident from Table 1, we performed four measurements in three weeks. The respirometry method included only three measurements because of the number of the measuring heads. It can be concluded that both methods have the same results for the same matrice.

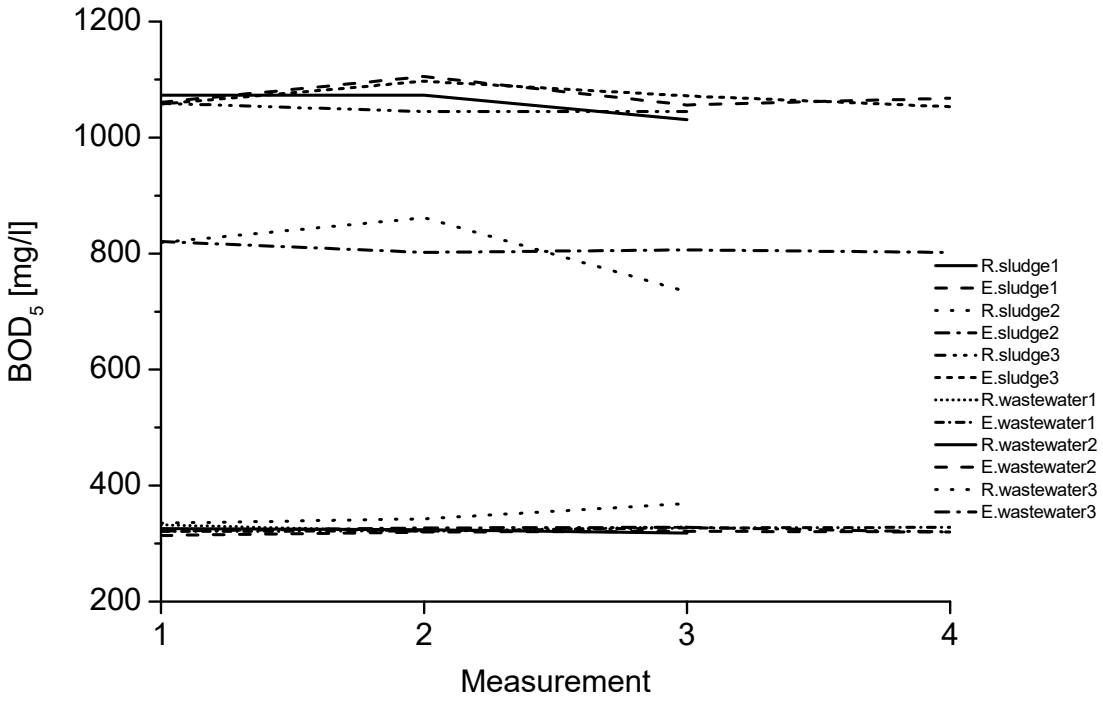
Table 1 Overview of measurement results BOD_5 particular methods and substrates.

Date	Measure	BOD_5 [mg/l]			
		R. waste water	E. waste water	E. sludge	R. sludge
30. 3. 2016	1	332	321.44	1061.07	1073
	2	321	321.81	1105.07	1073
	3	329	326.81	1056.20	1031
	4		328.15	1068.43	
6. 4. 2016	1	326	314.33	821.10	819
	2	324	320.11	801.77	862
	3	318	321.44	806.43	734
	4		320.11	802.38	
18. 5. 2016	1	335	321.81	1058.19	1059
	2	343	326.81	1096.86	1045
	3	369	328.15	1071.52	1045
	4		319.52	1053.07	

Legend: R. – Respirometry method of determining, E - Electrochemical method for determination

Progress of BOD value during the measurement is shown in Figure 4. The results indicate that the differences between the results are not statistically significant at a significance level of p = 0.05.

Figure 4 Results of comparison of methods for determination of BOD_5



Legend:R. – Respirometry method of determining, E - Electrochemical method for determination, 1, 2, 3, are days of measurement

Investment costs of equipment for electrochemical method for the determination of BOD_5. Price of Winkler flask is around € 17.6. For 3 measurements 6 bottles is needed. Altogether it is € 105.6. The

MultiMeter 3320 is € 1246.2, CellOx® 325 probe with calibration container € 775.5. Chemicals for preparation of diluted solution € 53.8. Altogether it is € 2181.1. Investment costs for the respirometry method. OxiTop® set with 6 bottles and measuring heads, induction stirrer, controller and software, total € 4965.1, (WTW 2016) (P-LAB 2016) (VWR 2016). For both methods purchase of thermostat and access to distilled water was not considered,

During BOD determination, we found that the least demanding on operation is respirometry method, since it is not necessary to dilute the sample and after sampling sample can be just fill up into the measuring bottles. The BOD determination by respirometry method is fully automatic, after five days data from controller are transferred to the computer. Electrochemical method is much more time-consuming. It is necessary tempered and aerate the dilution water and then dilute samples and fill it into the bottles. After that the samples are ready for measurement by immersing the electrode to the samples and writing the value of dissolved oxygen in sample. The same needs to be done after five days.

CONCLUSION

The aim of the paper was to compare the respirometry method and the electrochemical method of BOD_5 determination. It is clear from the results that the data obtained from the individual methods varies according to the different days of the measurement which is caused by diverse kinds of samples. Within a single measurement, the results are comparable. Both methods give very similar results but their investment costs are different. The electrochemical method is € 2181.1 and the respirometry method is € 4965.1, which is two times more.

The electrochemical method is applicable for quick measuring of the dissolved oxygen in the solution. Its convenience is a mobility, which means possibility of in situ measurement, for instance, inflow to the wastewater treatment plant. The problem is the necessary of calibration and degradation of permeable membrane which can cause errors in the measurement. This method is also more time demanding in comparison with the respirometry method.

On the other side, the respirometry method is easy and time undemanding. The samples do not have to be diluted and the electronic sensor with memory allows deducting the results even after 5 days. The disadvantage of this method is a necessity of preliminary knowledge of the BOD_5 range. Consequently, knowledge of the matrice analysed, and environment influence is needed. It is also essential to take care about airtight tightening of the heads on the bottle. This method is two times more expensive than the electrochemical method.

Measuring of BOD_5 in the laboratory is still in process together with measuring the matrices of drinking water and the standard solution. It is also planned to measure the methods volumetrically and spectrophotometry.

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THE HIGH PRESSURE INDICATION OF SPARK IGNITION (SI) ENGINE

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Abstract: This research aims to experimentally analyze and evaluate the influence of varying the spark timings (ST) on the cylinder pressure development and output parameters of a four-stroke spark ignition (SI) engine. Measurements were performed at stoichometric conditions for six different ignition timings at fixed compression ratio 9.5:1, wide throttle open and engine speed 2500 rpm. The main goal is determination of cylinder pressure curve due to piston top dead center (TDC). The correct variation in cylinder pressure is very important for optimal energy conversion in combustion chamber, which maximize the engine efficiency. Results showed that higher values of ignition timings increase the engine output power as well as the cylinder pressure. The proper adjustment of the engine is the key to achieve optimal engine parameters, which also proved this measurement.

Key Words: cylinder pressure, spark timing, engine output, torque

INTRODUCTION

Research, development and optimization of modern internal combustion engines is not possible without detailed knowledge of the processes inside the engine cylinder. Measurement and analysis of pressure development in the cylinder is the only source of data required for the optimization of efficiency, power output of the engine, fuel consumption, emissions and, last but not least, the lifetime period of the engine (Blažek 2012). Reciprocating piston internal combustion engines are basically heat engines in that they essentially convert the chemical energy from the air-fuel mixture into mechanical work and heat by means of combustion (Macek 2007). Manufacturers thus strive for the most efficient conversion of energy possible, and thus also increasing the mechanical work, which in turn increases the efficiency of the engine. Therefore, the aim is to create the homogenous air-fuel mixture in the combustion chamber, which must be ignited by the spark plug at the right moment (Beroun 2013). The correct ignition timing affects combustion, exhaust emissions and performance of the engine. The aim of the study is to evaluate the influence of the spark timing on the variation in cylinder pressure and output engine parameters.

MATERIAL AND METHODS

This measurement was performed at the engine test cell of Department of Technology and Automobile Transport of Mendel University in Brno. The testing engine is mounted on a test bench and connected to the electromagnetic eddy current dynamometer. Parameters of both devices can be seen in Table 1 respectively Table 2. The engine is controlled electronically via PC with software for calibration, diagnostic and validation of automotive systems. In-cylinder pressure data were measured via special measuring spark plug with Kistler piezoelectric pressure sensor, which allows cylinder pressure measurement without the effort of providing a separate measuring bore. This sensor is mounted flush with the wall of the combustion chamber to keep its natural frequency at about 65 kHz. This frequency level allows readings at high engine speeds and for knock control.

Engine specification

Experimental measurement was performed with four stroke turbocharged SI engine. The testing engine was fitted with fully programmable racing electronic control unit (ECU) Magneti Marelli. During

the measurement no aftertreatment and no engine accessories (alternator, air conditioning compressor and power steering pump) was used. Other selected parameters of the engine are available in Table 1.

Table 1 Selected parameters of measured engine (manufacturer´s data)

Specification of the Audi SI engine	
Manufacturer	Audi
Type	APU
Maximal power [kW / HP]	110 / 150
Aspiration of the engine	turbocharger with intercooler
Intercooling	air/water
Maximal boost pressure [kPa]	167
Number of cylinders (disposition)	4 (inline engine)
Number of valves (per cylinder)	20 (5)
Displacement (per cylinder) [cm^3]	1,781 (445)
Bore [mm]	81
Stroke [mm]	86.4
Conrod length [mm]	144
Compression ratio	9.5
Fuel	Natural 95
Maximum torque [Nm]	210
Cooling	fluid

Experimental devices

Devices can be divided into three sections – measured engine, dynamometer for measurement of output engine parameters (torque and brake power) and apparatus for engine combustion analysis (cylinder pressure)

- SI engine AUDI
- Electromagnetic eddy current dynamometer AVL DP 240 (see Table 2),
- PC with LabVIEW software
- Kistler devices for engine combustion analysis
  - Measuring spark plug with piezoelectric cylinder pressure sensor type 6118BFD16, which is mounted in cylinder head instead of original spark plug. Sensor is connected to KiBox,
  - Crank angle adapter set 2619A – connected to inductive sensor on a crankshaft and also to KiBox,
  - System for combustion analysis KiBox® To Go 2893AK1,
  - PC with Kistler software KiBoxCockpit – connected to KiBox via ethernet.

Table 2 Technical parameters of the dynamometer used

Manufacturer	Type	Max. power	Max. torque	Max. revolutions	Regulation
AVL	DP 240	$P_{max.}$ = 240 kW	$M_{tmax.}$ = 600 Nm	$n_{max.}$ = 10,000 rpm	speed and torque

Measurement methodology

This measurement is not determined by any standard. The engine combustion analysis was performed only for a first cylinder. The experiments were conducted at stoichiometric conditions for six different spark timings (see Table 3), wide throttle open and engine speed 2500 rpm. The spark timing is controlled electronically using an engine control unit (ECU). Evaluated parameters are brake power,

torque and cylinder pressure development. Torque (T) is measured via engine dynamometer and brake power (bp) is calculated from torque and engine revolutions (N) according to the relation:

$$bp = \frac{T \cdot 2 \cdot \pi \cdot \frac{N}{60}}{1000} \text{ [kW]} \quad (1)$$

where T is torque [Nm] and N is engine speed [rpm] measured via rev counter, which is part of the dynamometer

Table 3 Input measurement values

Engine speed [rpm]	Sparktiming (ST) BTDC [°CA]	Throttle [%]
2,500	18, 20, 22, 24, 26, 28	100

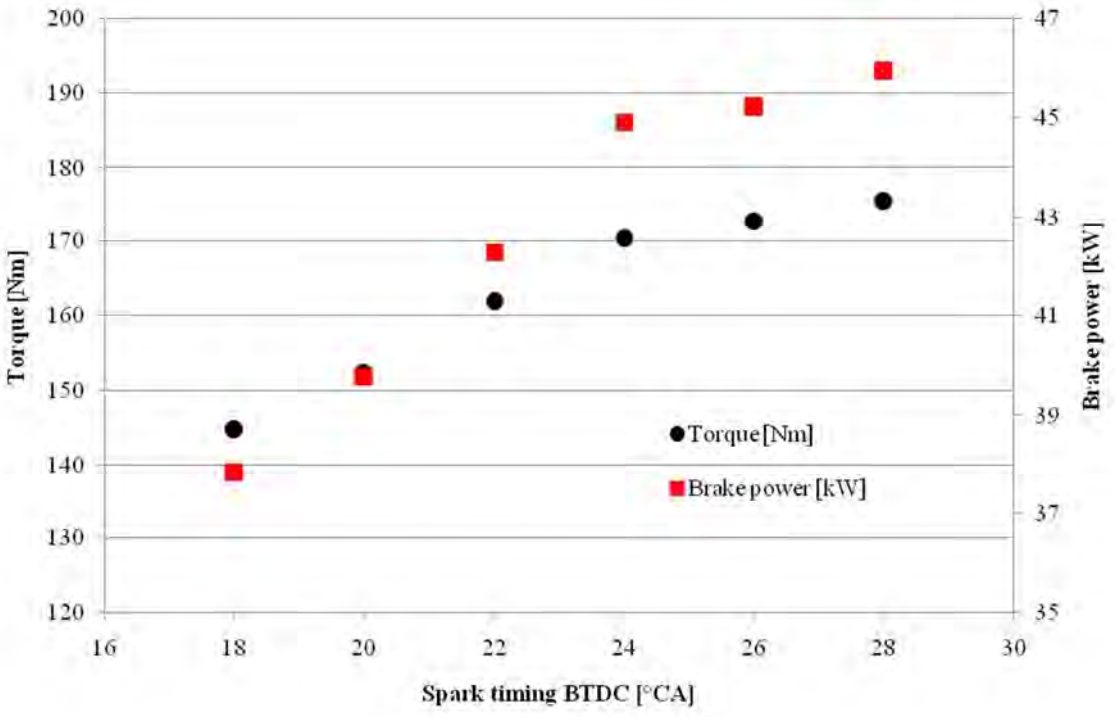
Output engine data (torque and brake power) were recorded for 5 second during each spark timing measurement via PC with LabVIEW software. The engine combustion analysis (cylinder pressure) includes data recorded from 100 cycles of the engine (one cycle = four strokes of the engine). Evaluation and data logging is performed by device for combustion analysis KiBox via PC with KiBoxCockpit software. Results represent mean values.

RESULTS AND DISCUSSION

Output engine parameters

Measurement results are represented by the load characteristic of the engine. Spark timing (ST) is used as independent variable and brake power and torque as dependent variable. Figure 1 shows the influence of ignition timing on the engine performance.

Figure 1 Variation of brake powerand torque with different spark timings



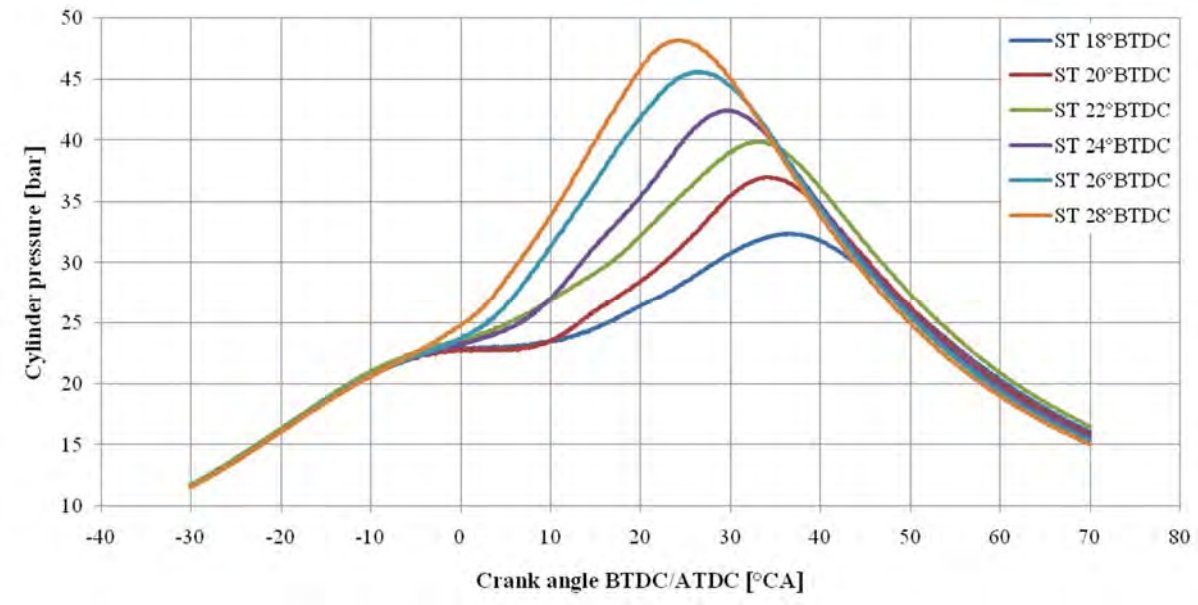
It is revealed that the spark timing influences both measured parameters. Results displayed that higher values of spark timings, increase the engine output parameters. Engine reaches the best parameters at ignition timing 28 °CA BTDC. These results suggest that maximal cylinder pressure should be reached at this value of spark timing, because of the highest engine performance. This supposition will be confirmed or refuted by cylinder pressure curves. Similar study published also

Binjuwair and Alkudsi (2016) on single cylinder four stroke engine with the same ignition timings, compression ratio and engine speed. Engine geometry parameters per cylinder (bore, stroke, displacement) are almost identical. They found out that the optimal power and torque is achieved at 28 °CA BTDC, which fully corresponds with results of this measurement. The authors also evaluated the influence of spark timing on brake specific fuel consumption (BSFC) and harmful emissions (CO, NO_x, THC and CO_2). They revealed that BSFC as well as THC, CO and CO_2 decreases with increasing of spark timing, while NO_x increases. The above mention parameters unfortunately were not measured, but is quite obvious, that could be reached the same results. This measurement could be the subject of further research.

Cylinder pressure curve (combustion analysis)

For most applications combustion analysis data is shown relative to top dead center (TDC) of the power stroke. The most important source of information in indication is the cylinder pressure curve. Both the signal level and the variation relative to the position of TDC are important in this regard. Heat supply from burning of air-fuel mixture results in a change of pressure in the cylinder. This variable can be measured by today's measurement technology with the required accuracy. Cylinder pressure variation is a representative indicator of the combustion process as well as the way of energy conversion in the engine. Cylinder pressures are displayed in relation to the crank angle position in the interval of 30 °CA BTDC and 70 °CA ATDC. The influence of spark timing on the cylinder pressure can be seen in Figure 2.

Figure 2 Development of cylinder pressure at various spark timings



Maximum cylinder pressure peak was obtained at spark timing 28° CA BTDC. Generally, it can be noticed that spark timing positively correlated with the variation in cylinder pressure. These results confirmed the hypothesis determined from engine performance diagram. Higher cylinder pressure causes better energy conversion in the engine as well as better combustion. When the ignition timing was retarded, the combustion would take place late in the expansion stroke. Forces acting on the piston due to combustion are then insufficient. Sayin (2012) published similar measurement on a four stroke single cylinder SI engine, but with lower compression ratio (8.5), maximum power output 7.7 kW and cylinder displacement 389 cm^3. The study evaluates the impact of three different spark timings (20, 23, 26 °CA BTDC) on cylinder pressure development with throttle wide open at 2500 rpm. He found out that maximal peak is reached at spark timing 23 °CA BTDC. This difference can be caused by different value of compression ratio, lower engine power and displacement and by atmospheric engine, which has lower values of the filling pressure. Almost the same measurement results, as can be seen in Figure 2, published Binjuwair and Alkudsi (2016). They revealed that higher values of ignition timings increase cylinder pressure, which is in accordance with our performed measurement. The values of maximal pressure in the cylinder are almost identical to values displayed in Figure 2. The difference between our

and their cylinder pressure curves is in maximal cylinder pressure peak, for all ignition timings, which is reached about an additional 7 °CA away from TDC in comparison with values measured by Binjuwair and Alkudsi (2016).

CONCLUSION

The effect of spark timing on performance and cylinder pressure development of SI engine have been investigated experimentally. The engine was operated at stoichiometric condition for six different spark timings (18 °CA BTDC, 20 °CA BTDC, 22 °CA BTDC, 24 °CA BTDC, 26 °CA BTDC and 28 °CA BTDC) at fixed compression ratio 9.5:1, wide throttle open and engine speed 2500 rpm. The results showed that the output engine parameters as well as combustion it the cylinder can be significantly improved with varying the spark timing. This parameter is the basic prerequisite for higher engine efficiency and higher power output of the engine at lower fuel consumption, since the chemical energy contained in the fuel is being optimally used for mechanical work of a crank mechanism.

The brake power, torque and cylinder pressure increases as the spark timing advances. The engine reaches the best parameters when the spark timing is adjusted on 28 °CA BTDC according to the results of this measurement.

ACKNOWLEDGEMENTS

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DATA MINING OF VEHICLE CONTROL UNITS

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Abstract: On this article is described the method of automobile control unit data mining. The article is divided into several categories. One part contains the theory of data mining, describing the history of its origin of the method and its division. The main focus is on the communication of control units and diagnostic testers in CAN bus vehicle buses. The data mining method focuses on specific objectives in this article. The objectives are described in the practical part of this article. On the practical part is described the connection of diagnostic hardware to the control unit of the BOSCH engine marked EDC17CP20. The communication sample is subtracted from the CAN bus with hardware. The sample is then analyzed and interpreted. For example, it displays the VIN (Vehicle identification number) from the engine control unit, CAN bus filters and other parameters. The results are then reviewed in the original diagnostic tool SuperVAG.

Key Words: data mining, CAN bus, control unit, actuators, emission

INTRODUCTION

Data mining is the fastest growing business intelligence sector today. It enables us to extract more complex and useful information from stored data than mere charts and basic outlines. In terms of statistics, it is about finding correlations, or investigating relationships and data patterns. The objective is to analyze data dependencies, identify trends and predict the future development, if the type of data allows it. The first application of data mining was carried out in the 1960s. The subsequent development of computers and computing technology, and the introduction of electronic data collections, led to the emergence of large data files. It became necessary to process large amounts of data from different companies and businesses, which could be used for further development. The information obtained was then used to increase company profits, etc. Standard statistical methods are not suitable for such data volumes. The aim was to find methods that are capable of finding even complex nonlinear relationships without restrictive conditions. This task was a direct result of the need for a new method using the processing power of computers to find structures instead of statistical parameters.

The term data mining began to appear more frequently in the 1990s. In 1991 Frawley wrote the first definition of data mining: "Data mining is the nontrivial extraction of implicit, previously unknown, and potentially useful information from data." Data mining has now become an independent statistics branch, and it is also used in the automotive industry.

Data mining can be used in the automotive industry. Modern control units have a clearly defined structure of building components. For example, the control unit Motronic consists of several parts. Figure 1 shows a block diagram of building components of the engine unit.

Figure 2 shows the first sample of data mining of control unit BOSCH EDC15P+. On the right you can see the order number of the part (038906012AP). Another parameter is 1.9 l. This value represents the engine capacity. Position R4 means that it is an inline-four engine. EDC is an abbreviation that describes the electronic control of the diesel engine (Electronic Diesel Control). The last label 2807 is the number of the software program of the control unit. This number enables us to determine whether there is an available update for the engine control unit. The practical part presents the data mining process in control unit EDC17CP20.

MATERIAL AND METHODS

CAN (Controller Area Network) is a serial (data) bus developed by Bosch. The development of this bus began in 1983 and it was officially introduced in 1986. In 1992, the first car with a CAN bus from Mercedes-Benz was introduced to the market, but the use of CAN buses for control unit diagnostics came later. The objective of CAN buses was to create a protocol that would lead to a reduction in the size and weight of cabling. Another goal was to ensure the secure transmission of information between the sensor, control and power components of automotive systems. All transmission requests that are not carried out are processed in the system according to their priority, even under insufficient transmission by the bus. The CAN protocol doesn't establish its own layers and physical medium, so the bus can work with voltage, current or light for the desired application. The main advantages of the CAN protocol include low latency, error control, setting priorities and continuous monitoring of the system. Figure 1 shows a block diagram of building components of the engine unit (Konrad 2011).

Figure 2 shows the first sample of data mining of control unit BOSCH EDC15P+. On the right you can see the order number of the part (038906012AP). Another parameter is 1.9 l. This value represents the engine capacity. Position R4 means that it is an inline-four engine. EDC is an abbreviation that describes the electronic control of the diesel engine (Electronic Diesel Control). The last label 2807 is the number of the software program of the control unit. This number enables us to determine whether there is an available update for the engine control unit. The practical part presents the data mining process in control unit EDC17CP20 (Konrad 2011).

Figure 1 Block diagram of ECU Motronic

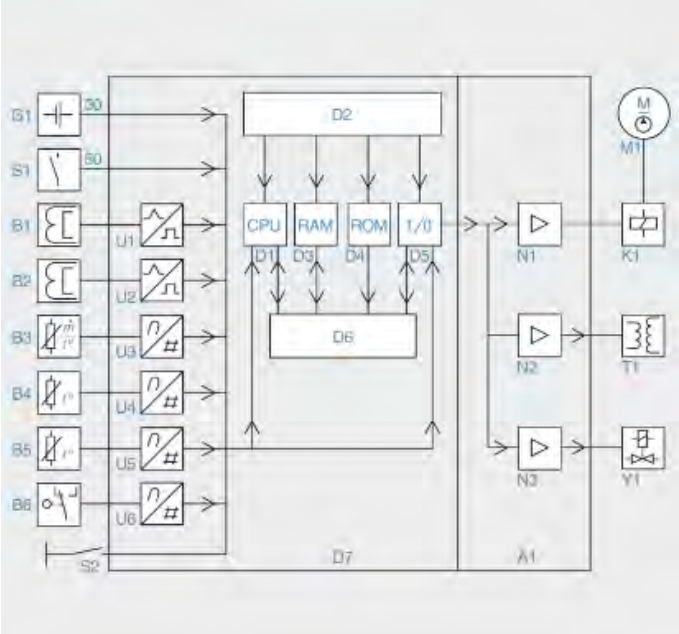


Figure 2 Data from EEPROM

```
00076C30 | 7D 79 8D A0 7E 79 8E A0  7F 79 8F A0 80 79 90 A0 | }y  ~yŽ  y  €y
00076C40 | 81 79 91 A0 E1 79 F1 A0  E2 79 F2 A0 E3 79 F3 A0 |  y' áyñ âyò ãyó
00076C50 | E4 79 F4 A0 E5 79 F5 A0  30 33 38 39 30 36 30 31 | äyô åyõ 03890601
00076C60 | 32 41 50 20 31 2C 39 6C  20 52 34 20 45 44 43 20 | 2AP 1,91 R4 EDC
00076C70 | 20 53 47 20 20 32 38 30  37 20 32 38 53 41 34 32 |  SG  2807 28SA42
00076C80 | 33 31 20 30 32 38 31 30  31 30 31 32 36 20 45 42 | 31 0281010126 EB
00076C90 | 47 57 44 31 30 30 48 45  58 30 33 38 39 30 36 30 | GWD100HEX0389060
```

CAN network connection in the VW Group

The VW Group uses different variations of the CAN bus. The first variation is a CAN bus for the comfort system with a transmission rate of 62.5 kBit/s, followed by a drive unit CAN bus with a transmission rate of 500 kBit/s. The drive unit CAN bus is now used in all models. Models from the year 2000 and up use "new" CAN buses for the comfort system and informatics, each with a transmission

rate of 100 kBit/s. The new CAN bus for the comfort system/informatics is capable of exchanging data with the drive unit CAN bus through an instrument panel called Gateway (Diagnostic communication 2015).

CAN bus features

The CAN bus has a two-cable cabling system with a transmission rate of 100 kBit/s (comfort system/informatics) or 500 kBit/s (drive unit). The comfort system/informatics CAN bus is also referred to as a Low-Speed-CAN bus, and the drive unit CAN bus is called a High-Speed-CAN. The CAN bus is parallelly connected to all control units of the CAN system. Both wires of the CAN bus are called CAN-High and CAN-Low wires. The physical layer consists of two cables twisted together. These cables are called Twisted Pair cables and they are shown in Figure 3.

Figure 3 Twisted Pair

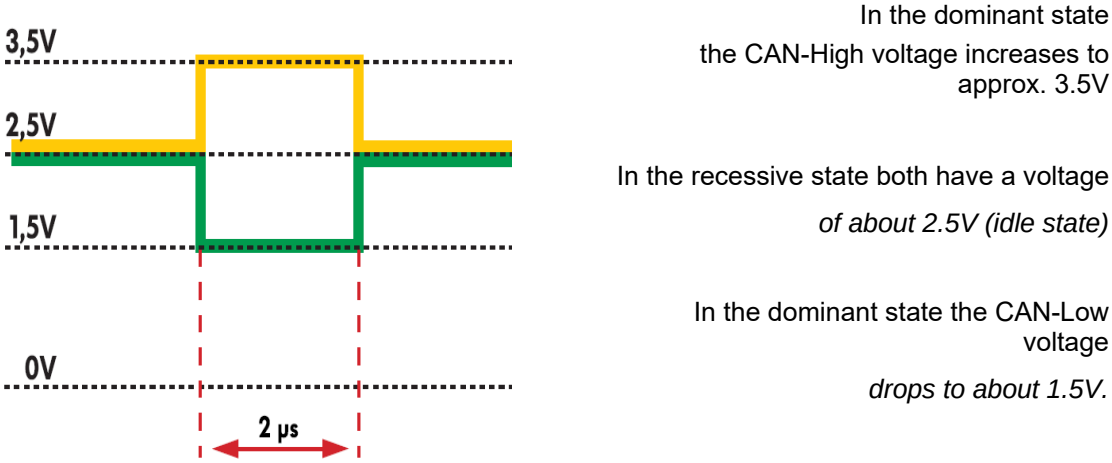
Data exchange between control units takes place throught these two cables. This data includes engine revolutions, fuel tank level and speed. The CAN cables can be identified in the cable harness by their orange color. CAN-High cables of the drive assembly CAN bus are also marked with a black identification color. The comfort system CAN bus cable has a red identification color, the CAN-High cable has a green identification color, and the informatics CAN bus cable has a purple identification color. CAN-Low cables always have a green identification color (Konrad 2011).

Differential data transmission on the drive assembly CAN bus

In order to achieve high transmission reliability, the CAN bus system uses two-cable cabling (Twisted Pair) with differential data transfer. The wires are called CAN-High and CAN-Low.

Voltage changes in CAN cables when changing between the dominant and recessive state are given in the example of the drive unit CAN bus. In an idle state both cables are at the same defined value, which is called the recessive level. In the drive assembly CAN bus this value is about 2.5 V. The idle state is also referred to as the recessive state, because it can be changed by each connected control unit. In the dominant state the voltage in the CAN-High cabling increases by a defined value of at least 1 V in the drive unit CAN bus; the CAN-Low voltage decreases by the same value. This means that the voltage in the CAN-High cabling of a drive unit CAN bus in its active state increases to at least 3.5 V (2.5 V + 1 V = 3.5 V), and the voltage in CAN-Low cabling drops to a maximum of 1.5 V (2.5 V – 1 V = 1.5 V). This results in a 0 V difference between CAN-High and CAN-Low in the recessive state, and at least 2 V in the dominant state. Figure 4 shows the course of a signal on the drive unit CAN bus (Diagnostic communication 2015).

Figure 4 CAN bus period



RESULTS AND DISCUSSION

Data mining of BOSCH EDC17CP20 control unit

The control unit EDC17CP20 from BOSCH was selected for the data analysis using data mining. The task was to build a measurement system that can read data between the control unit, the diagnostic tester and the PC.

After it's connected to the on-board network (12 V), the control unit begins transmitting to the CAN bus. This results in a set of data that must be processed. Figure 5 shows part of the communication with the CAN bus. If no CAN bus filters are set up, you will get data from all control units.

Figure 5 CAN bus communication with ECU

```
80 00 00 0A 01 FF FF FF 00 03 08 00 00 00 00 05 00 00 FF FF 00 02 68 00 80 00 00 00 80 00 7F
80 00 00 0A 01 FF FF FF 00 03 08 00 00 00 00 05 00 00 FF FF 00 02 68 00 80 00 00 00 80 00 7F
80 00 00 0A 01 FF FF FF 00 03 08 00 00 00 00 05 00 00 FF FF 00 02 68 00 80 00 00 00 80 00 7F
80 00 00 0A 01 FF FF FF 00 03 08 00 00 00 00 05 00 00 FF FF 00 02 68 00 80 00 00 00 80 00 7F
80 00 00 02 01 FF FF FF 00 03 08 00 00 00 00 05 00 00 FF FF 00 02 68 00 80 00 00 00 80 00 7F
80 00 00 02 01 FF FF FF 00 03 08 00 00 00 00 05 00 00 FF FF 00 02 68 00 80 00 00 00 80 00 7F
80 00 00 02 01 FF FF FF 00 03 08 00 00 00 00 05 00 00 FF FF 00 02 68 00 80 00 00 00 80 00 7F
80 00 00 02 01 FF FF FF 00 03 08 00 00 00 00 05 00 00 FF FF 00 02 68 00 80 00 00 00 80 00 7F
```

All data is transmitted through the bus and can be captured using the equipment shown in Figure 6. During the transmission from the unit to the bus, we can use a diagnostic tester to send a command that asks the control unit for a specific application. If the tester sends a command, the engine control unit responds to the tester. This data transmission cannot be analyzed unless we connect a device that captures the communication with the CAN bus. The data was captured by a CAN converter by National instruments. We used software from National instruments to interpret the data into a text document (Diagnostic communication 2015).

Figure 6 Data mining scheme ECU BOSCH EDC17CP20

Legend: 1 – ECU EDC17CP20, 2 – OBD connector, 3 – OBD reduction; 4 – Multiplex 7I, 5 – CAN BUS converter, 6 – PC

The diagnostic tester sends the following service command 02 16 03 85 85 85 85 85 00 07 224. This command can be interpreted in the hexadecimal system 02 10 03 55 55 55 55 55 00 07 E0. The advantage of converting between digital systems is that we don't have to work with high decimal numbers. The service command is called a telegram and can be analyzed in individual parts. For example, the value 7 224 (7 E0) is called a CAN bus filter, and it can be used to only filter communication with the engine control unit. If we don't set up the filter correctly, we will only see the data shown in Figure 7.

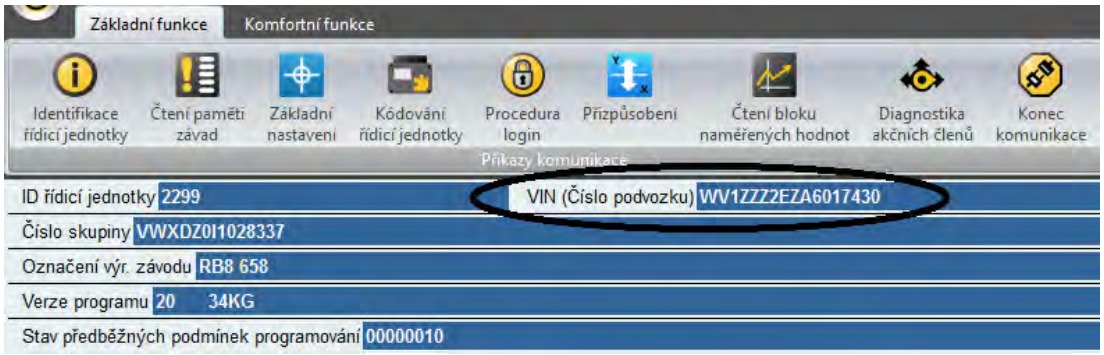
Figure 7 CAN bus data

```
57 56 31 00 03 CA 00 80 00 00 0A 01 FF FF FF 00 03 08 00 00 00 00 05 00 00
FF FF 00 02 68 00 27 5A 5A 5A 32 45 5A 41 00 03 CA 00 18 36 30 31 37 34 33 30
00 03 CA 00 B9 00 03 CC 00 80 00 00 0A 01 FF FF FF 00 03 08 00 00 00 00 05 00 00 FF FF 00 02 68 00
```

Here we can see all the communication on the CAN bus. Without filters it is hard to extract the results. For example, the vehicle identification number VIN can be seen in the highlighted area in Figure 7. If we manually assemble the highlighted areas, we get the result 57 56 31 5A 5A 5A 32 45 5A 36 30 31 37 34 33 30. After converting it from its hexadecimal form to ASCII characters, we get the text WV1ZZZ2EZ6017430.

Verification of the control unit identification is performed in the SuperVAG diagnostic tool. The software loads the identifiable elements of the control unit and checks the vehicle's VIN. The result is shown in Figure 8.

Figure 8 Vehicle identification number (VIN)



With the SuperVag diagnostic tool and measurement system we were able to achieve the goal of this article. The inserted CAN tool from National Instruments captured the communication from the engine control unit and translated it into default form. The CAN tool captured the entire communication on the CAN bus. Orientation in the communication is very difficult. We manually retrieved the identification and used the data mining method. By assembling several parts of the telegram we achieved the result 57 56 31 5A 5A 5A 32 45 5A 36 30 31 37 34 33 30. Interpretation of the result is in the hexadecimal system. By translating the information into ASCII characters, we get the value WV1ZZZ2EZ6017430. This set of characters is the VIN, see Figure 7 and Figure 8.

CONCLUSION

On this article is described the method of automotive control unit data mining. The article consists of a theoretical and practical part. The theoretical part of the history and use of data mining is described in the field of statistics. The practical part deals with communication between the control unit and the diagnostic adapter, with particular attention paid to the CAN bus. The physical layer that consists of the Twisted Pair data cabling is described on the article. It also talks about the data bus from Volkswagen. This data bus is unique and its different speeds are described in this article. The objective of the article is to verify vehicle identification in two ways and to use the data mining method. The Figure 7 shows the control unit identification. This identification is compiled manually. The sample was obtained with the CAN converter. Here we applied the data mining method. Verification was carried out by the original diagnostic software SuperVAG. All diagnostic protocols used by the control unit EDC17CP20 on the protocol KWP2000/CAN bus are implemented here. The SuperVAG diagnostic software read the complete unit identification and was compared with the data mining method.

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COMPARE TENSILE TEST OF COMPOSITE AND ALUMINIUM MATEIRALS BY ACUTSTIC EMISSION

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Abstract: Paper deals with possibilities of non-destructive testing (with acoustic emission) for identification and characterization of distinct stages in course of mechanical stress loading in aluminium alloys EN AW 7075 and modern composite materials, especially fibrous polymer composites with carbon support. In experimental part of paper we propose method for continuous acoustic signal recording, on-flight data assessment, and measurement of stressed material response to applied mechanic stress in real time. Partial results from ongoing research were consecutively implemented into mentioned method, namely calibration process of acoustic emission detection on measuring apparatus. Observation of material response to mechanic stress load with thoroughly designed technique of detection, processing, and assessment of acoustic emission signal provides the valuable information on material response to stress load and physic interpretation of measured data enables new insight into processes accompanying the occurrence of cracks in solid materials. Above mentioned emission signals provide lead on indication of micro-fissure emergence in stressed material internal structure

Key Words: aluminium alloy, acoustic emission, composite material, non-destructive testing, tensile test

INTRODUCTION

Aluminium alloys are important and widely used construction material (aside of steels); they exhibit highly desirable combination of physical, mechanical, chemical and technological properties, which enable application of aluminous materials in almost all fields of industry. In terms of composite materials we recognize heterogenous materials composed of two or more distinct phases, which are different in their mechanic, physical, chemical and technological properties. Composite materials are designed for enhancement of material applications in fields of extremely durable machine part fabrication, where classic materials perform insufficiently (Nettles 1990).

Characteristic of every material are its mechanical properties, which determine its suitability for functional designation and practical use. Due to persistent development and innovation the requirements for mechanic properties of used material are constantly rising (Fasana et al. 2007). Metal alloys are irreplaceable for their specific properties and constant research of mechanical properties enhancement possibilities is a leading force of industry. Therefore it is highly important to enrich the knowledge on mechanic resistance thresholds and destructive process mechanisms and progression (Zhang et al. 2012).

The corner-stone of this paper is insight and research into ongoing processes in internal structure of material subjected to gradual stress loading, where structural changes are accompanied with acoustic emission (AE) impulses, that are related to micro-fissure indication in stressed materials. These impulses occur in loading process, which precedes the stages of macroscopic material damage and its destruction (Kapadia 2012).

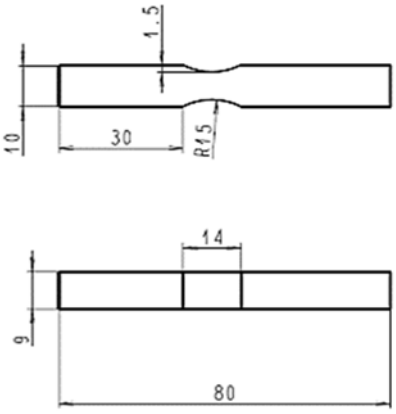
Acoustic emission was confirmed to be a viable instrument for identification of destructive process course. Stated method could be feasibly employed in diagnostic and monitoring of stressed material structural integrity and prediction of their structural collapse (Dickinson 1990). Presented results give a complex overview of AE implementation in material engineering and research of degradation processes in tested materials.

MATERIALS AND METHODS

For tensile testing sample set the parameter and dimensions were determined in accord with technical standard ČSN EN ISO 6892-1, which defines the dimensions and characteristics of measured objects for tensile testing. Sample dimensions are 10 × 9 mm, (Figure 1). Tested alloy EN AW 7075 is used predominantly in automobile and aerospace industry. Due to Mg content these alloys report the highest durability after heat treatment from the entire spectrum of aluminium alloys. Aside of Zn and Mg other alloying elements present are Cu, Cr, Mn, Fe, and Si.

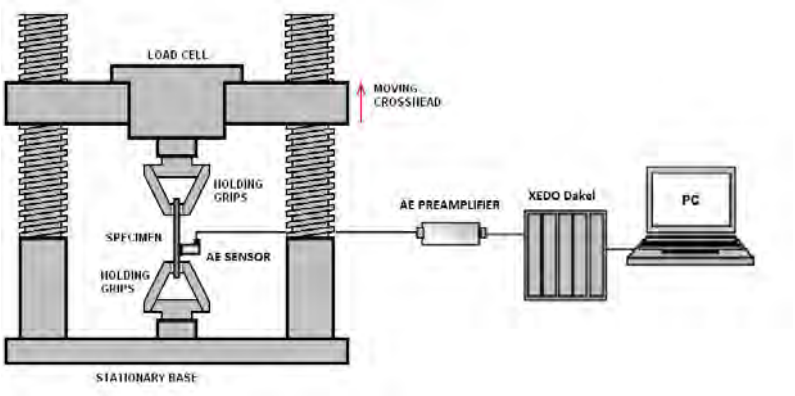
Tested samples were supplied by PREFA KOMPOZITY Inc., which provided a material commonly used in commercial composite manufacturing of carbon fibre materials with grammage 350 g/m^2.

Figure 1 Layout and dimensions of testing samples



Universal testing apparatus ZDM 5/51 was used for testing of alloy and composite samples (Figure 2). According to technical standard ČSN EN ISO 527-4 the tensile load is transferred to tested object by shearing when fixed into terminal clamps. Measured samples were fixed into self-locking jaws and loaded with jaws shift by 5 mm/min. From previously conducted experiments this velocity was determined as suitable for AE signal recording.

Figure 2 Schematics of ZDM 5/51 testing apparatus and AE sensor fixation



IDK-09 piezoelectric sensor was fixed on the sample surface for each individual measurement. AE signals were recorded and analyzed with Dakel XEDO measuring system with one piezoelectric sensor fixed with spring-loaded clamp in upper part of sample. Contact surface of the sensor was covered with ultrasonic gel. In course of AE signal measurement root mean square (RMS) of acoustic signal was observed with count (C) of signal level overshoot (C1 and C2). This parameter identifies so called signal effective value. In case of AC voltage the RMS equals to DC voltage value providing the same power with application of resistive load. RMS unit is mV. This value elucidates the quantitative characteristics of measured AE events, i.e. energy quantum. Counts of preset C1 and C2 levels overshoots characterize the behaviour of signal in relation with its time course and intensity. Collapse of sample structure

corresponds to the RMS curve endpoint, which indicates the stop of measuring apparatus at the highpoint of loading force (Koktavy 2004).

RESULTS AND DISCUSSION

In comparison of both sample sets in tensile testing, it is obvious that results show high variability. Figure 3 provides the overview of data recorded in measurement of aluminium alloy tensile strength thresholds and composite thresholds. Individual curves suggest that aluminium alloy reports significantly lower values than composite material. Shearing limit R_e is hinted in the (Figure 3), which occurs in the interval of loading between 1.2–1.3 kN. This drop is relatively stable among all samples and occurs in a certain force interval. Therefore we conclude that no mechanical influence of testing apparatus (e.g. flattening of sample in fixing clamps in course of force load) caused this irregularity. Endpoints of tested samples show relatively small indentation from fixing clamps. Curves of composite samples report considerably steep characteristics and report no typical signature of soft material. From mentioned characteristics it is obvious that hit length is a viable characteristic of degradation progress namely in cases where the material or its vicinity is accessible for measurement.

Following graph demonstrates the measurement results (Figure 4). The obtained results indicate the suitability of AE method for detection of structural integrity changes inside the material. Majority of AE emission hits detected in course of composite testing is dedicated to rapid development of fissure and transfer of released deformational energy in form of hits.

In composite material the individual stages of disturbance were detected (change of mechanic properties, separation of fibres, extraction of fibres from matrix, snapping of fibres, delamination).

On the basis of time-course signal analysis it was determined that individual shapes of hits are characteristics due to kind of material and character of breaking behaviour of aluminium alloy (breaking of some particles and their flaking from matrix at the crack extension) and some of them may be attributed to plastic deformation. Lower RMS value in aluminium alloy is caused by brittle fracture and lower recorded activity.

Figure 3 Tensile test results

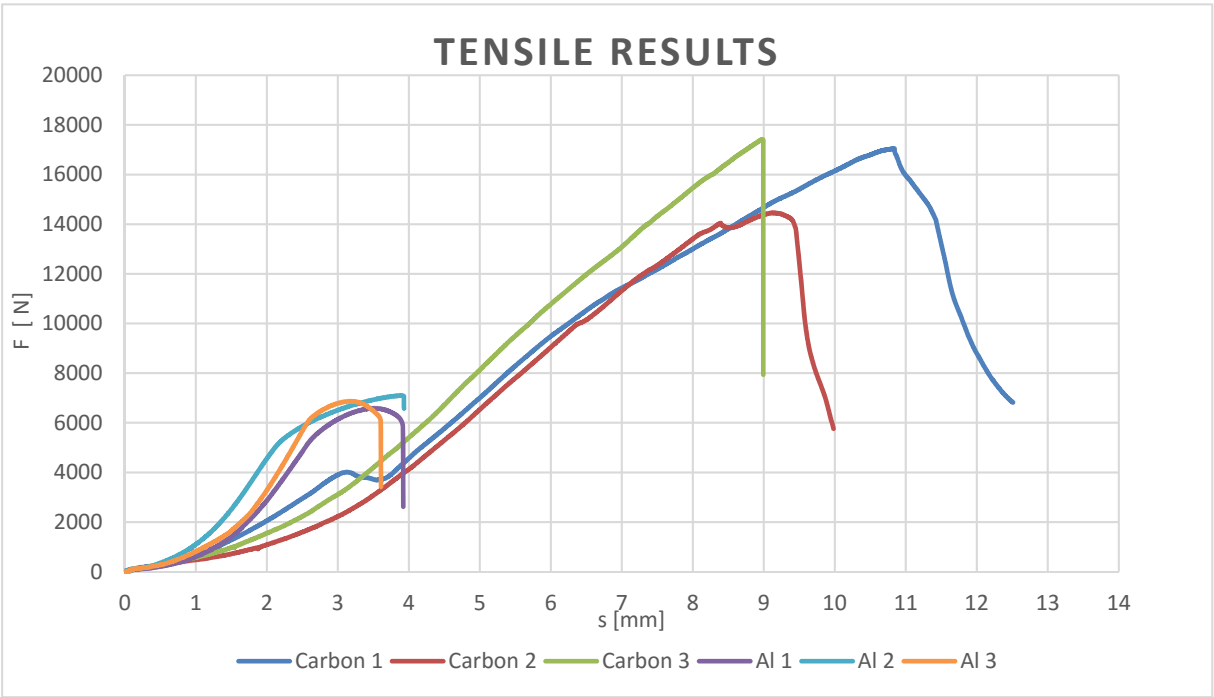


Figure 4 Values of tested samples

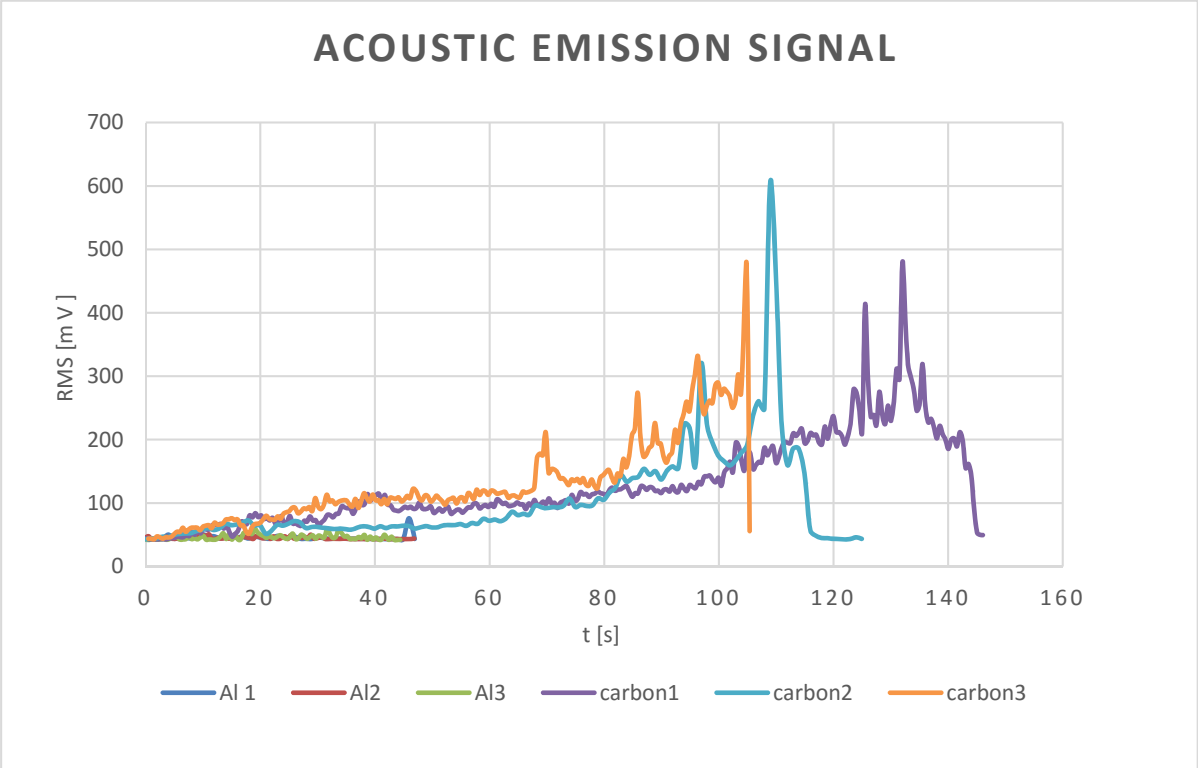
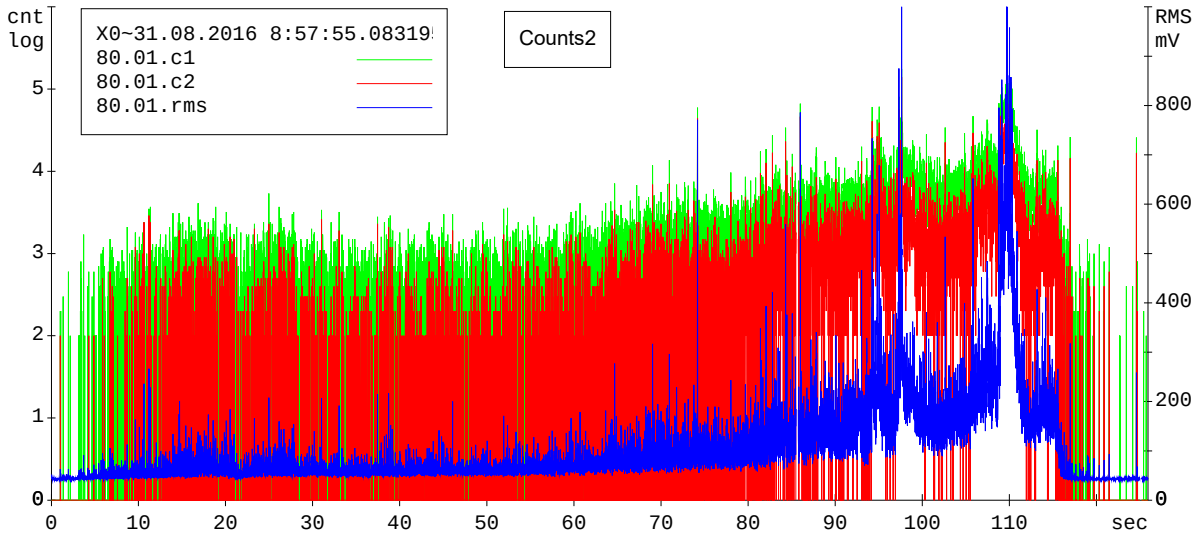


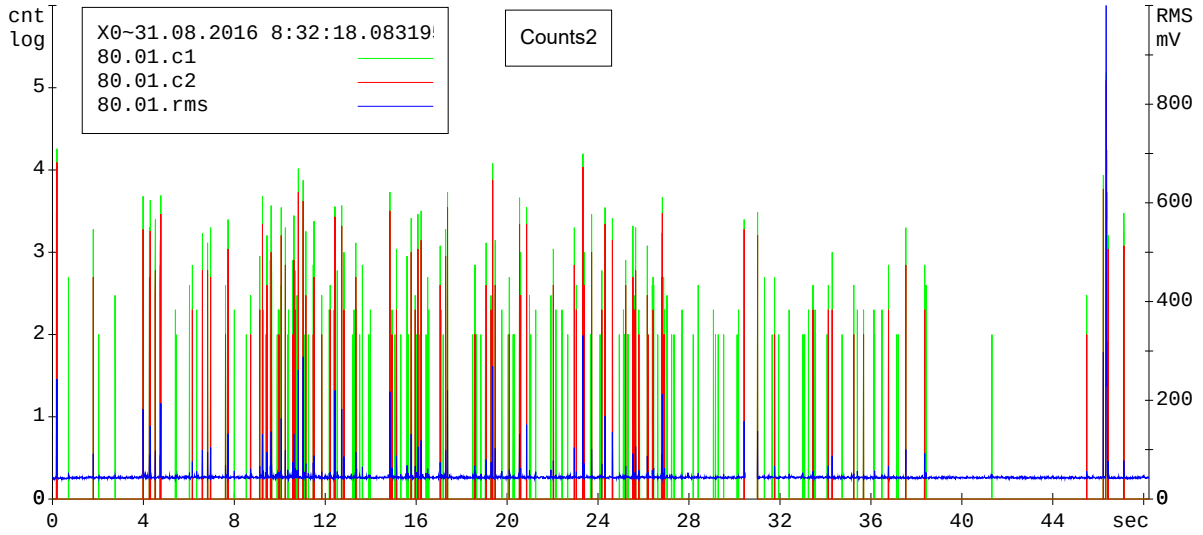
Figure 5 Overshoots of Count levels at carbon 2 sample



(Figure 5) represents the Count 1 and Count 2 courses, i.e. quantities of present threshold levels overshoots on sample Carbon 2. The record shows dramatic increase of signal in initial phase. Entire graph area reflects highly frequent increases of signal, which are attributed to emergence of damage trace with onset of continuous matrix destruction – occurrence of micro fissures of matrix. Considerable overshoots occur here, which points to emission of greater energy quanta, which are related to significant damage in material structure.

Distinctive deformation threshold is passed after 60s, when support fibres and matrix show inconsistent deformation patterns, leading to interphase separation of fibres from matrix, which could progress further to certain distance along the fibre. This period continues to the moment, when signal shows maximum oscillation. Then fibres break only after reaching the maximum transformation limit. In course of crack progression in direction perpendicular to support fibres (under sufficient force load) the fibres finally break, which is considered as a point of composite structure collapse.

Figure 6 Overshoots of Count levels at A1 sample



(Figure 6) reports detection of curves with low amplitude (up to 50mV). RMS remains mostly unchanged in these samples. Signal course reports occasional overshoots, when energy quantum is released in short bursts. Test recording is accompanied with acoustic activity, related to changes in material micro structure. Spatial shift of dislocations takes place, which results to change of mechanic properties. Major hit quantity is recorded at the emergence of a crack in final part of recording (45s). Analyzed data allow us to claim that aluminous alloys show shorter test course periods and simultaneously lesser amplitudes. The course of measurement demonstrated that few seconds before crack emergence in plastic component the AE signal dissipated. Then one final massive energetic burst occurred, which concluded the recording.

Following figures show the sample surfaces where the crack took place. It is obvious to certain extent how the cracking progressed throughout the sample. (Figure 7) shows the fracture surface of aluminium alloy. It is a typical fracture in fragile material, where major surface area is finely granulated. (Figure 8) depicts the fracture surface of composite material with visibly ragged surface, plastic deformation of basic material is also observable. Fibres are torn out of composite matrix.

Figure 7 Fracture surfaces of Al 1 sample

Figure 8 Fracture surfaces of Carbon 1 sample

CONCLUSION

This paper deals with employment of AE measurement in tensile tests of aluminium alloy (EN AW 075) and carbonaceous composite material in comparison. Non-destructive testing with AE reported a valuable insight in behaviour of both distinctive materials under stress and assessment of internal structure damage, as well as viability of method to visualize the stress load effect in real time.

Acquired data show that AE method is sensitive enough to discover emerging weakness in material structure before occurrence of actual fracture. This is highly desirable in actual material application in industry. AE testing is simple and reliable method with wide application possibilities in

selected branches of industry, where ability to detect the changes in material structure provides advantage for prevention of safety hazards, malfunctions of machinery and subsequent economical losses.

It was demonstrated that AE signal recording acquired in material tensile testing is highly different in cases of carbon fibre composite compared to aluminium alloy. Most favourable AE parameters which provide insight into microscopic changes in material structure is RMS, course of maximum emission periods and energy levels of AE hits. Further possibilities of research include AE signal analysis in frequency area. Technical aspect worthy of consideration is influence of composite fibre direction in relation to tensile force load.

ACKNOWLEDGEMENT

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Section – Applied Chemistry and Biochemistry

PEPTIDE BLOTTING AND LIQUID EXTRACTION SURFACE ANALYSIS FOR PROTEIN DETECTION AND IDENTIFICATION: PROOF OF CONCEPT

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Abstract: We demonstrate that peptides of in-gel digested proteins separated by polyacrylamide gel electrophoresis can be transferred directly onto a C18 disk and analyzed via Liquid Extraction Surface Analysis mass spectrometry. This concept, if further optimized, could facilitate a rapid identification of proteoforms separated by 1D or 2D electrophoresis.

Key Words: LESA, peptide blotting, MS

INTRODUCTION

Liquid Extraction Surface Analysis (LESA) is one of the mode of operation for the TriVersa NanoMate, an automated chip-based nanoelectrospray source for mass spectrometry. LESA enables simple, direct nanoESI mass spectrometric analysis from a v ariety of surfaces, including thin tissue sections, TLC plates and other planar separation media. Previously, it has been shown that noncovalently bound protein complexes can be directly probed via liquid extraction surface analysis from dried blood spot samples and that the intact hemoglobin complex can be sampled directly from thin tissue sections of mouse liver (Griffiths and Cooper 2016). However, the analysis of intact proteins is still limited and even high-end instruments can't offer the sufficient resolution and sensitivity to address a complex mixture.

Electrophoretic methods provide invaluable resolution and a cost effective fractionation of a complex proteome sample (e.g. Černý et al. 2014). Available studies that compare gel-based and gel-free "shotgun" quantitative proteomics have demonstrated that a significant portion of proteome changes detected by a gel-based technique occur on the proteoform level and are not observed in the later approach (e.g. Černý et al. 2013, Baldrianová et al. 2015). However, the identification of separated proteoforms from a gel requires a multistep procedure that includes gel excision, washing, digestion, peptide extraction and desalting steps. In effect, a protein identification is usually limited only to a small subset of protein bands or spots and thus the most of the resolved proteome is not identified. Here, to address this issue, we tested the combination of peptide blotting and LESA connected to a mass spectrometer (MS).

MATERIAL AND METHODS

Protein extraction and separation

App. 100 mg of barley kernels were homogenized in Retsch Mill. The resulting fine powder was suspended in SDS-sample buffer (100 mM DTT, 2% SDS, 0.1% bromophenol blue, 50 mM TRIS, pH 6.8), boiled for 10 min and separated by SDS-polyacrylamide gel electrophoresis (PAGE); TRIS-glycine PAGE; stacking gel - 5%; resolving gel - 11%; 50 V for 10 min and 150 V for 60 min; Mini-PROTEAN system, Bio-Rad).

Gel washing, digestion and blotting

The whole gel was washed with distilled water, fixed in acetonitrile (5 min) and dried on air. Dried gel was overlaid with 5 ml of trypsin solution (20 μg, Promega, MS-grade; 50 mM ammonium bicarbonate), incubated for 20 min at 4 °C and digested overnight at 37 °C. The gel with digested

proteins was rinsed with 0.1% formic acid (FA) and blotted onto EMPORA C18 disk by a semi-dry blotting (30 min, 5 mA/cm^2; Bio-Rad).

LESA-MS analysis

Advion TriVersa NanoMate was operated in the LESA mode. The surface extraction was achieved by dispensing and aspirating of 2 µl of the extraction solvent (0.1% FA in acetonitrile). The extraction time was set to 5 s and the sample was delivered to MS at constant flow for 10 min at 1,400 V. Ion trap was operated in CID mode with the following acquisition parameters: MS - enhanced resolution mode with the scan range 400–2,000 m/z; ICC target 400,000; 3 spectra averages; SPS target mass 900 m/z; MS/MS - XtremeScan mode with the scan range 100 m/z to 2× mass of the precursor; ICC target 500,000; Smart Isolation, 20 precursors, no spectra averaging and the fragmentation amplitude at 60%.

Identification of peptides extracted from C18 disk by LESA

The measured spectra were processed by Bruker's Data Analysis 4.1 as described previously (e.g. Cerna et al. 2016) and the resulting MGF files were searched against barley protein database by Sequest HT with the following parameters: trypsin; max two missed cleavage sites; mass tolerance 0.3 Da; up to three dynamic modifications including Met oxidation, Asn/Gln deamidation, Lys methylation, N-terminal acetylation.

RESULTS AND DISCUSSION

Protein separation, digestion and transfer to C18 disk

To test the feasibility of peptide blotting and LESA-MS, we separated a representative complex proteome sample extracted from homogenized barley seeds by SDS-PAGE. The resulting gel was fixed and dried by acetonitrile and then soaked with 5 ml of 0.0004% (w/v) MS-grade Trypsin and digested overnight. The gel with digested peptides was overlaid with 0.1% formic acid and blotted onto a C18 disk. The successful blotting was confirmed by observing the transfer of colored bands of a protein marker (Bio-Rad). The whole protocol takes less than 24 h (Table 1).

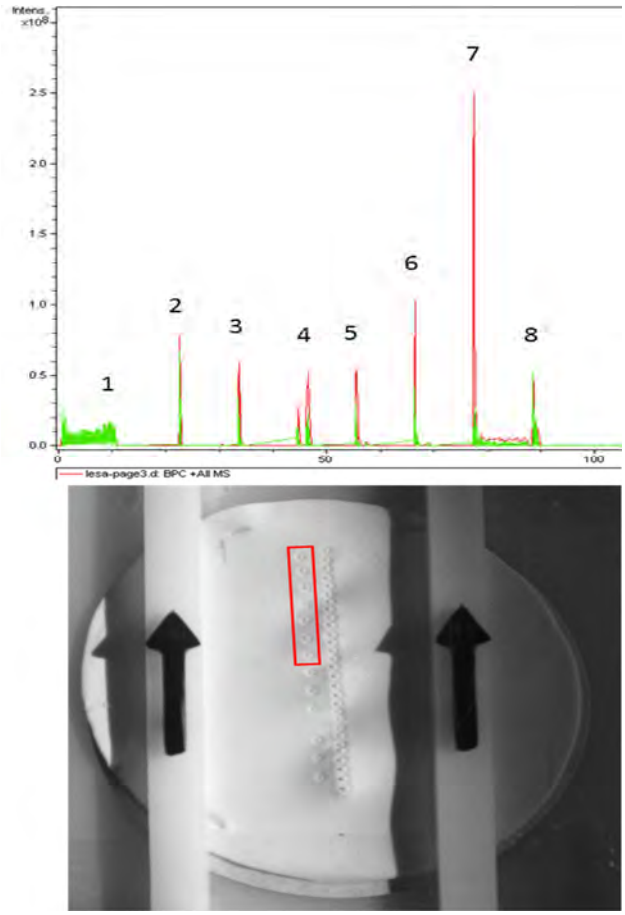
Table 1 Overview of the experiment

Method	Details	Time [h]
Protein extraction	Retsch Mill MM400, boiled in SDS-sample buffer	0.5
SDS-PAGE	5% stacking gel, 11% resolving gel; 150 V for 60 min;	1.0
Digestion	Trypsin, 20 µg (Promega)	15.0
Blotting	Washed with 0.1% FA; 30 min blotting (5 mA/cm^2); EMPORA C18 disk	0.5
LESA-MS	TriVersa NanoMate (Advion) and amaZon Speed ion trap (Bruker)	5.0

Identification of peptides extracted from C18 disk by LESA

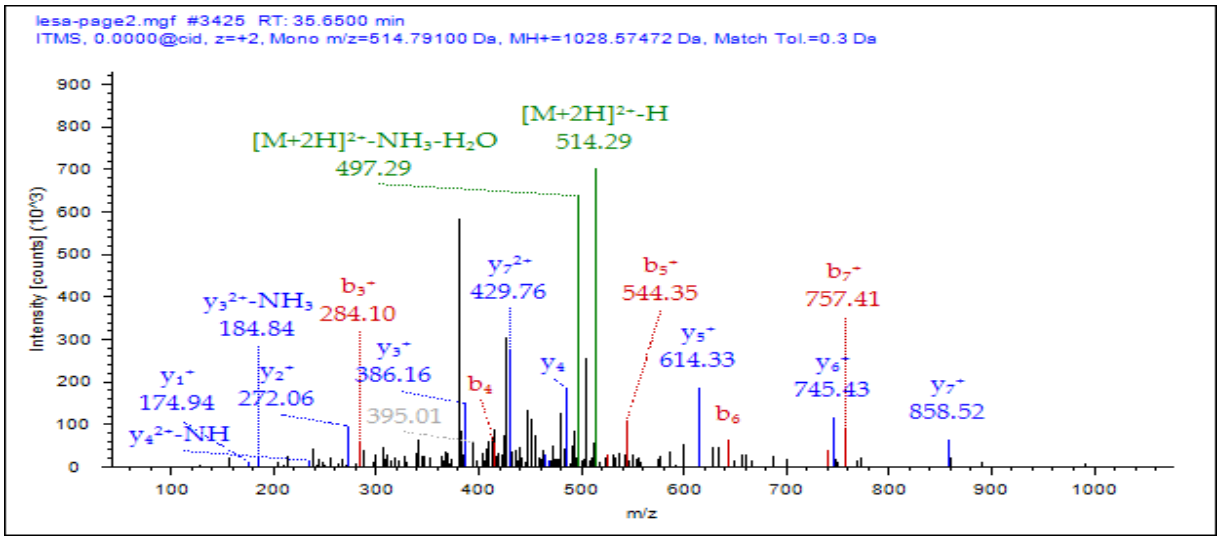
The C18 disk was taped to the LESA holder and analyzed by LESA connected to the mass spectrometer as described in Materials and Methods and the resulting spectra were analyzed (Figure 1). Altogether, 34 barley peptides were successfully identified in LESA-MS spectra, including peptide VAIMEVNPR detected in the third extraction (Figure 2).

Figure 1 C18 disk after the LESA analysis and the corresponding MS chromatogram



The number of identified peptides correlates with a short extraction period (5 s) and would likely improve with a more sensitive/faster MS analyzer. Further, the solution employed in LESA (0.1% formic acid in acetonitrile) provides optimal conditions for peptide elution, but the amount of coeluting peptides suppresses the signal. A stepwise extraction gradient could improve the results, but would require a modification in the Advion software that would allow multiple extractions out of a single spot. We also found that the average amount of hydrophobic residues in identified peptides is 43%, which indicates that those more hydrophilic peptides could have migrated deeper into the disk or that the transfer time was too long to retain them in a C18 disk.

Figure 2 Representative peptide spectrum measured by LESA-MS



CONCLUSION

Our experiments proved that the semidry Western blot can be adapted for a peptide transfer and employed as a desalting step prior the ESI analysis. If further optimized, its combination with the LESA could provide a high throughput platform for identification of proteins separated by SDS-PAGE.

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USING ENERGY DISPERSIVE FLUORESCENCE SPECTROMETER FOR SOIL SUBSTRATES AND BEDROCK DIFFERENTIATION

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Abstract: Chemical composition of soil was studied within post mined area of limestone quarry Mokrá, south Moravian region, the Czech Republic. According to fieldwork research six quarry walls were selected for detail study. From one to four sampling sites were determined within each quarry wall. Overall fifteen sampling sites (10*10m) were determined from central part where Devonian limestone dominates to the east part where transition to Drahany Kulm formation occurs. Three soil samples from each sampling site were analyzed by Energy dispersive fluorescence spectrometer (ED–XRF, DELTA soil). This relatively new method was tested for brief characterization of soil substrate and bedrock type. For this purpose the samples were only sieved (2 mm) a dried. This time and cost reducing method confirmed two types of the bedrock in quarry Mokrá and corresponded with supposed mineral composition. Chemical composition of macro and microelement revealed any anomaly which can dramatically inhibit plant growth.

Key Words: bedrock, ED – XRF, element composition, limestone quarry, preparation of soil samples

INTRODUCTION

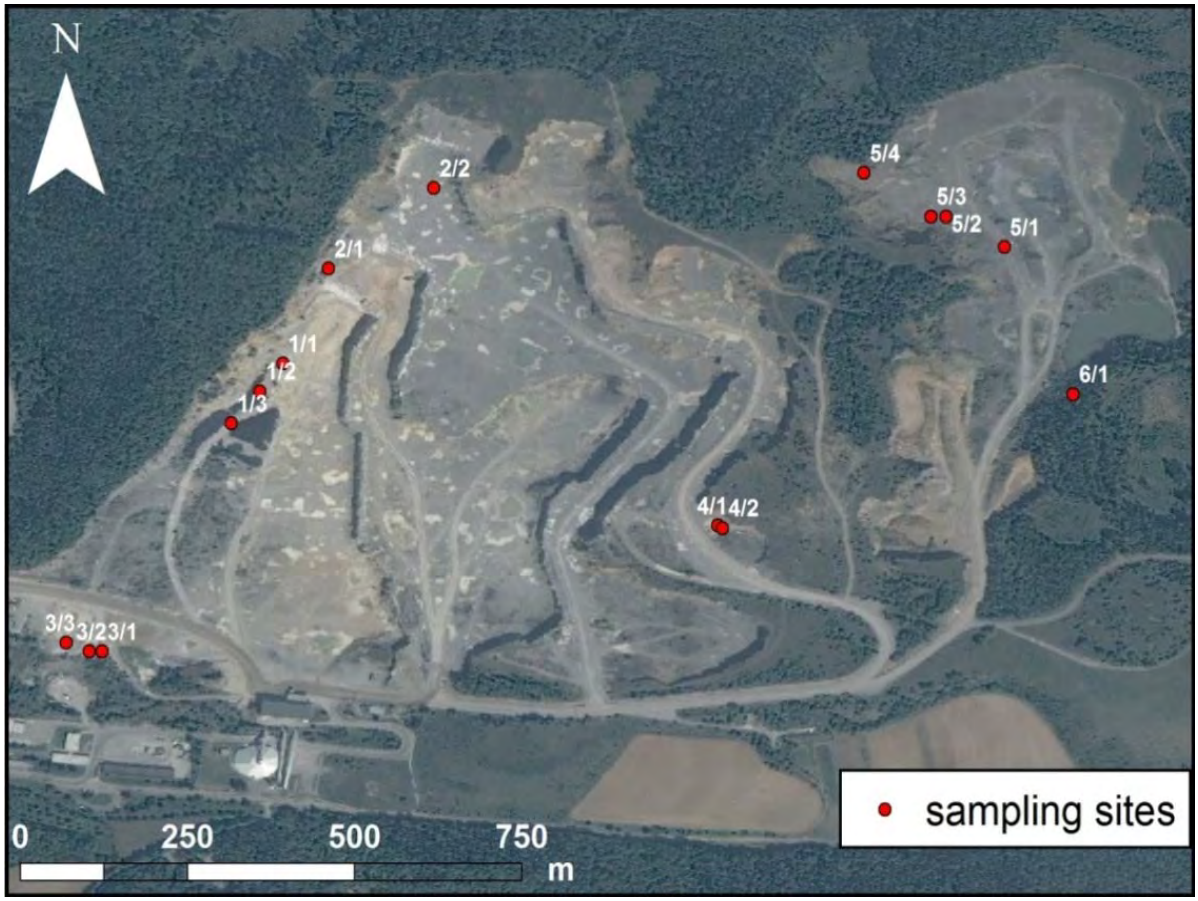
Soil chemical composition is not among commonly used soil analysis however in quarries and post mined area is commonly used (Sahuquillo et al. 2003). Most of the methods are based on geological measurements and doesn´t have special methodology for soils. Methods used are: inductively coupled plasma-optical emission spectroscopy (ICP-OES), inductively coupled plasma mass spectrometry (ICP-MS), and flame atomic absorption spectrometry (FAAS) (Welz and Sperlig 1999). For these methods it is required to dissolve soil sample in strong acids together with standard, what is expensive and time consuming way (Šulcek and Povondra 1989, Krakovská and Kuss 2001). Relatively new method energy dispersive fluorescence spectrometer require compressing of the sample to special tablets (Parsons et al. 2013, Sahnd and Wendler 2014), however in this case it was tested for simplified measurement in field and laboratory, without complicated sample preparation. For the purpose of differentiation of soil substrate and bedrock, this method is supposed to be sufficient.

There are 17 essential elements required by plants subdivided into the macronutrients and micronutrients. Macronutrients are required in relatively large amount represented by C, H, O, N, P, K, Ca, Mg, S and Cl. Micronutrients B, Cu, Fe, Mn, Mo, Ni and Zn are required in relatively small amount (White 2006). Lack of the essential elements can inhibit plant growth even can cause death of the plant. Such situation may also happen if some element occurs in excess, especially in the case of micronutrients which are essential in very low concentrations (Aubert and Pinta 1977, Richter 1996, Ajibola and Rolawanu 2000, Chesworths 2008). Other important group of elements with possible impact to ecosystem formation are toxic heavy metals: Cd, Pb, Cr, Hg and As. Relatively low concentrations can cause inhibition of plant growth (Alloway 1995, White 2006).

MATERIAL AND METHODS

Limestone quarry Mokrá is situated near the Brno city in south Moravian region. The quarry is divided into three parts. West and Central open pits consists mainly from Devonian limestone which transit to the Drahany Kulm formation in the East open pit (Sekanina and Musilová 2011). The formation consists mainly from clay schist, conglomerates and sandstones. Six spontaneously recovered quarry walls were selected numbered from the central to the east part (see figure 1). From one to four sampling sites were defined within each quarry wall according to soil substrate heterogeneity.

Figure 1 Central and east part of the quarry Mokrá with studied sampling site

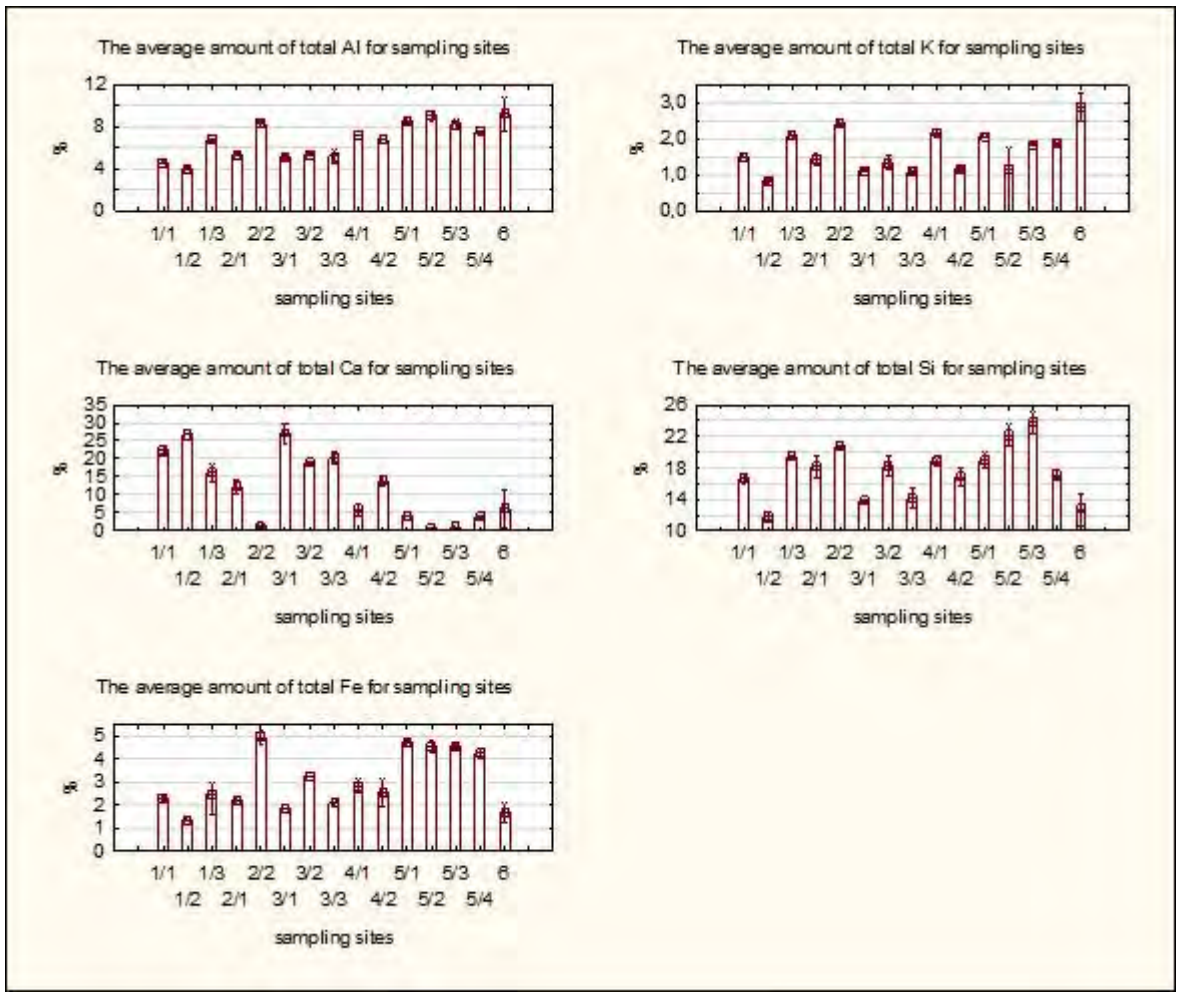


Three soil samples were picked from each sample site. The upper horizon was removed and maximal depth was 0.1m. The samples were dried and sieved to fine earth soil (2 mm). Five millimeters width layer and the area of 3*3 cm were analyzed by Energy dispersive fluorescence spectrometer (ED–XRF, DELTA soil, Mendel University in Brno). The calibration was made in „Geochem mode“ for quantitative analysis of these elements: K, Ca, S, Cl, Ti, Cr, Mn, Fe, Ni, Cu, Zn, Hg, As, Pb, Se, Rb, Sr, Y, Zr, Mo, Ag, Cd, Sn, Sb, V, Co, Au, W, Bi, Th, U, La, Ce, Nd, Pr, Nb, Ta and Ba.

RESULTS AND DISCUSSION

For better evaluation of obtained results elements considered in this study were divided into two groups according to Hruška and Jelínek (1998): macroelements and microelements Macroelements were represented by Al, Si, K, Ca, and Fe (see figure 2). The data were processed in statistical program STATISTICA 12. Data were subjected to statistical peculiarities investigation and basic assumptions were verified. The aim of these analyzes was to reveal the degree of symmetry and kurtosis of the data; detect outlier values and verify a normality of the data and their independence on the selection. All statistical methods and the subsequent creation of graphs were made in accordance with Meloun and Militký (2006). According to S-H test the data show normal distribution. There are statistically important differences between studied sample sites.

Figure 2 Concentrations of analysed macroelements in soil samples



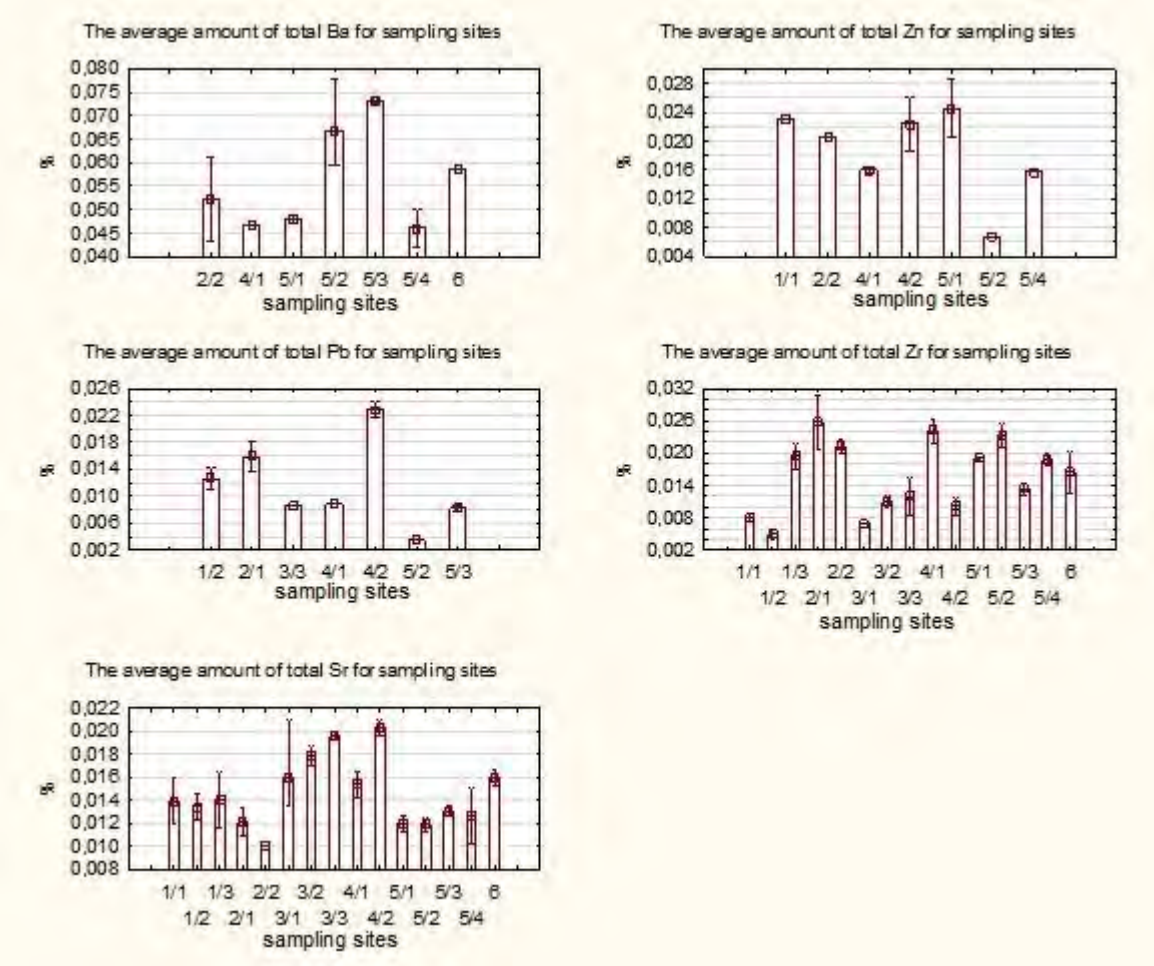
Measured concentrations of Al varies from 4.7% on sampling site 1/2, 3/1 and 3/3 up to 8.7% on sampling site 2/2. Higher values were measured also at sampling site 5/1, 5/2 and 6. Certain forms of Al show toxic effect to living organisms (for example Driscoll 1980, Yokel R. A. 2000). Measured concentrations do not indicate which form of Al is present in soil, but due to high soil reaction toxic forms are not supposed to occur. Concentrations of Si ranges from 13.4% on sampling site 1/2 up to 22%+-0.5% measured on sampling sites 5/2 and 5/3. Concentrations of these two elements are in positive correlation and probably indicate presence of clay minerals and products of their weathering. Concentration of potassium (K) can also be result of clay minerals occurrence and into some degree correlates with Al and Si content. Potassium is very important macroelement in plant nutrition (Richter 1996). Plants require potassium in available form, which represents 0.8% (sandy soils) to 3% (rich chernozem) of total potassium (Richter 1996, Pokorný et al. 2007). In measured samples available potassium is supposed to be present in middle to high amount evaluated according to Pokorný et al. (2007). This assumption was confirmed by laboratory analysis (Mehlich II method) when available K varied from 60mg/kg to 120mg/kg. The highest concentrations of Fe were measured on sampling sites 2/2, 5/1 and 5/2 with the values close to 5%. The lowest concentrations were measured on sampling site 1/2 where Fe content decreases below 2%. Higher Fe content can indicate occurrence of clay minerals of illite group while low Fe content probably show to clay minerals of caolinite group (Kynický 2015, personal communication). Average concentrations of Fe in soil range from 0.7–7% (Richter 1996). Solubility of Fe decrease with higher value of soil reaction which together with high concentration of $CaCO_3$ can cause precipitation of iron and its deficiency for plants (Richter 1996).

The lowest concentrations of Ca show quarry wall number 5 (sampling site 5/2, 0.74%) and sampling site 2/2. Most of the quarry walls show concentrations from approximately 10% to 24%.

Average content of Ca in the Czech soils varied from 0.15–6% (Richter 1996). Measured values higher than 15% are relatively extreme and confirm initially forming soils on limestone bedrock while low values correspond to Drahany Kulm formation consisting mainly of clay schist, sand stones and conglomerates. Low concentration of Ca and high concentration of K on the sampling site 2/2 is supposed to reflect small lens of the Kulm formation in Devonian limestone. Same trend was confirmed by measurement of available Ca by Mehlich II method with significantly lower values on quarry wall number five and sampling site 2/2.

Measured microelements are represented by Zn, Sr, Zr, Ba and Pb (see figure 3). According to S-H test the data show normal distribution. There are not statistically important differences between studied sample sites.

Figure 3 Measured concentrations of microelements in soil samples



Zinc is essential trace element with average values in the Czech Republic 0.0010–0.03% (Richter 1996). Detectable concentrations of Zn were measured in seven samples in concentrations from 0.002% to 0.02%. Solubility of zinc decreases with increasing values of soil reaction (Richter 1996). Deficiency of Zn may occur in these soils while toxic effect is not supposed. Measured concentrations of Rb varied from 0.006% to 0.02%. Percentage of Sr showed relatively homogenous character close to value 0.015% and reflects presence of marine sediments in limestone (Kynický 2015, personal communication) This value is lower than limit concentrations in soils of the Czech Republic (Beneš 1994, Ďuriš 2005). Concentrations of Zr varied from 0.01% up to 0.03%. Measured concentrations do not exceed average values of global soils measured by Vinogradov (1954), Bowen (1979), Kabata-Pendias (2001). These values indicate presence of zircon minerals which are typical accessories in sedimentary rocks. Zircon minerals are very resistant to weathering process as well as Nb containing minerals and do not poses risk for organisms (Kynický 2015, personal communication). Barium was detected on seven sampling

sites in concentration from 0.01% to 0.08%. The highest value exceeds average values of world soil measured by Vinogradov (1954), Bowen (1979), Kabata–Pendias (2001). However the concentration is not extreme and risk for organisms is not supposed. Occurrence of Ba can be explained by substitution of Ba for Ca. Detectable concentrations of toxic metal Pb were measured on seven sampling sites. The highest concentrations were measured within sampling site 4/2 (0.015%). This concentration is higher than average value in the Czech republic which range from 0.0005% to 0.005% and also exceed average concentrations for global soils which ranges from 0.0001 to 0.0035 (Vinogradov 1954, Bowen 1979, Kabata–Pendias 2001). However the concentration is not higher than maximal concentrations given by Czech law 13/1994 Sb. This sampling site is situated near the road which may explain measured value. For comparison maximal concentration measured in soils in capital city Prague was 0.042% (Ďuriš 2005). However high soil reaction decrease solubility of Pb and possible intake by plants (Richter 1996) and toxic effect is not supposed. Other elements (Co, Cu, V, S etc.) were detected in very low concentrations and usually occur below detection limit.

CONCLUSIONS

Limestone quarry Mokrá situated on the transition of Devonian limestone and Drahany kulm formation was tested for time and cost reducing measurement by ED – XRF spectrometer. The transition is situated in the east part of the quarry (Sekanina and Musilová 2011) while central part consist of Devonian limestone. Measured values of total Ca are significantly lower in the east part of the quarry. Same trend was confirmed by analysis of available amount of Ca by Mehlich II method. Content of Si, Al and K is higher in the east part of the quarry and correspond to mineral composition of clay schist, conglomerate and sandstones which are dominating for Drahany formation. Available content of K was calculated according to Pokorný et al. (2007) and then confirmed by laboratory analysis (Mehlich II). For XRF measurements soil samples were only dried and sieved. However for brief reconnaissance of bedrock type, this method is supposed to be applicable although the accuracy is decreasing without special preparation of soil samples.

Concentrations of selected microelements revealed any significant anomaly which can dramatically inhibit plant growth and due to high content of carbonates no toxic effects are supposed.

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PHYSICOCHEMICAL INVESTIGATION OF STABILITY OF APOFERRITIN WITH ENCAPSULATED DOXORUBICIN

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Abstract: The many negative side effects of drugs used for treatment of highly diverse diseases, such as cancer, lead in recent years to the development of ways how to target the drug selectively to the diseased tissue while avoiding the healthy cells. Nanocarriers, made from various materials, can serve as a suitable platform for this targeted drug delivery. Herein, we evaluate the long-term stability of nanocarrier based on ubiquitous protein apoferritin with encapsulated doxorubicin. Various properties of the nanocarrier were observed over the course of 12 weeks while stored at various temperatures, such as premature drug release, optical properties of the encapsulated drug, nanocarrier size and surface zeta potential. The nanocarrier showed very good stability for up to 12 weeks with the best results observed with nanocarrier prepared in water and stored at dark at 4 °C.

Key Words: absorbance; fluorescence; nanomedicine; surface zeta potential

INTRODUCTION

With no universal cure for cancer, the cause behind the death of every 4th person in the developed world, various anti-tumour agents are used in the treatment, as well as still being developed (American Cancer Society 2016). The drawback of these conventional cancer treatments are its many negative side effects (Wagland et al. 2016). Doxorubicin (DOX), an anthracycline antibiotics discovered in 1969, is just one of the examples for this (Arcamone et al. 1969). While it is used in the treatment of diverse cancer types, both those of blood elements (leukemia, Hodgkin's lymphoma) and solid tumours (breast, lung, sarcoma, bladder or ovaries) (Laskowska et al. 2016, Li et al. 2016, Vassilakopoulos et al. 2016), this is often at the expense of many side effects. These include less severe ones, such as nausea, diarrhoea, hair loss or hyperpigmentation (Panchuk et al. 2016). The most dangerous side effect of DOX, cardiotoxicity, exhibits in up to 10% of patients treated with this drug (Mazevet et al. 2013). The cardiomyopathy caused by a cumulative dose of DOX can lead to congestive heart failure (Holmgren et al. 2016). Patients treated with DOX and other anthracycline antibiotics are often co-treated with a cardioprotective agent dexrazoxane. However, this can lead to higher rate of secondary malignancies or even acute myelogenous leukemia (Cruz et al. 2016).

Due to the negative side effects that many drugs possess, various research groups in the recent years have been focused towards the development of nanoparticles able to transfer drugs selectively to the diseased tissue, with minimal effect on healthy tissue (Jeong et al. 2016). So far, nanocarriers made from many different materials have been researched, including both inorganic and organic. Each approach has its benefits and drawbacks. Inorganic particles are easier to manufacture, but are often toxic to the organism. Organic particles, especially those derived from molecules already found in the

body, are more suitable (Landesman-Milo and Peer 2016). One of the nanodrugs already used in clinical practice, is liposome-encapsulated DOX, sold under the name of Myocet. This drug has a very limited stability once DOX is encapsulated within the liposome – only 24 h at 2–8 °C (Alphandery et al. 2015). Therefore, there is a need for a more stable nanocarrier, providing similar toxicity to diseased tissue while protecting the healthy tissue.

Recently, we developed nanocarrier based on the ubiquitous protein apoferritin (APO), a 12-nm self-assembled hollow cage whose function is to store and transport iron ions in an organism. We loaded APO with DOX (Blazkova et al. 2013, Tmejova et al. 2013) and modified its surface with antibodies targeted to an antigen specific for prostate cancer. This targeted nanocarrier retained toxicity for prostate cancer cells similar to that of free DOX, while 43% of healthy cells were spared from the toxic effects of free DOX (Dostalova et al. 2016). In this work, we decided to evaluate the stability of APO nanocarrier over the course of 3 months. The stability was investigated using the fluorescence spectrometry for evaluation of prematurely released DOX from APO structure and changes in size and zeta potential of the whole nanocarrier.

MATERIAL AND METHODS

Chemicals

All chemicals of ACS purity were obtained from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated. The pH was measured using pH meter WTW inoLab (Weilheim, Germany).

Encapsulation of DOX into APO and its storage

9600 µL of mg/mL DOX was added to 960 µL of 50 mg/mL horse spleen APO and 4800 µL of water. 120 µL of 1 M hydrochloric acid was added to decrease the solution pH and dissociate the APO. The solution was mixed for 15 min. 120 µL of 1 M sodium hydroxide was added to increase the pH and encapsulate the DOX inside APO (creating APODOX). The solution was mixed for 15 min and divided into 2 equal parts. The parts were diafiltrated three times with water or phosphate buffered saline (PBS, pH 7.4; 0.137 M NaCl + 0.0027 M KCl + 0.0014 M KH_2PO_4 + 0.0043 M Na_2HPO_4), respectively, using Amicon® Ultra - 0.5 mL 3K (Merck Millipore, Billerica, MA, USA) at 6000 g for 15 min and filled to 24000 µL with the same solvent used for diafiltration. The samples were divided into 300 µL aliquots and stored for 12 weeks at -20; 4; 20 and 37 °C and dark with the 4 and 20 °C stored also under ambient light for 12 h every day.

Characterization of nanocarrier changes during storage

Every week of storage, aliquots from all storage conditions were collected and prematurely released drug molecules were removed by diafiltration with the respective solvent using Amicon® Ultra - 0.5 mL 3K at 6000 g for 15 min. The amount of released drug was determined by measurement of free DOX fluorescence compared with fluorescence of the whole sample. The fluorescence measurement was performed using Tecan Infinite 200 PRO (Tecan, Männerdorf, Switzerland) with excitation wavelength of 480 nm and emission wavelengths of 515–815 nm. The encapsulated drug was evaluated by absorbance measurement using Tecan Infinite 200 PRO with wavelengths of 230–850 nm.

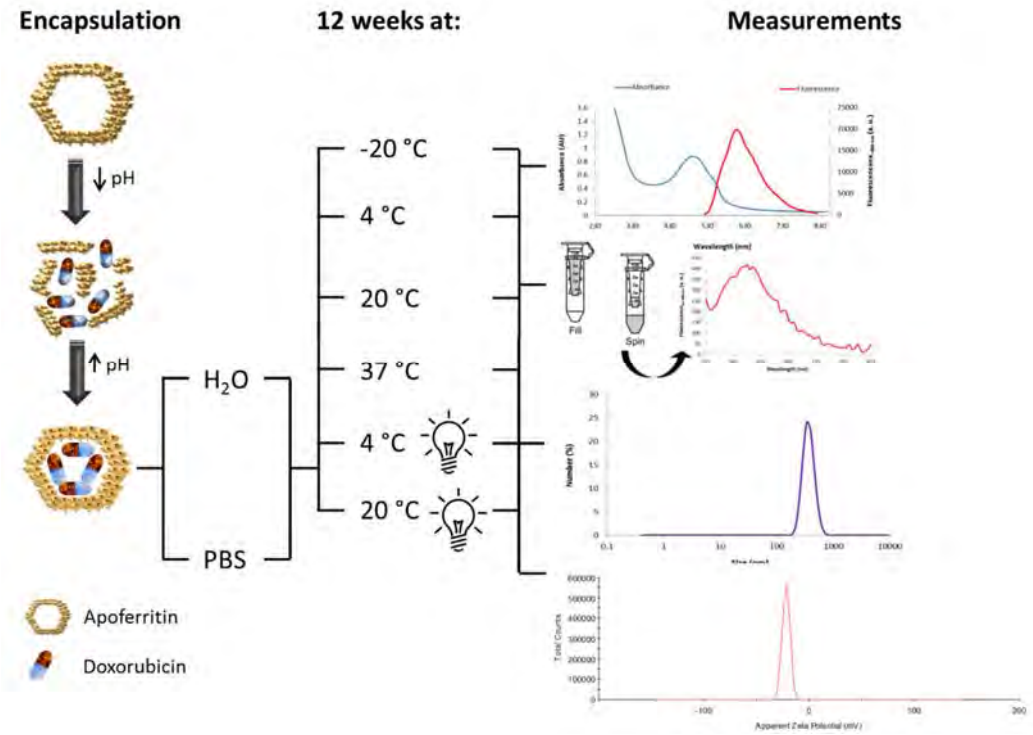
The average size of the nanocarrier was determined by quasielastic dynamic light scattering with Zetasizer Nano ZS instrument (Malvern Instruments Ltd., Worcestershire, UK). The nanocarrier prior to removal of released drug and diluted 100× with distilled water was placed into polystyrene latex cell and measured at a detector angle of 173°, wavelength of 633 nm and temperature of 25 °C with the refractive index of dispersive phase 1.45 and 1.333 for the dispersive environment. For each measurement, disposable cuvettes type ZEN0040, were used, containing 50 µL of sample. The equilibration time was 120 s. The measurements were performed in hexaplicates.

The surface zeta potential of the nanocarrier diluted 20× was measured using the Zetasizer Nano ZS instrument. For each measurement, disposable cells DTS1070 were employed. The number of runs varied between 20 and 40 and calculations considered the diminishing of particles concentration based Smoluchowsky model, with a F(κa) of 1.50 and an equilibrating time of 120 s.

RESULTS AND DISCUSSION

Experiment layout

Figure 1 Layout of the experiment.



The aim of this work was the evaluation of the stability of natural APO nanocarrier with encapsulated DOX (APODOX). The drug encapsulation mechanism of APO is based on its structural responsiveness to the surrounding environment (pH in the case of horse spleen APO). In neutral pH, APO forms a 12-nm icosahedral hollow cage made of 24 protein subunits (Haussler 2003). When acidified, it disassembles into its subunits and these can be mixed with any drug of choice. After adjustment of the pH back to neutral, the subunits spontaneously reassembly to again form the icosahedral structure, with the drug encapsulated within the hollow cavity. This physical entrapment allows for encapsulation of any drug large enough to not leak through the small pores in APO shell (Kim et al. 2011).

Figure 1 shows the layout of the experiment in this work. The APODOX was prepared in two solvents – water and PBS. These were aliquoted and kept for 12 weeks at various temperatures at dark (-20; 4; 20 and 37 °C). The samples at 4 and 20 °C were also simultaneously kept under ambient light to evaluate the influence of light on the stability of the nanocarrier. Every week, aliquots from the different conditions were collected and chosen measurements aimed to help evaluate the stability were performed.

Properties of nanocarrier at the start of the experiment

Table 1 Nanocarrier properties at the start of the experiment.

Solvent	Water	PBS
Premature DOX release (%)	6.4	6.2
Encapsulated DOX absorbance at 480 nm (AU)	0.2	0.2
Encapsulated DOX fluorescence (a. u.)	24905	31137
Nanocarrier size (nm)	12/43.8	12/68.1/342
Surface nanocarrier zeta potential (mV)	-27.9	-29.5

The properties of freshly prepared nanocarrier in the two solvents were evaluated at the start of the experiment (see Table 1). One of the most undesirable properties of a nanocarrier is the premature release of its cargo, whether in patient's organism or during its storage. The undesired cargo release can lead to increased toxicity for healthy cells (Yang et al. 2015). Due to the nature of APO drug encapsulation; there is low premature release of the drug molecules from the nanocarrier (6.4% for nanocarrier prepared in water and 6.2% for nanocarrier prepared in PBS, respectively). This is probably caused by the fact that some drug molecules may be adsorbed to the surface of nanocarrier instead of encapsulated in its cavity (Konecna et al. 2014).

Due to the structure of doxorubicin, it is possible to easily detect it using its absorbance maximum at 480 nm and its emission maximum at 600 nm (Konecna et al. 2014). The amount of encapsulated DOX in APO prepared in water and PBS was comparable, with 0.2 AU absorbance at 480 nm in both cases, which revealed encapsulation efficiency of 68%. However, the nanocarrier prepared in PBS exhibited higher fluorescence.

Other very important properties of the nanocarrier are its size and surface zeta potential. Both of these properties are indicative of the nanocarrier ability to enter individual cells (Stewart et al. 2016). The size of majority of APODOX particles prepared in water was 12 nm, with occasional aggregates of 43.8 nm and a zeta potential of -27.9 mV which shows the stability of freshly prepared particles. While the size of majority of APODOX particles prepared in PBS was also 12 nm, there was higher amount of 43.8-nm aggregates and occasionally even aggregates of 342 nm. The zeta potential was also indicative of the nanocarrier stability (-29.5 mV).

Stability evaluation of the nanocarrier

Table 2 Changes observed in APODOX nanocarrier stored for 12 weeks in water. (-)… lower value of observed parameters compared with freshly prepared samples. (+) … higher value of parameters compared with freshly prepared samples.

Temperature of storage (°C)	-20	4		20		37
Stored at dark/under light	Dark	Dark	Light	Dark	Light	Dark
Change in premature DOX release (%)	-2.3	-2.0	-2.3	-3.6	-3.4	-4.1
Change in encapsulated DOX absorbance at 480 nm (%)	+5.6	+18.1	+11.1	+32.2	+32.6	+25.6
Change in encapsulated DOX fluorescence (%)	+9.2	+16.0	+3.4	+3.3	+7.8	+5.7
Change in nanocarrier size (%)	+303.8	+25.0	+151.8	+67.5	+123.4	+68.4
Change in nanocarrier surface zeta potential (%)	-5.5	-4.4	-12.4	-26.1	-20.8	-11.4

The measurements from each individual week were averaged to show the influence of individual storage conditions on the nanocarrier. Table 2 shows the changes observed in nanocarrier prepared in water. Even though the undesired premature release of DOX from the APO structure was very low in the freshly prepared samples, it was even further lowered in all the stored samples. The best results were obtained with APODOX stored at 37 °C. The observed absorbance and fluorescence of the encapsulated DOX was higher in all stored samples compared with the freshly prepared samples. However, this did not correlate with the percentage of prematurely released DOX. Significant differences in nanocarrier size in samples stored at various conditions were observed. The samples stored at -20 °C showed a formation of aggregates of up to 300% larger size than were observed in freshly prepared samples. The most stable size throughout the storage was observed in samples stored at 4 °C and dark with only 25% increase of size. The formation of aggregates, combined with different amount of prematurely released drug can explain the changes of DOX absorbance and fluorescence. The surface zeta potential was less negative in all observed samples, showing lower stability of the nanocarrier. The best results were observed in samples stored at 4 °C and at dark.

Table 3 Changes observed in APODOX nanocarrier stored for 12 weeks in PBS. (-)… lower value of observed parameters compared with freshly prepared samples. (+) … higher value of parameters compared with freshly prepared samples.

Temperature of storage (°C)	-20	4		20		37
Stored at dark/under light	Dark	Dark	Light	Dark	Light	Light
Change in premature DOX release (%)	+1.1	-0.4	+0.4	-0.5	+1.0	+1.4
Change in encapsulated DOX absorbance at 480 nm (%)	+11.3	+21.2	+26.8	+28.6	+37.6	+30.6
Change in encapsulated DOX fluorescence (%)	+8.1	+1.7	+0.5	-10.8	-5.2	-10.1
Change in nanocarrier size (%)	+125.7	+362.0	+318.3	+405.2	+486.5	+244.3
Change in nanocarrier surface zeta potential (%)	-9.6	-4.8	-4.2	-27.2	-27.5	+6.0

Table 3 shows the changes observed in nanocarrier prepared in PBS. In contrast to the results obtained with nanocarrier stored in water, the samples stored in PBS showed slightly higher percentage of premature drug release than was observed in freshly prepared samples. The only storage conditions in which the premature release was slightly lower were 4 and 20 °C at dark. The observed absorbance and fluorescence of the encapsulated DOX was also higher in all stored samples compared with the freshly prepared samples. However, as the percentage of prematurely released drug was in many cases increased, these results did not correlate. Significant differences in nanocarrier size in samples stored at various conditions were observed, even more so than in the samples in water. The largest aggregates were observed in samples stored at 20 °C (486.5% larger than in freshly prepared samples for storage under light and 405.2% for storage at dark). The best results were obtained for samples stored at -20 °C with aggregates of up to 125.7% larger size than those in freshly prepared samples. The surface zeta potential was less negative in most observed samples, showing lower stability of the nanocarrier, with the exception of samples stored at 37 °C. It can be concluded that the observed stability of nanocarrier kept in water was higher than that stored in PBS.

CONCLUSION

The experiment presented in this work dealt with the evaluation of stability of a nanocarrier for anti-cancer drugs, based on naturally occurring and versatile protein apoferritin. Overall, the presented nanocarrier showed high stability for up to 12 weeks with the optimal results were obtained with nanocarrier stored in water at 4 °C and dark.

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DETERMINATION OF S-ADENOSYLMETHIONINE AND S-ADENOSYLHOMOCYSTEINE IN TWO PROSTATIC ADENOCARCINOMA CELL LINES

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Abstract: Studies of processes leading to cancer tumour progression are one of the major foci of current cancer research. Therefore, it is important to shade the light on the metabolic pathways involved in cancerogenesis. In the present study we have chosen as a model two prostatic adenocarcinoma cell lines (PC-3 and LNCaP) for the comparison of the metabolites involved in tumour growth. Applying the HPLC-MS method, we determined and compared the concentration levels of S-(5'-adenosyl)-L-homocysteine (SAH) and S-(5'-adenosyl)-L-methionine (SAM), that are involved in the tumour growth processes. SAM/SAH ratio, a methylation index describing the aggressiveness of tumour cells, was determined here. The methylation index was 0.4 and 1.2 for PC-3 and LNCaP cells, respectively, pointing out that the PC-3 cells are more aggressive than LNCaP cells. This finding is of great importance for our following studies.

Key Words: SAM, SAH, methylation index, PC-3, LNCaP, metallothionein

INTRODUCTION

Prostate cancer is the most common noncutaneous cancer in men in the Czech Republic. An estimated prevalence in years 2012–2017 is 50.4% (Ferlay et al. 2013). The processes leading to prostate cancer progression are still poorly understood. Therefore the studies of metabolomic pathways involved in the tumour growth are of a high importance and could subsequently lead to the discovery of new approaches to tumour growth inhibition. Metallothionein (MT), the cysteine rich metalloprotein, is one of the potential markers for tumour disease development. There is important evidence that increased content of heavy metals and MTs in tumour tissues is connected to the increased invasiveness and metastasizing of a tumour (Gumulec et al. 2014).

The focus of our current research is on metallothionein biomarkers and their distribution in biological tissues. In our previous study we have successfully used MALDI-TOF MSI (matrix assisted laser desorption/ionization time-of-flight mass spectrometry imaging) to acquire 2D spatial distribution of chicken metallothionein MT-1 in chicken embryo (Guran et al. 2015). Recently, we have also used MALDI-TOF MSI to determine spatial distribution of human metallothionein MT-1X in PC-3 tumour xenografts (Heger et al. 2016). With respect to these results we discussed other factors, which are linked to MT expression and tumour growth. In closer look onto metabolic processes involved in the production of MT, we have found that the homocysteine produced in methionine cycle from SAH (Figure 1) is further transformed into cysteine (Figure 2), which is, *inter alia*, used in synthesis of cysteine-rich proteins, such as MTs (Hughes et al. 2009, Shlomi and Rabinowitz 2013). One of the most important processes in tumour cells is a methylation of DNA, proteins, peptides and other biomolecules. S-(5'-adenosyl)-L-homocysteine (SAH) and S-(5'-adenosyl)-L-methionine (SAM) are intermediates in methionine cycle and participate in methylation processes. SAH is formed by SAM demethylation with

a help of nicotinamide N-methyl-transferase. This enzyme is overexpressed in some types of tumours and therefore it can enhance cancer aggressiveness by draining methyl groups from SAM. The SAM/SAH ratio is one of the parameters used for describing the aggressiveness of tumour cells. Moreover, the so-called methylation index, expressed as ratio SAM/SAH, is connected with tumour development (Shlomi and Rabinowitz 2013). These findings inspired us to gain some information about SAM and SAH concentration levels in two prostatic adenocarcinoma cell lines (PC-3 and LNCaP). We have used HPLC-ESI-QqTOF MS for determination of SAM and SAH as it is one of the most suitable methods for metabolomics (Wu and Li 2016).

The aims of this study were i) to develop/adapt and validate HPLC-ESI-QqTOF MS method for analysis of SAM and SAH in cells, especially in PC-3 and LNCaP cell lines, ii) use the method to determine concentrations of SAM and SAH in PC-3 and LNCaP cells, and iii) to determine the methylation index (SAM/SAH). The obtained results will be used in future experiments to find whether the concentration levels of MTs are in correlation with methylation index.

Figure 1 Methionine cycle describing how SAM and SAH are involved in methylation processes. THF is tetrahydrofolate, 1MNA is 1-methylnicotinamide. Adapted with permission from Shlomi and Rabinowitz (Shlomi and Rabinowitz 2013).

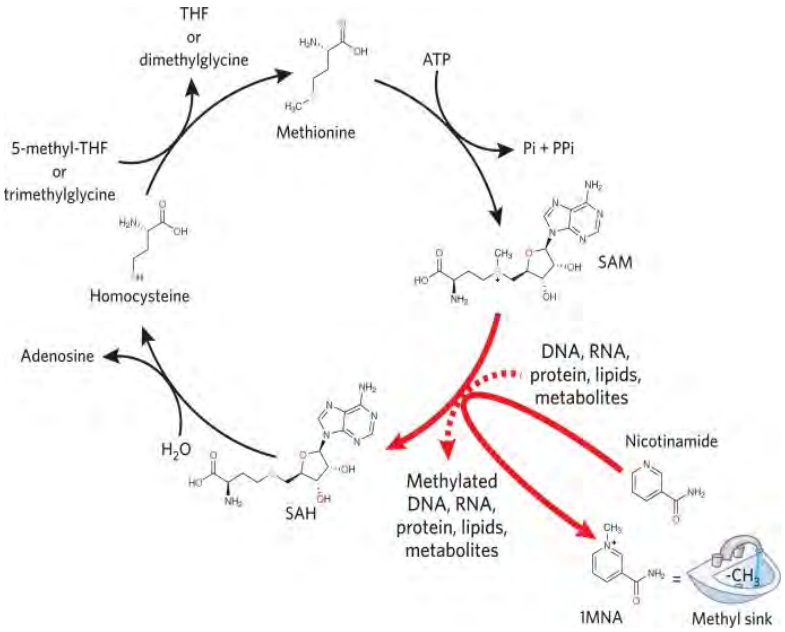
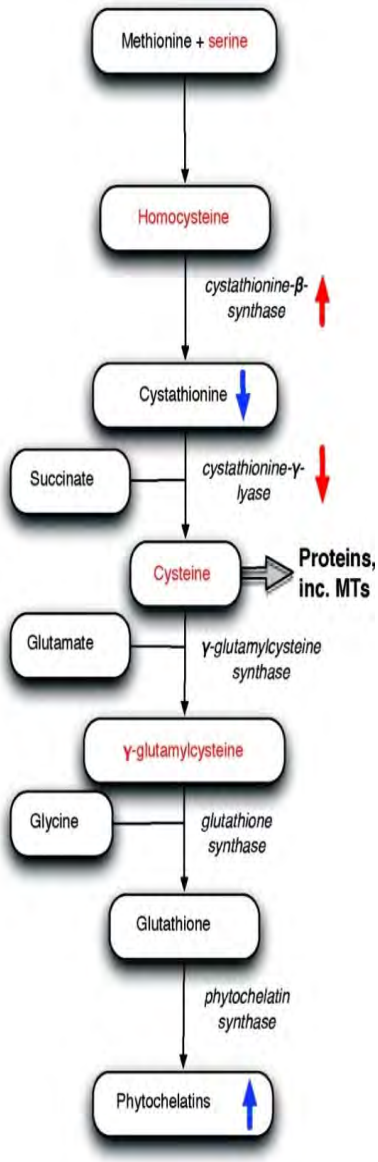


Figure 2 (on right) Metabolic processes resulting from methionine cycle connected with expression of metallothioneins. Adapted with permission from Hughes et al. (Hughes et al. 2009).



MATERIAL AND METHODS

Chemicals

All chemicals used for HPLC-ESI-QqTOF MS were purchased in LC-MS quality from Sigma Aldrich (St. Louis, MO, USA). Other chemicals were purchased from the same company in ACS quality, unless otherwise noted. As SAM standard was used S-(5'-adenosyl)-L-methionine iodide (mass ratio of SAM in this substance is 0.7564). As SAH standard was used S-(5'-adenosyl)-L-homocysteine (crystalline).

Cell culture and harvesting

The PC-3 human cell line, established from a grade 4 androgen-independent prostatic adenocarcinoma, was purchased from the Health Protection Agency Culture Collection (Saltsbury, UK). The cells were maintained as monolayer culture in the Ham's F-12 medium containing 7% fetal bovine serum (FBS) and penicillin (100 U/ml) and streptomycin (0.1 mg/ml). Cells were grown at 37 °C in a humidified incubator Galaxy® 170 R (Eppendorf, Hamburg, Germany) with 5% CO_2. Then, the cells at ~80% confluence were harvested using Trypsin/EDTA. One million cells were pelleted by centrifugation at 200 × g for 10 min at 4 °C. Finally, pellets were frozen and stored at -80 °C until further use.

The LNCaP human cell line was established from a lymph node metastatic lesion of prostatic adenocarcinoma. Cells were maintained as monolayer culture in the RPMI-1640 with 10% FBS. Medium was supplemented with penicillin (100 U/ml) and streptomycin (0.1 mg/ml), and the cells were maintained at 37 °C in a humidified incubator Galaxy® 170 R (Eppendorf, Hamburg, Germany). Afterward, the cells at ~80% confluence were harvested, washed four times with PBS (pH 7.4) and Trypsin/EDTA. Cells were pelleted by centrifugation at 200 × g for 10 min at 4 °C and counted using Countess IIFL Automated Cell Counter (Life Technologies, Carlsbad, CA). Finally, pellets were frozen and stored at -80 °C until further use.

SAH and SAM extraction

A following extraction protocol was adapted from Stevens et al. (Stevens et al. 2010) and slightly modified by not using a vacuum centrifuge for extract concentration. Methanol with acetic acid (1M) in a volume ratio 80:20 was selected as extraction solvent. According to the literature, this mixture yields good extraction recoveries for both SAM and SAH (Stevens et al. 2010). Firstly, 600 µl of the extraction solvent were added to the frozen pellets. Then the sample was slowly thawed on ice. Subsequently, the cells were shock-frozen in liquid nitrogen and thawed on ice again. The freeze/thaw cycle was performed three times and the sample was vortexed between each cycle. The sample was centrifuged at 9000 × g for 5 min at 4 °C and the supernatant was transferred to a 1.5 ml glass vial. The pellet was washed twice with 200 µl of extraction solvent and all supernatants were combined.

Evaluation of SAM and SAH extraction recoveries

The following protocol for evaluation of extraction recoveries for SAM and SAH was adapted from Stevens et al. (Stevens et al. 2010). Cell pellets were spiked in quadruplicate with three different concentration levels and extracted as was described in previous paragraph. The concentrations of spikes were selected for each analyte based on its intracellular concentration range found in literature. If c is a mean concentration of each analyte found in literature, then the following concentrations were spiked: c, 0.5c, and 2c.

HPLC-ESI-QqTOF MS method

Cell extracts were separated on C18 reverse phase column Phenomenex Kinetex EVO (5 µm particles; 150 × 4.6 mm) in HPLC system consisted of two pumps ESA Model 584 and an autosampler ESA Model 542 (ESA Inc., Chelmsford, USA). Flow rate was 0.5 ml/min. Injected sample volume was 20 µl. Mobile phase A consisted of water with 0.1% formic acid. Mobile phase B consisted of methanol with 0.1% formic acid. The following gradient was programmed: 0 min 0% B; 4 min 30% B; 8 min 40% B; 8.1 min 100% B; 13 min 100% B; 13.1 min 0% B; 18 min 0% B; 18.1 min STOP. HPLC was coupled with ESI-QqTOF mass spectrometer Bruker Maxis Impact (Bruker Daltonik GmbH, Bremen, Germany). The following parameters of mass spectrometer were used: End plate offset potential 500 V; capillary potential 3500 V; nebulizer gas (N_2) pressure 0.3 MPa; dry gas (N_2) flow rate 12 l/min; drying temperature 300 °C. Mass range was set from 50 to 3000 *m/z*. Prior to HPLC-MS analysis a mass spectrometer was calibrated using ESI-TOF Tuning mix (Sigma Aldrich, St. Louis, MO, USA).

Statistical evaluation

The HPLC-ESI-QqTOF MS method was calibrated on SAM and SAH standard working solutions prepared in used extraction solution at concentrations 0.04, 0.2, 1, 5 and 25 µM. Each calibration solution was measured five times. Linear regression with least squares approach was used for calculating regression equation and constructing calibration curve. Dixon's Q test was used for identification of

possible outliers among results. The linearity of calibration curve and a significance of intercept *a* (*y* = *bx* + *a*) was tested.

For determination of SAM and SAH concentrations in cell extracts, the method of standard additions at constant volume was used. Four standard additions were used for each cell extract. Into five glass vials were added 100 µl of cell extract and 0, 10, 20, 30 and 40 µl of SAM and SAH standards at concentration 500 µM (both) prepared in extraction solution. Finally, each vial was filled up to 1 ml with extraction solution and all solutions were measured three times.

For data analysis was used Microsoft Excel 2010 (Microsoft, Redmond, WA, USA). Statistical significance for all tests was accepted at $p < 0.05$.

RESULTS AND DISCUSSION

In order to analyze SAH and SAM in PC-3 and LNCaP cells, the confirmation of suitability of HPLC-ESI-QqTOF MS method was performed by extensive literature search. Several publications using HPLC and ESI-MS methods for SAM and/or SAH analyses were found (Iglesias González et al. 2015, Klepacki et al. 2013, Stevens et al. 2010). Therefore the method similar to the one used by Stevens et al. (Stevens et al. 2010) was selected for optimization on SAM and SAH standards. In Figure 3A is shown typical chromatogram of SAM and SAH standard calibration mixtures with mass spectra of SAM and SAH. In Figure 3B are shown calibration curves for SAM and for SAH. Basic analytical parameters of method calculated from calibration analysis, with measured extraction recoveries, are shown in Table 1.

*Figure 3 Calibration of HPLC-ESI-QqTOF MS method on SAM and SAH standards. (**A**) A typical chromatogram of SAM (purple line) and SAH (blue line) standard calibration mixture. Under the chromatogram are shown mass spectra of SAM and SAH. (**B**) Calibration curves for SAM (purple points) and SAH (blue points) in a linear range from 0.04 to 25 µM.*

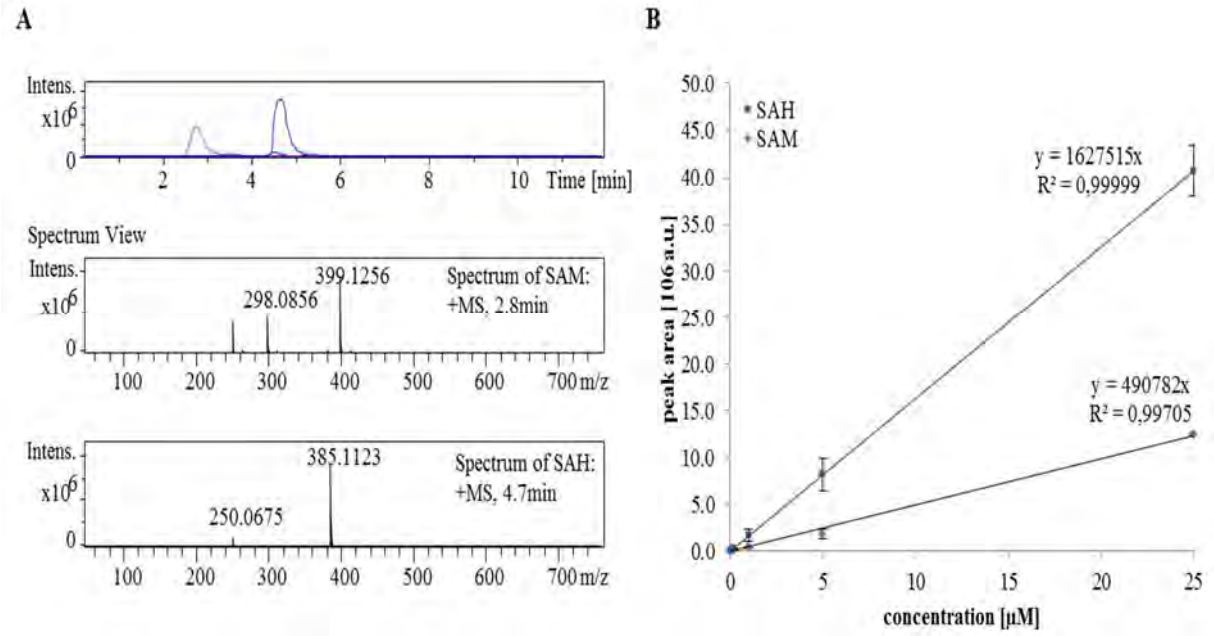


Table 1 Analytical parameters for HPLC-ESI-QqTOF MS method used to determine SAM and SAH concentrations. Number of measurements per one calibration sample was 5.

	MW	Regression equation	Linear dynamic range [µM]	LOD [µM]	LOQ [µM]	Extraction recovery [%]
SAM	398.14	y = 490782x	0.04–25.00	0.08	0.27	85–103
SAH	384.41	y = 1627515x	0.04–25.00	0.01	0.02	83–102

Linear range covers the usual concentration range of SAM and SAH in extracts from million cells (Stevens et al. 2010) pointing out that this method is suitable for measuring SAM and SAH in cell extracts. As it is mentioned in material and methods section, the determination of SAM and SAH in cell extracts was done by the method of standard additions at constant volume. In Table 2 are shown determined concentrations of SAM and SAH in PC-3 and LNCaP cells.

Table 2 Determined concentrations of SAM and SAH in PC-3 cells and LNCaP cells by HPLC-ESI-QqTOF MS method and corresponding methylation indexes (SAM/SAH). In total were used three million PC-3 cells and three million LNCaP cells. Number of measurements per one sample with each standard addition was 3.

PC-3 cells	c [μM/10^6 cells]	c [μmol/10^6 cells]	SD [μM/10^6 cells]	SD [μmol/10^6 cells]	RSD [%]
SAM	3.58	0.004	0.19	0.0002	5
SAH	9.58	0.010	0.12	0.0001	1
SAM/SAH	0.4				
LNCaP cells	c [μM/10^6 cells]	c [μmol/10^6 cells]	SD [μM/10^6 cells]	SD [μmol/10^6 cells]	RSD [%]
SAM	18.63	0.019	0.38	0.0004	2
SAH	15.87	0.016	0.43	0.0004	3
SAM/SAH	1.2				

As it is shown in Table 2, the determined concentration of SAM was higher nearly six times in LNCaP cells than in PC-3 cells. The concentration of SAH in LNCaP cells was higher at least 1.5x. The most important information is obtained by calculation of the methylation index, which was 1.2 for LNCaP cells and 0.4 for PC-3 cells indicating that PC-3 cells are probably more aggressive than LNCaP cells. This finding is in correlation with higher metastatic potential of PC-3 than of LNCaP (MuraliKrishna et al. 2005).

CONCLUSION

In this study, the concentration levels of SAM and SAH in PC-3 and LNCaP cells were analyzed by HPLC-ESI-QqTOF MS and corresponding methylation indexes (the ratio SAM/SAH) were calculated. It was found that methylation index for LNCaP cells was 1.2 and for PC-3 cells was 0.4. This means that PC-3 cells are more aggressive than LNCaP cells. These findings are of a high importance for our future experiments focused on correlation between methylation index and expression of metallothioneins. Moreover, in future experiments the UPLC-MS method will be used to improve (decrease) the time of the analyses and those minimize the consumption of chemicals.

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SEED STORAGE PROTEINS IN FOUR CONTRASTING PLANT SPECIES

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Abstract: Seed proteome analysis is a challenging task. Here, we compare seed proteome composition of four contrasting plant species - *Arabidopsis thaliana*, *Hordeum vulgare*, *Helianthus annuus* and *Solanum lycopersicum*. We found a strong negative correlation between seed protein content and the amount of detectable proteins in the LC-MS analysis.

Key Words: seed proteome, LC-MS, abundant proteins

INTRODUCTION

Plant seed composition may vary significantly depending both upon plant species and the external conditions during the seed development. The ratio between three major components - lipids, carbohydrates and proteins - may vary significantly. For example, starchy seeds like barley (*Hordeum vulgare*) contain predominantly carbohydrates that represent over 70% of seed's mass. Sunflower (*Helianthus annuus*), a representative of oilseeds, relays more on lipids, which form over 50% of its content. Some plant species, like *Arabidopsis*, employ a different propagation strategy and produce a large amount of seeds, with a lower amount of storage compounds but a higher percentage of protein (Table 1).

Table 1 Average percent composition of seeds (based on Bewley et al. 2013, Beisson et al. 2007, Li et al. 2006, Molina et al. 2006, Ohlrogge and Browse 1995, Persia et al. 2003)

Plant	Protein [%]	Lipids [%]	Carbohydrates [%]	Other [%]
Barley	12	3	76	9
Tomato	28	25	35	12
Arabidopsis	30	37	20	13
Sunflower	21	51	20	8

Seed proteins represent 10–30% of seed mass. Most of this content is formed by storage proteins. These are mostly used as a source of energy, but can also be important for the seed protection. Here, we compared storage proteins of four different plant species: *Arabidopsis thaliana*, barley, tomato (*Solanum lycopersicum*) and sunflower. These plants represent (i) albuminous (barley) and exalbuminous (*Arabidopsis*, tomato, sunflower) seeds; (ii) monocots and dicots; and (iii) species with a high and low amount of storage nutrients.

MATERIAL AND METHODS

Seed material

Barley (cv. Sebastian), tomato (cv. Moneymaker), sunflower (line HA412HO) and *Arabidopsis thaliana* (Col-0) seeds were homogenized (Retsch Mill MM400), aliquoted and stored at -80 °C.

Protein extraction and LC-MS analysis

Total protein extracts were prepared by acetone/TCA/phenol extraction (Černý et al. 2014, Novák et al. 2015) from app. 300 mg of ground tissue. The resulting protein pellets were solubilized and digested with an immobilized trypsin (Promega) overnight, desalted by C18 SPE and analyzed as

described previously (Baldrianová et al. 2015). Briefly, tryptic digests corresponding to 5 μg of protein extract were dissolved in 0.5% (v/v) formic acid in 5% (v/v) acetonitrile, and then analyzed by nanoflow C18 reverse-phase liquid chromatography using a 15 cm column (Zorbax, Agilent), a Dionex Ultimate 3000 RSLC nano-UPLC system (Thermo) and an UHR maXis impact q-TOF mass spectrometer (Bruker). Peptides were eluted with a 120-min, 4% to 40% acetonitrile gradient and spectra were acquired at 2 Hz (MS) and 10 to 20 Hz (MS/MS) using an intensity-dependent mode with a total cycle time of 7 s.

Protein identification

The measured spectra were extracted by Bruker's Data Analysis 4.1 and processed as described previously (e.g. Cerna et al. 2016). In brief, recalibrated MGF files were searched against *Arabidopsis* (TAIR 10), tomato (ITAG 2.4), barley (3/2012) or sunflower (10/2014) protein sequence database by Sequest HT, MS Amanda and Mascot 2.4 with the following parameters: Enzyme - trypsin, max two missed cleavage sites; Mass tolerance - 35 ppm (MS) and 0.1 Da (MS/MS); Modifications - up to three dynamic modifications including Met oxidation, Asn/Gln deamidation, Lys methylation, N-terminal acetylation, Ser/Thr/Tyr phosphorylation.

Protein quantitation

The protein abundance was estimated by calculating normalized number of peptide spectral matches (PSM) (Černý et al. 2013).

RESULTS AND DISCUSSION

Major storage proteins identified in seeds of *Arabidopsis*, barley, sunflower and tomato

Four types of seeds were analyzed to decipher the differences in the composition of their proteomes. First, we noted that the number of identified proteins varied between species. In total, we identified 422, 1 061, 1 309 and 458 for *Arabidopsis*, barley, sunflower and tomato, respectively. The same extraction method and similar starting amount of material was used and the total protein yield correlated well with the expected protein amount (Table 1). Seed proteome diversity seems to be lower in *Arabidopsis* and tomato (~ 500 identified proteins). This likely corresponds to the amount of seed protein, which is much higher for tomato and *Arabidopsis* (app. 30%). Thus, the highly abundant storage proteins represent the major component of the seed and interfere with the detection of a lower abundant ones. Indeed, in *Arabidopsis*, three cruciferin proteins (CRU1-CRU3) represent 50% of seed proteome (Figure 1). A similar proportion of tomato seed proteome is filled by five legumine- and vicilline-like proteins. In contrast, 50% of seed protein content is formed by 16 and 54 proteins in sunflower and barley, respectively. These proteins function both in seed storage and primary metabolism. However, in barley, a big portion of high abundant proteins still remains uncharacterized.

Seed protein content negatively correlates with the amount of detected proteins

To analyze the relation between the seed protein content and amount of proteins that form its 50%, we plotted the data and extrapolated respective dependency (Figure 2). We found that the number of proteins decreases with the square value of the protein content and dependency seems to be polynomial.

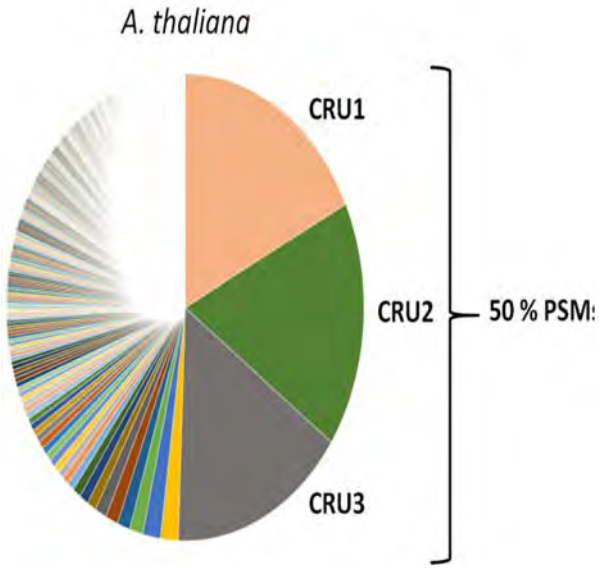
Resulting data are beneficial for optimization of seed proteome analysis of different plant species

The seed proteomic analysis is limited by the employed methodology and the proteome coverage suffers thanks the presence of high abundant proteins. Thus, it would be beneficial to remove or deplete these proteins which are not directly involved in the regulation of seed germination progress. For this purpose, a huge number of fractionation methods (based on seed anatomy and composition) has been developed (e.g. Wang et al. 2015). These methods, and especially their combination, increase the number of detected proteins. However, the majority of these methods is not specific and may cause a loss of some proteins of interest. The comparative analysis of the most abundant proteins presented in this work could be used for the development of specific separation methods and by monitoring the rations between these abundant proteins, the method efficiency can be evaluated. Further, our results indicate that the most beneficial depletion in *Arabidopsis*, tomato and sunflower seed proteomics would be based on the removal of seed storage proteins belonging to 11S cupin protein family. These proteins

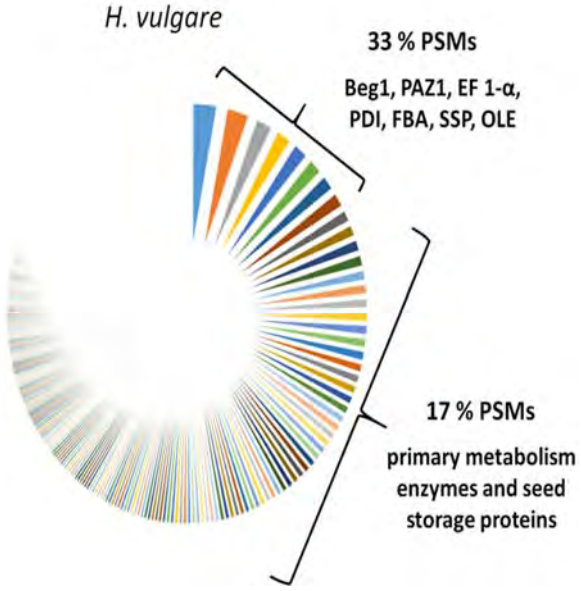
are relatively homologues and thus, the development of a cheap anti-cupin antibody could be feasible. In contrast, barley seed proteome composition is more complicated and an antibody-based depletion would not be cost effective. Thus, we suggest that to improve barley seed proteomics, a combination of separation methods must be used instead, including e.g. strong cation exchange chromatography (SCX), size exclusion chromatography or precipitation-based techniques.

Figure 1 Seed proteome composition

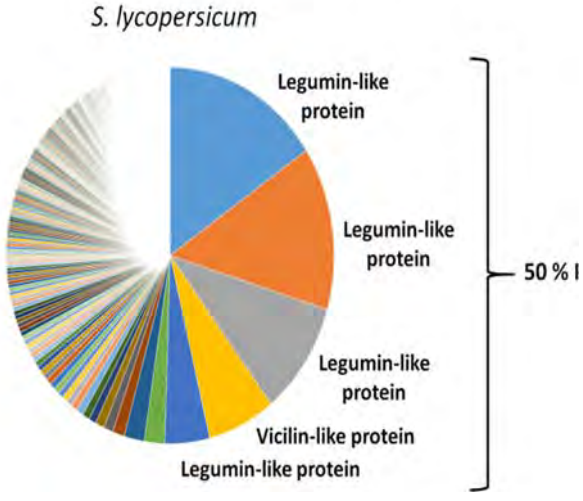
A) Arabidopsis



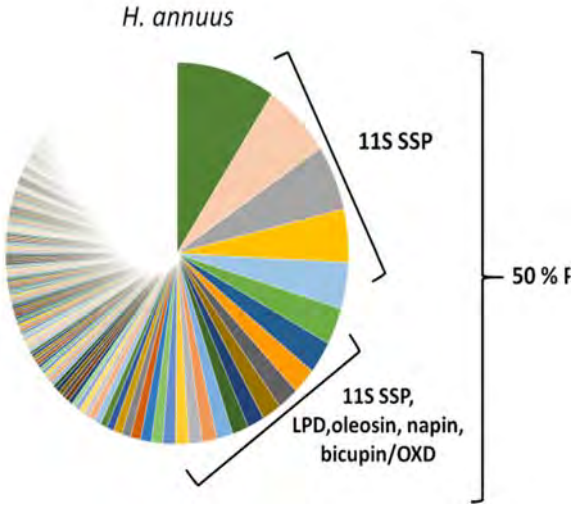
B) Barley



C) Tomato

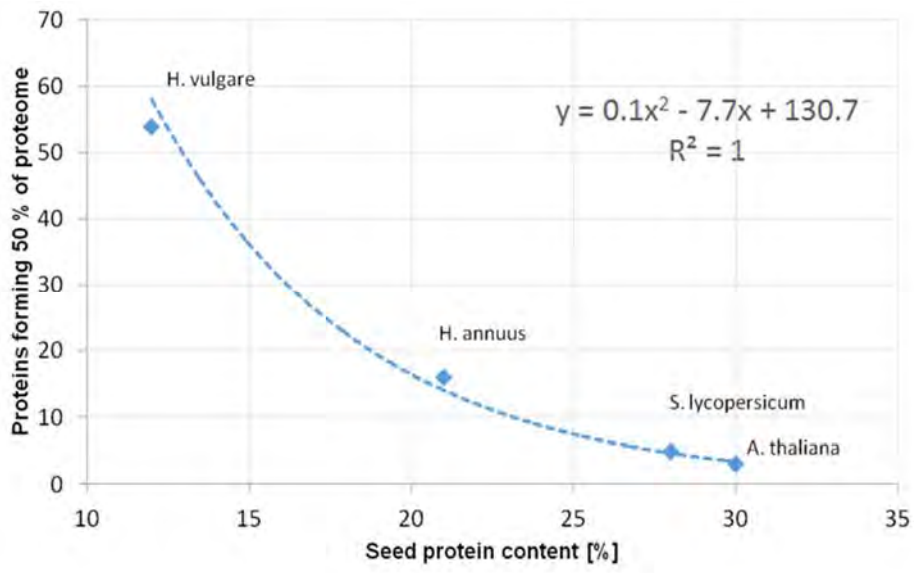


D) Sunflower



(CRU - cruciferin; EF 1 - elongation factor 1; FBA fructose-bisphosphate aldolase; SSP - seed storage protein, a member of cupin protein family; PDI - protein disulfide-isomerase; OXD - oxalate decarboxylase; LPD – lipid transfer protein; OLE – oleosin; Beg1 – embryo globulin; PAZ1 – serpin Z4)

Figure 2 Number of identified proteins forming 50% of seed proteome in seeds with different protein content



CONCLUSION

Protein content of seeds varies dramatically between species. After comparing low, medium and high-protein seeds, we can conclude that the higher proportion of proteins intended for the nutrition of the germinating seedling results in a seemingly lower diversity in seed proteome. In addition, this decrease in diversity corresponds with a significant increase in the amounts of storage proteins. Thus, in order to increase the number of identified proteins, seeds that contain more than 20% of protein should be subjected to proteome equalization or other method that would achieve the depletion of these major components.

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NEUROBLASTOMA HOMING PEPTIDE SCREENING USING UNREFINED HOMOLOGY STRUCTURE OF NOREPINEPHRINE TRANSPORTER

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Abstract: The norepinephrine transporter (hNET) is a potential target for many antidepressants and for neuroblastoma therapeutics. The entrance channel of hNET and also dopamine transporter (DAT) serve as candidate site for targeting by large peptides e.g. α-helix-based. Targeting peptides, also known as homing peptides, are used to direct the delivery of cargo to specific cell types. Peptides of known secondary structures such as α-helix and β-sheet have predictable and stable folding. In this study, approx. 27 peptides, with predictable secondary structures, were evaluated by 20 dockings predictions on unrefined hNET homology model and DAT crystal structure using molecular mechanics (total ~1080 models). As anticipated, peptide size was detrimental for docking in channel space, whereas peptide isoelectrics point did not affect docking. Peptide's initial non-bonded energy affected docking while overall peptide free energy and initial electrostatic energy did not. Two α-helices showed favorable docking in channel of hNET; namely, GASNGINAYL and SLWERLAYGI with binding energy of -106.2 kJ/mol and -128.6 kJ/mol, respectively. Prior to *in vitro* and *in vivo* applications, future work will focus on development of refined accurate model of hNET and application of solvated docking (in presence of water). This study provides new insight to the development of helix-based therapeutic peptides.

Key Words: targeted therapy, norepinephrine transporter, homing peptide, molecular mechanics, docking

INTRODUCTION

Targeting peptides, also known as homing peptides, are used to direct the delivery of cargo (drugs) to specific cell types. Many homing peptides have been investigated for applications in diagnosis and treatment of different types of cancer (van den Berg and Dowdy 2011; Svensen et al. 2012). Current methods of new targeting peptide discovery rely on screening of phage display peptide libraries and one-bead one-compound libraries (Gautam et al. 2014). Peptide-receptor interactions can provide valuable insight for the development of novel and more potent therapeutics. Molecular docking is a primary method for computational evaluation of protein-protein interactions. Docking is comprised of three steps: First, representation of the system (structures of receptor vs. ligand) followed by conformational-space search. Finally, there is an evaluation step which involves ranking or scoring of potential solutions (Halperin et al. 2002). Methods for evaluation of docking can be classified to either empirical (e.g. based on wet lab experimental data) or knowledge-based methods (e.g. based on statistical analysis of distances between atom types in ligand receptor interface). Molecular mechanics (MM) based on evaluation of free binding energy can be an alternative approach for scoring of docking processes; limited only for high throughput of ligand-receptor investigations (dozens or hundreds) because they are computationally expensive (Huang et al. 2006).

Norepinephrine transporter (hNET) is one of the most promising targets for treatment of neuroblastoma (Matthay et al. 2012). It belongs to SLC6 family of sodium dependent neurotransmitter

transporters involved in translocation of small amino acid or amino acid-like substrates, including serotonin, dopamine, norepinephrine, GABA, taurine, and creatine (Pramod et al. 2013). The first choice to design hNET targeting peptides would be based on mimicking cell-surface interacting proteins with hNET. Unfortunately, all known interacting proteins such as 14-3-3 protein, syntaxin1A, proteinphosphatase 2A (PP2A), PICK1, and Hic-5 bind the intracellular domains of hNET (Sung et al. 2005). Early studies on dopamine and serotonin transporters suggested that they form oligomers (Torres et al. 2003). Due to this, the reasonable approach was to use peptides mimicking hNET itself, presuming that such peptides will have self-affinity to the same molecule. Since linear peptides are highly unpredictable, the secondary structure; particularly alpha helix, was the primary choice for design of stable predictable 3D molecule. Alpha helix is the classical basic element of protein structure. One helix can have more influence on the stability and organization of a protein than any other individual structure element. It is composed of 3.6 residues per turn, with a hydrogen bond between the CO of residue n and the NH of residue n+4 (Richardson 1981).

The aim of this study was to design peptides of known secondary structures that are able to bind the norepinephrine transporter (hNET) for targeted therapy.

MATERIAL AND METHODS

Building of Structure Models

The structure of hNET (Uniprot ID: P23975) was constructed using SWISS-MODEL (Bordoli et al. 2009). The X-ray crystal structure of dopamine Transporter (PDB ID: 4M48), (Penmatsa et al. 2013) with 2.96Å resolution, 59.9% sequence identity and 0.94 coverage was used as model template. MolProbity, (Davis et al. 2007) was used to check the quality of hNET model. Model quality check was only used to assess the weaknesses in the constructed models. No energy minimization, refinement or deliberate change in structure were made. The focus of this work was to develop screening method as a proof of concept. We have recently analyzed and conveyed guidelines for developing accurate model of hNET (Haddad et al. 2016). Future work will focus on employing such model in homing peptide design. Peptide sequences were selected from hNET sequences with known α-helix or β-sheet secondary structures. PepFold, (Maupetit et al. 2009) was used to construct peptide models. Peptide model was acceptable for further analysis when de novo structure resulted in predicted secondary structure conformation. Rejected structures were mostly due to disruptions caused by PRO residue in the middle of the peptide.

Molecular Docking

Preparation for docking was done using “dock prep” tool in UCSF Chimera version 1.10.2 (Pettersen et al. 2004). Addition of hydrogens was performed with consideration to possible hydrogen bonds. Protonation states were as follows: GLU, ASP and LYS were charged. HIS was unspecified and determined by method. The assignment of charges was performed according to AMBER ff14SB force field (Maier et al. 2015). All docking experiments were performed using GRAMM-X Protein-Protein Docking Web Server v.1.2.0 (Tovchigrechko and Vakser 2006). Approximately 20 alternative predictions per docking were considered feasible load for screening by molecular mechanics computations.

Molecular Mechanics

Molecular Mechanics (MM) force field energy calculation was performed using GROMOS96 forece field, (Scott et al. 1999) *in vacuo*, in DeepView/Swiss-PDB Viewer v4.1.0 (Guex and Peitsch 1997). Results in text format were transferred to Microsoft Excel for analysis. Accordingly free energy of each amino acid residue was calculated by the equation:

$$E = \Delta G_{bond} + \Delta G_{angles} + \Delta G_{torsion} + \Delta G_{improper} + \Delta G_{nonBonded} + \Delta G_{electro}$$

Where ΔG_{bond} is the energy of covalent bonds, ΔG_{angles} is the energy of bond angles, $\Delta G_{torsion}$ is the energy resulting from torsion forces, $\Delta G_{improper}$ is the energy resulting from improper clashes, $\Delta G_{nonBonded}$ is non-covalent bond energy of van der Waals, and $\Delta G_{electro}$ is energy from electrostatic interaction (ionic bonds and Hydrogen bonds).

ΔΔG Energy of binding (E^{bind}) *in vacuo* was calculated according to the equation:

$E^{bind} = E^{R+P} - E^{R} - E^{P}$

Where $E^{R+P}$is ΔG of docking complex, E^{R} is ΔG of hNET receptor, and E^{P} is ΔG of peptide ligand.

Based on distribution of binding energy results (N=1080 docking models, range from -201.0 to $+4.4 \times 10^{19}$), models were classified in three categories: models with E^{bind}< 0 kJ/molwere considered favorable, models with low binding energy (0 <E^{bind}< 1000 kJ/mol) were considered unfavorable, whereas models with E^{bind}> 1000 kJ/mol were considered highly unfavorable.

RESULTS AND DISCUSSION

hNET Structure Model

The human hNET structure constructed by SWISS-MODEL, (Bordoli et al. 2009)was evaluated taking in consideration the x-ray-based drosophila DAT (PDB ID: 4M48), (Penmatsa et al. 2013)as control reference. Molprobity scores were 2.96 and 1.96 for hNET SwissModel and the x-ray based DAT model, respectively. All 8 Ramachandran outliers in hNET were in the poorly modeled extracellular domain (EL2) whereas no outliers were reported in DAT. Approximately eight rotamer outliers were reported in hNET, whereas in DAT ~12 rotamer outliers were reported. In hNET, the rotamer outliers were either hidden, intracellular or in the EL2 except for TRP80 and THR381 which were in channel. Another worth mentioning remark in the model is the bad clashes which were seven folds more frequent in the hNET model compared to DAT model. We have recently analyzed and conveyed guidelines for developing accurate model of hNET (Haddad et al. 2016). Future work will focus on employing such model in homing peptide design.

Molecular Docking and Molecular Mechanics

Peptides with predictable secondary structures (particularly alpha helix based) can be candidates for homing/targeting carriers of cargo due to their more rigid nature and seclusion as independent protein domains when compared to unpredictable linear peptides. Here, Twenty six α-helix and one β-sheet peptide structures were tested for their affinity to the human hNET transporter and drosophila DAT transporter. Peptide sequences were selected from hNET (Uniprot ID: P23975) based on their secondary structure, and then *de novo* models were constructed using PepFold. The affinity of each peptide to hNET and DAT transporters was investigated by performing 20 docking predictions via GRAMM-X, and evaluated using molecular mechanics GROMOS96 force field. The GRAMM-X server allows for a maximum of 300 dockings per trial and 10 trials per day, therefore this study was only limited by the computational challenge of molecular mechanics. Models were classified in three categories: models with negative binding energy (E^{bind}) were considered favorable, models with low binding energy (0 <E^{bind}< 1000 kJ/mol) were considered unfavorable, whereas models with E^{bind}> 1000 kJ/mol were considered highly unfavorable. In total, 1080 resulted models were evaluated. The sequences and docking results of 27 peptides in hNET and DAT are shown in (Table 1). Upon docking, no observable change in binding energy was attributed to covalent binding free energy($\Delta\Delta G_{bond}$, $\Delta\Delta G_{angles}$, $\Delta\Delta G_{torsion}$, or $\Delta\Delta G_{improper}$). This can be explained by the rigid nature of GRAMMX docking that requires no direct change in covalent bonds. Binding energy attributed to van der Waals ($\Delta\Delta G_{nonBonded}$) as well aselectrostatic ($\Delta\Delta G_{electro}$) interactions contributed variably.

As anticipated, peptide size was detrimental for docking in channel space of hNET and DAT (Figure 1a). Peptides composed of less than 13 amino acids showed very high frequency of docking in channels of hNET and DAT, while overall charge of peptide (pI) did not show any correlation (Figure 1b).Therefore,molecular weight is one of the major considerations for the cargo that can be linked to such homing peptides. Peptide's initial non-bonded energy affected docking while overall peptide free energy and initial electrostatic energy did not (Figure 1c-e). Two helices showed favorable docking in channel of hNET; namely, GASNGINAYL and SLWERLAYGI with binding energy of -106.2 kJ/mol and -128.6 kJ/mol, respectively (Figure 1g-f). For GASNGINAYL, the effect of van der Waals interaction ($\Delta\Delta G_{nonBonded}$ = -179.9 kJ/mol) compensated for electrostatic repulsion ($\Delta\Delta G_{electro}$ = +67.3 kJ/mol). The ΔΔG contributed by the peptide were -50.5 kJ/mol suggesting that the electrostatic repulsion was higher from amino acid residues of hNET than residues of peptide.In the case of SLWERLAYGI both nonbonded and electrostatic interaction contributed to binding ($\Delta\Delta G_{nonBonded}$ = -90.2 kJ/mol and $\Delta\Delta G_{electro}$ = -43.3 kJ/mol). The ΔΔG contributed by the peptide residues was-62.1

kJ/mol. GASNGINAYL peptide was also docked in channel of DAT in two models (E^{bind} = -139.8 and -88.0 kJ/mol) whereas three models of SLWERLAYGI showed docking in channel of DAT (E^{bind} = -192.3, -58.5 and -12.8 kJ/mol). Five other peptides showed energy favorable dockings in channel of DAT: IDAATQIFFSL (E^{bind} = -201.0 and -152.8 kJ/mol), TFWAVVFFVMLLALG (E^{bind} = -52.8 kJ/mol), TFSTFLLALFC (E^{bind} = -96.4 kJ/mol), and IYVLTLLDT (E^{bind} = -72.9 kJ/mol). These findings are confronted with two main limitations: The accuracy of structural models and the docking in vacuum space. Water solvent and possibly other salt ions are reported to play major role in receptor-ligand interactions. Exploration of docking in the presence of water can now be done using HADDOCK approach (van Zundert et al. 2016). In this study, the two main determinants in binding, namely van der Waals and electrostatic interactions are sensitive to both temperature and ionic strength (particularly influenced by pH), respectively. Temperature and ionic strength are factors that should be considered in differences between hypothetical and experimental binding parameters.

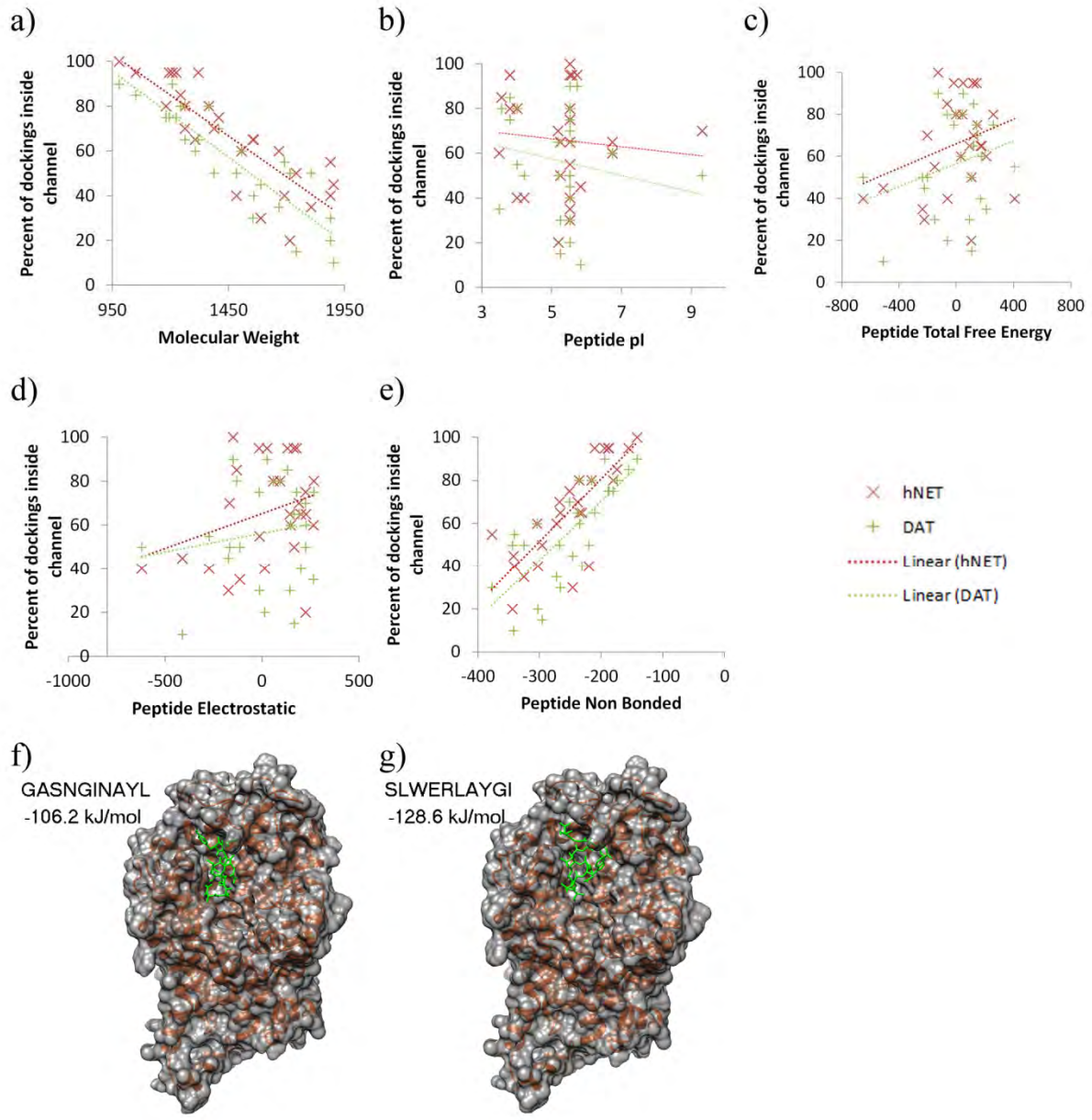
Table 1 Peptide models and summary of docking in channel entrance of hNET and DAT.

No.	Peptide	2°	Amino Acids	MWt	pI	Dockings in channel N=20		Favorable dockings in channel	
						hNET	DAT	hNET	DAT
1	IDFLLSVVGFA	α	11	1180	3.80	16	15		
2	MPLFYMELALGQYN	α	14	1690	4.00	8	11		
3	GVGYAVILIALYVG	α	14	1408	5.52	15	14		
4	YAVILIALYVG	α	11	1194	5.52	19	15		
5	NVIIAWSLYYLFS	α	13	1589	5.52	6	9		
6	IAWSLYYLFS	α	10	1262	5.52	16	16		
7	WQLLLCLMVVVIVLY	α	15	1805	5.52	7	10		
8	YFVLFVLLVHGVT	α	13	1507	6.74	12	12		
9	YFVLFVLLVHG	α	11	1307	6.74	13	12		
10	GASNGINAYL	α	10	979	5.52	20	18	1	2
11	IDAATQIFFSL	α	11	1225	3.80	19	15		2
12	YRDALLTSSINCITSFV	α	17	1903	5.83	9	2		
13	VSGFAIFSILGYMAHE	α	16	1742	5.24	10	3		
14	GFAIFSILGYMAHE	α	14	1556	5.24	13	6		
15	INCITSFVSGFAIFSILG	α	18	1889	5.52	8	4		
16	ITSFVSGFAIFSILG	α	15	1559	5.52	13	8		
17	TFWAVVFFVMLLALG	α	15	1714	5.19	4	10		1
18	LDSSMGGMEAVITGLAD	α	17	1667	3.49	12	7		
19	TFSTFLLALFC	α	11	1262	5.18	14	13		1
20	IYVLTLLDT	α	9	1050	3.80	19	17		1
21	GTSILFAVLMEAI	α	13	1365	4.00	16	16		
22	VDRFSNDIQQMM	α	12	1484	4.21	8	10		
23	YWRLCWKFVS	α	10	1388	9.31	14	10		
24	AFLLFVVVVSII	α	12	1320	5.57	19	13		
25	PLTYDDYIFP	β	10	1243	3.56	17	16		
26	WANWVGWGIALSSMVLV	α	17	1889	5.52	11	6		
27	SLWERLAYGI	α	10	1207	5.72	19	18	1	3

CONCLUSION

It is possible to design homing peptides using approach of molecular docking and evaluation using molecular mechanics. Here, two α-helices showed favorable docking in channel of hNET; namely, GASNGINAYL and SLWERLAYGI. It is important to develop accurate homology model of hNET, to confirm these findings prior to *in vitro* and *in vivo* analysis.

Figure 1 Molecular docking inside channel of neurotransmitter transporters



(a) Role of peptide size. (b) Role of peptide charge/isoelectric point. (c) Role of peptide total initial free energy. (d) Role of peptide initial electrostatic free energy. (e) Role of peptide initial non bonded free energy. (f) Docking of GASNGINAYL at channel of hNET. (g) Docking of SLWERLAYGI peptide at channel of hNET. Peptides are shown in green. hNET is shown in orange ribbon and transparent grey surface.

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UTILIZATION OF SELENIUM NANOPARTICLES WITH SCHIFF BASE CHITOSAN AS ANTIBACTERIAL AGENTS

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Abstract: Bacterial infections and the increasing resistance of bacteria to antibiotics are included among the major global health problems. Therefore selenium nanoparticles in complexes with chitosan and selenium nanoparticles with Schiff base chitosan were synthesized and tested as potential antibacterial agents. Selenium nanoparticles with chitosan showed the average particle size of 29.4 nm with zeta potential of -44.3 mV. The standardized disc diffusion method has been used for susceptibility testing of selenium nanoparticles with chitosan and derivates on *Staphylococcus aureus, Escherichia coli* and methicillin-resistant *Staphylococcus aureus* (MRSA). Chitosan selenium nanoparticles show inhibitory effect on gram-negative bacteria *Escherichia coli* only, whereas chitosan Schiff bases inhibit growth of all bacterial strains tested. The use of selenium nanoparticles in combination with chitosan Schiff bases appears to be a good way for the reduction of bacterial infection.

Key Words: selenium nanoparticles, chitosan, nosocomial infections, antimicrobial activity, MRSA

INTRODUCTION

Nosocomial infections are undesirable complication of health care undertaken in hospitals (Valaperta et al. 2010). They extend the period of patient treatment, cause an increase in morbidity and mortality and finally cost increases for medical care (Chudobova et al. 2014). Gram-positive *Staphylococcus aureus* (*S. aureus*) and gram-negative *Escherichia coli* (*E. coli*) are major and basic bacterial pathogens (Tong et al. 2015). With rapid use of antibiotics to treat infectious diseases the inhibitory effect is decreased and the effect is also manifested by bacterial resistance to antibiotics (Carvalho and Santos 2016). From this reason it is necessary to look for new antibacterial agents for inhibition of bacterial growth (Kopel et al. 2015, Nawas et al. 2016).

Nanoparticles are small molecules with diameters from 1 nm to 100 nm (Snoddy and Jayasuriya 2016). Synthesis of non-toxic and highly pure nanomaterials allows the new directions for their use as antimicrobial agents against pathogenic bacteria (Chudobova et al. 2013, Chaudhary et al. 2016).

Nowadays, polymers are increasingly used as suitable drug carriers. They are used for slow release of the active ingredient, to increase solubility and for targeted delivery (Kumar 2000). Chitosan has excellent biological properties; it is non-toxic, biocompatible and biodegradable. Due to its perfect properties, is used in fields including biomedicine, agrochemistry and cosmetics (Anitha et al. 2014). Only chitosan own antibacterial properties against gram-positive (*S. aureus*), but also gram-negative bacteria (*E. coli*). The exact mechanism is not fully elucidated. Prerequisite is to change the permeability of cell membranes, causing escape of intracellular content, which leads to cell lysis. Another mechanism can be penetration of chitosan into the cells and subsequently binding to the DNA and partial inhibition of RNA and protein synthesis (Zheng and Zhu 2003).

MATERIAL AND METHODS

Chemicals

All the chemicals were supplied by Sigma-Aldrich (St. Louis, MO, USA) in ACS purity unless noted otherwise. The deionized water was prepared by using reverse osmosis equipment Aqual (Aqual s.r.o., Brno, Czech Republic). The water was further purified by using Milli-Q Direct QUV equipped with the UV lamp. The pH was measured by using pH meter WTW inoLab (Weilheim, Germany).

Synthesis of SeNPs with chitosan and chitosan Schiff bases

Selenium nanoparticles were prepared according to published method (Chudobova et al. 2014, Cihalova et al. 2015). Briefly, to sodium selenite solution was added 3-mercaptopropionic acid and pH was adjusted to 7 by addition of NaOH. The concentration of selenium was 160 μg/mL.

Chitosan solution was prepared by dissolving chitosan (5 g) in 500 mL of water and addition of acetic acid (5 mL).

C-ACP

The Schiff base was prepared by mixing 2-acetylpyridine (0.59 mL) with chitosan solution (50 mL), heating at 80 °C for 1 h and neutralization with NaOH.

C-PA

It was prepared similarly to C-ACP, only 2-pyridinecarboxaldehyde (0.59 mL) was used instead of ACP.

C-SA

The same method as above, only salicylaldehyde (0.66 mL) was used.

C-Se, C-ACP-Se, C-PA-Se, C-SA-Se

The solutions were prepared by mixing of 5 mL of chitosan (Schiff bases) solutions with 5 mL of selenium solution. The final concentration of selenium is 80 μg/mL and the solutions were used for treatment of bacteria.

Nanoparticles characterization

The SeNPs with chitosan and chitosan Schiff bases were characterized using measurement of particle sizes and zeta potentials by Dynamic Light Scattering (DLS) (NANO-ZS, Malvern Instruments Ltd., Worcestershire, U.K.). The parameters of the measurement were as follows: temperature 25 °C, absorption coefficient 10^{-3} and equilibration time 120 s. In each case, the measurement duration depended on the number of runs, which varied between 20 and 40.

Cultivation of *S. aureus*, methicillin-resistant *S. aureus* (MRSA) and *E. coli*

S. aureus (NCTC 8511), MRSA (ST239) and *E. coli* (NCTC 13216) were obtained from the Czech Collection of Microorganisms, Faculty of Science, Masaryk University in Brno, Czech Republic. The bacterial strains were cultivated in Luria Bertani (LB) into 50 mL Erlenmeyer flasks for 24 h on a shaker at 130 rpm and 37 °C. Antibiotic oxacillin (3μg/mL) was added in to the MRSA for cultivation.

Testing of antibacterial activity

The antimicrobial effect of selenium nanoparticles in complexes with chitosan and chitosan Schiff bases was determined using the measurement of the inhibition zones. Agar surface in Petri dish was covered with a mixture of 100 μL of 24 h bacterial cultures in the exponential phase of growth, and 3 μL of LB medium. Excess volume of the mixture on the Petri dishes was aspirated. Discs (Ø 1 cm) were mixed with SeNPs complexes in 80 μg/mL concentration in Eppendorf tubes. Soaked discs were then laid on a Petri dish. Petri dishes were insulated against possible external contamination and placed in a thermostat (Tuttnauer 2450EL, Israel) set at 37 °C for 24 h. After 24 h of incubation, the inhibition zones were measured and photographed in each Petri dish (Richtera et al. 2015).

RESULTS AND DISCUSSION

Figure 1 Representative samples of SeNPs with chitosan and its derivates

Legend: A) C-Se; B) C-ACP-Se; C) C-PA-Se; D) C-SA-Se

Size and zeta potential of SeNPs

The stability behavior of the selenium nanoparticles were determined by using size distribution and ζ-potential measurements (Figure 2). It was found that chitosan modified selenium nanoparticles have an average size of 29.4 nm and zeta potential was detected at -44.3 mV. The obtained results confirm that selenium nanoparticles modified with chitosan are highly stable. The results on selenium nanoparticles with chitosan Schiff bases are given in Table 1. These results demonstrated that the complexes are less stable and they probably aggregate. The high aggregations are observed in the case of all selenium nanoparticles with chitosan Schiff bases. The modification of selenium nanoparticles with chitosan Schiff bases caused the change of charge from negative to positive which allows better interaction with negatively charged bacterial membrane (Qi et al. 2004).

Figure 2 A) Size of C-Se. B) Zeta potential distribution of C-Se.

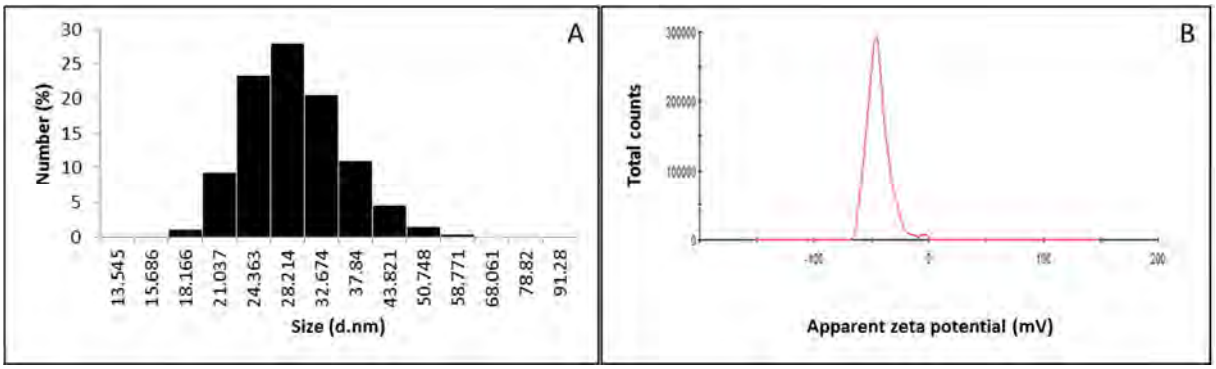


Table 1 Characterization of selenium nanoparticles and their complexes with chitosan and its derivates

Sample	Size (nm)	Zeta potential (mV)
C-Se	29.4 ± 3.5	-44.3 ± 2.4
C-ACP-Se	586.9 ± 116	13.4 ± 1.4
C-PA-Se	144.5 ± 40.6	8.48 ± 0.2
C-SA-Se	178.1 ± 41.9	15.8 ± 0.9

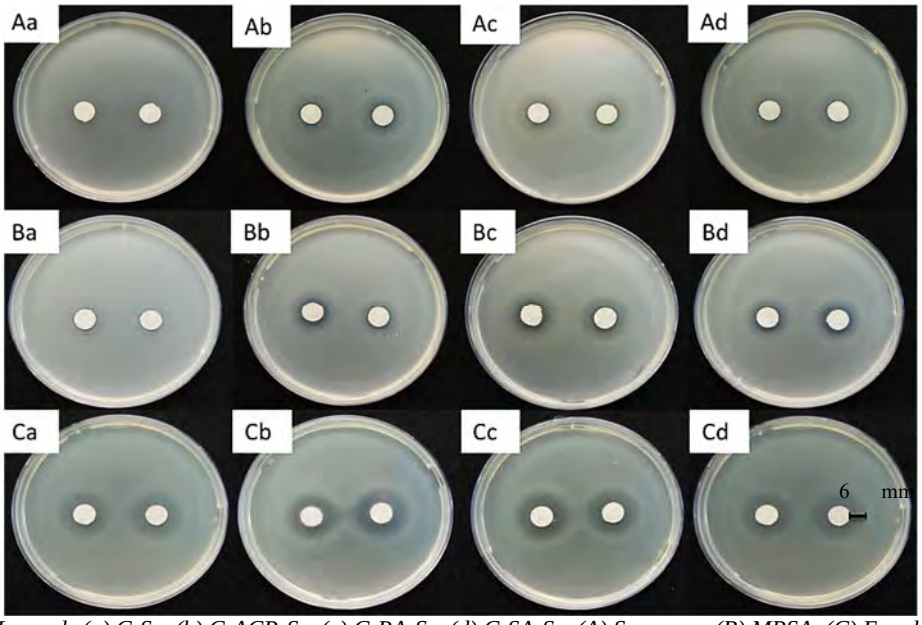
Testing of antibacterial activity

Antibacterial activity of selenium nanoparticles with chitosan and selenium nanoparticles with chitosan Schiff bases was determined using disc diffusion method and expressed in terms of the size of the inhibition zone (mm). SeNPs with chitosan and SeNPs with chitosan Schiff bases were applied on different bacterial strains: G^+ (*S. aureus*) and G^- (*E. coli*), bacterial strain resistant to antibiotics (MRSA). Effect of selenium nanoparticles with chitosan and selenium nanoparticles with chitosan Schiff bases on bacterial strains is shown in Table 2 and Figure 3. The highest inhibitory effect after 24 hours of incubation can be seen after the addition of C-PA-Se on *E. coli* (Figure 3).

Table 2 Measurement of inhibition zones (mm)

Sample / Bacteria	C-Se	C-ACP-Se	C-PA-Se	C-SA-Se
Staphylococcus aureus	0	5	4	3
MRSA	0	3	3	3
Escherichia coli	6	6	7	6

Figure 3 Characterization of resistance of bacterial strains by using standardized disc diffusion method



Legend: (a) C-Se, (b) C-ACP-Se, (c) C-PA-Se, (d) C-SA-Se, (A) S. aureus, (B) MRSA, (C) E. coli

CONCLUSION

The SeNPs with chitosan and SeNPs with chitosan Schiff bases were synthesized. Almost all compounds show good antibacterial activity. The best inhibitory effect showed on *Escherichia coli* (G^- bacteria) after application of all selenium nanoparticles with chitosan and selenium nanoparticles with chitosan Schiff bases. These SeNPs with chitosan and SeNPs modified with chitosan Schiff bases appear to be a good kind for treatment against G^- bacterial strains.

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USING CHROMIUM MODIFIED CARBON PASTE ELECTRODE FOR HEAVY METAL IONS DETERMINATION

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Abstract: This paper describes the preparation and electrochemical application of chromium (III) oxide modified carbon paste electrode for simple and high sensitive simultaneous determination of zinc, cadmium, lead and copper ions. Square wave anodic stripping voltammetry which is very sensitive for metal ions was selected as electrochemical detection method. This method was optimized with respect to deposition time, deposition potential, frequency and amplitude. The detection limits were found to be 30.3 µg/L for Zn^{2+}, 3 µg/L for Cd^{2+}, 24.2 µg/L and 3 µg/L for Pb^{2+} and Cu^{2+} respectively. The potential for simultaneous detection of these heavy metal ions by the chromium modified carbon paste electrode was also demonstrated. Using this modified carbon paste is also possible to detect mercury ions, detection limit was found to be 18.2 µg/L.

Key Words: carbon paste electrode, heavy metals, chromium, voltammetry

INTRODUCTION

Contamination of heavy metal ions is one of the most serious environmental problems. Environmental pollution is biggest in point source areas such as foundries, mining and smelters (Gouda and Al Ghannam 2016, Ramnani et al. 2016, Tchounwou et al. 2012). But other sources of contamination also come from agriculture, pharmaceutical and cosmetic industries. Some of the metals are naturally in the human organism (March et al. 2015, Tchounwou et al. 2012). However, even their slightly elevated concentrations are in most cases toxic and carcinogenetic. Heavy metal ions are not biodegradable and tend to accumulate in living organisms, which leads to chronic diseases and disorders of the organism. Therefore, it is important to trace actual concentration of heavy metal ions in all parts of the environment (Bailey et al. 1999, El Tall et al. 2007, Nouacer et al. 2015, Yantasee et al. 2004, Zhu et al. 2014). For soil, drinking water, ground water and waste water, there are regulations that determined the limit concentration of pollutants (including heavy metal ions). Recommended values of the pollution (selected metals) of wastewater discharged into public sewers in Czech Republic are mentioned in Table 1.

Table 1 Recommended values of the pollution of wastewater discharged into public sewers (Groda et al. 2007)

Metal	Recommended maximum [mg/L]
Cadmium	0.2
Copper	0.5
Lead	0.1
Mercury	0.005
Zinc	2.0

For the detection of heavy metal ions absorption or emission spectroscopy and electrochemical methods belong among the most commonly used quantitative methods. Mainly electrochemical methods are one of the most used techniques for environmental pollutants determination (Pujol et al. 2014, Sánchez et al. 2012). Their advantages are high sensitivity, easy operation and transferability. Nowadays, many efforts have been made to develop sensors for online monitoring of heavy metal ions *in situ* (Nejdl et al. 2015). In such cases, relatively inexpensive printed sensors and small, portable potentiostats are ordinarily used. In recent years considerable growth of screen printed electrode sensitivity is caused by designing and using of various modifications. Like an intermediate element, between laboratory measurements and *in situ* measurements on printed sensors, carbon paste electrodes are often used. The carbon paste electrodes have a wide range of benefits - they are easy to prepare and use, and can be easily modified (March et al. 2015, Švancara et al. 2009, Tchounwou et al. 2012). Results of this paper are based on our previous research (Richtera et al. 2016).

MATERIAL AND METHODS

Chemicals

All used chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). High purity deionized water (Milli-Q Millipore 18.2 MΩ/cm, Bedford, MA, USA) was used throughout the study. Chromium (III) oxide was prepared from the thermal reaction of ammonium dichromate, according to following formula:

$$(NH_4)_2Cr_2O_7 \rightarrow Cr_2O_3 + N_2 + 4\ H_2O$$

Chromium (III) oxide was washed 7 times, to prevent trace contamination from heavy metals ions.

Electrode preparation

To prepare the modified carbon paste, 100 mg expanded graphite and 25 mg chromium (III) oxide were mixed with 300 µL paraffin oil in a mortar with a pestle for 25 minutes. This mixture was thereafter transferred with spatula into the electrode body. The body was made from teflon and its inner diameter was 2.5 mm.

Electrochemical determination of ions

Electrochemical detection of Zn, Cd, Pb and Cu ions was carried out using three electrode system connected with 663 VA Stand (Metrohm, Herisau, Switzerland). Ag/AgCl/3 M KCl electrode was used as a reference electrode and platinum as an auxiliary electrode. As working electrode chromium modified carbon paste electrode was used. Prior to each measurement approximately 0.1 mm of paste from carbon paste electrode was wiped on a filter paper for new surface. Software NOVA 1.8 (Metrohm, Herisau, Switzerland) was employed for data evaluation. Square wave anodic stripping voltammetry (SWASV) was performed in the presence of 2 M acetate buffer, pH 5. The parameters of the measurement were as follows: initial potential of −1.3 V, end potential +1.3 V, deposition potential −1.3 V, deposition time 300 s, voltage step 5 mV, pulse amplitude 150 mV, frequency 150 Hz and equilibration time 5 s, volume of measurement cell 4 mL. The dosage was 3.7 mL samples and 300 µL of buffer.

RESULTS AND DISCUSSION

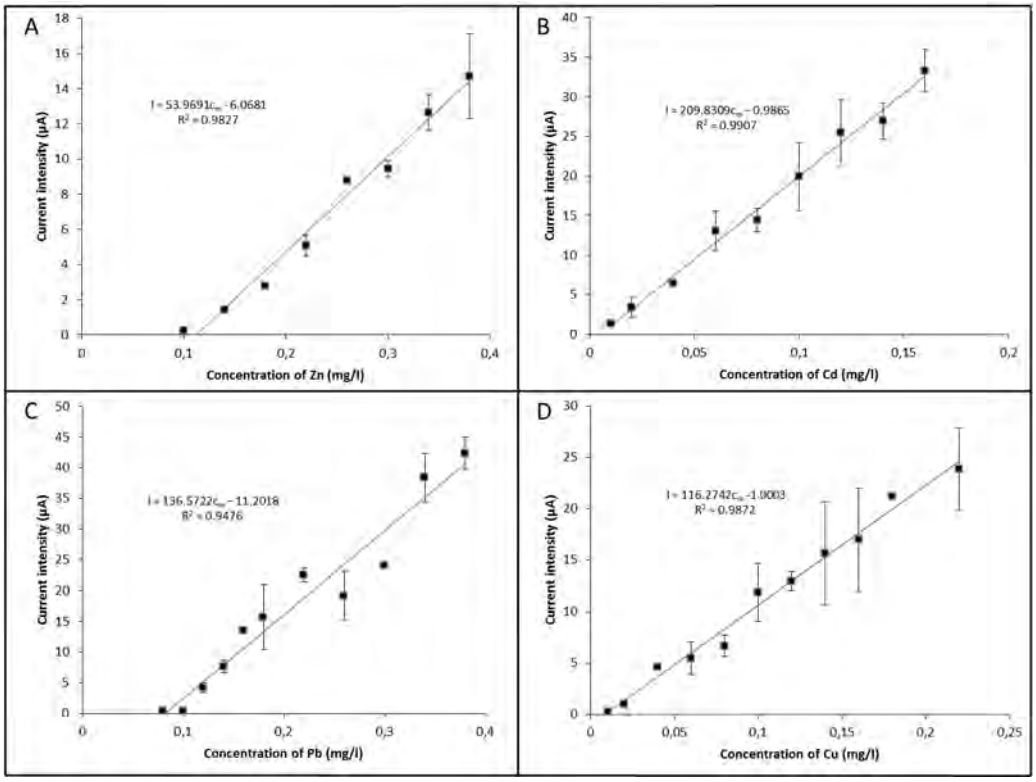
Electrochemical detection of zinc, cadmium, lead and copper ions

Carbon based materials bind to each other substances (such as heavy metal ions), because these materials are used to prepare the paste electrodes. Their big advantage is that the carbon paste is non-toxic, environmentally friendly, and as the material has a large potential for many applications. In recent years, carbon pastes are classified as intermediate element between fixed carbon electrodes and printable carbon ink-based sensors (March et al. 2015, Švancara et al. 2009). Nowadays modified carbon pastes are used, because they frequently increase the detection limit of the substances studied (Švancara et al. 2009).

In this study a modification in the form of chromium (III) oxide was used. It was confirmed that this modification increases the sensitivity towards zinc, cadmium, lead and copper ions. First, calibration curves were measured under optimal parameters for a given modification.

The graph in figure 1A represent the calibration curve for zinc ions, error bars were calculated from the standard deviations of the measurements. The dependence of current on concentration of Zn^{2+} ions demonstrate very good linearity in a range 100–80 µg/L (R^2 = 0.9827), with a limit of detection of 30.3 µg/L. Graph 1B shows the calibration line of cadmium. The calibration plot show an excellent linearity in a range 10–160 µg/L (R^2 = 0.9907), with low detection limit of 3 µg/L Cd^{2+}. Detection limit of lead was found to be 24.2 µg/L with good linearity in a range 80–380 µg/L Pb^{2+} (R^2 = 0.9476). The graph in figure 1D shows very good linearity in a range of 10–220 µg/L Cu^{2+} (R^2 = 0.9872), the limit of detection was found to be 3 µg/L of copper ions.

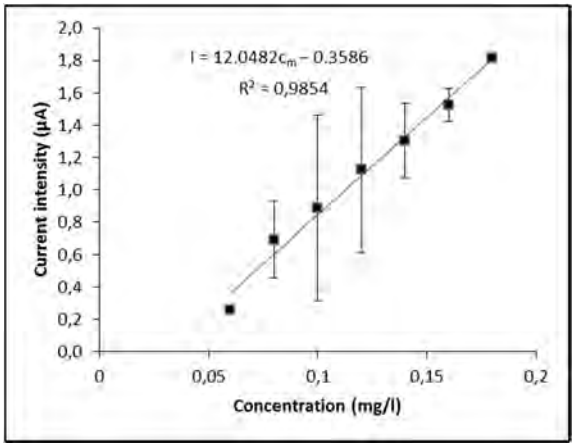
Figure 1 Calibration lines obtained using SWASV in acetate buffer pH 5. (A) Calibration line of zinc ions. (B) Calibration line of cadmium ions. (C) Calibration line of lead ions. (A) Calibration line of copper ions.



Electrochemical detection of mercury ions

Mercury is one of the most toxic metals. Its concentration should be in the waste waters very low. Therefore it is important to detect the metal in the lowest possible concentrations. The limit of detection of Hg^{2+} was determined to be 18.2 µg/L. The calibration plot was linear in the narrow range 60–180 µg/L . This modification is not suitable for the measurement of mercury. Mercury can be detected, but quantification is difficult due to big measurement error. Further optimization will be examined.

Figure 2 Calibration line of mercury using SWASV in acetate buffer pH.

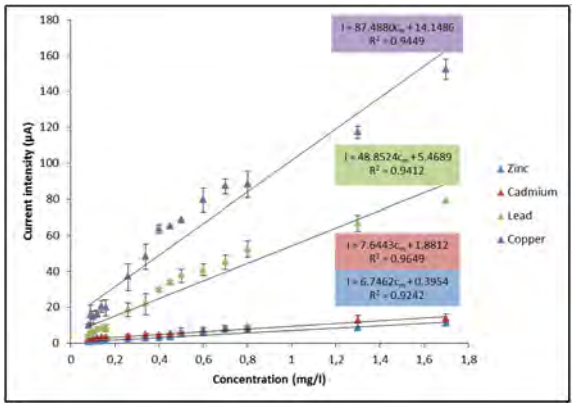


Simultaneous electrochemical detection of zinc, cadmium, lead and copper ions

Final step of our study was simultaneous measurement of metal ions, this procedure was supposed to identify possible interference between metal ions. Figure 2 shows the calibration curve of simultaneous metal detection. The results indicate that the metals are really interacting with each other. For example zinc is affected in the mixture such that its limit of detection is increasing (24 µg/L). Sensitivity to copper and lead increased too (for lead almost 20 x).

It is therefore clear that if we are together in mixture zinc, lead and copper, we are able to use this modified paste get much better detection limits. This study presents only basic insight into possible interferences. Interference with other ions will be examined.

Figure 3 Calibration curves recorded simultaneous detection of zinc, cadmium, lead and copper in acetate buffer pH 5.



CONCLUSION

The unique simultaneous analysis of zinc, cadmium, lead and copper ions has been successfully performed using chromium modified carbon paste electrode. This electrode modification showed good stability and high sensitivity. We are able to simultaneously detect selected heavy metals at very low concentrations. The preparation of the electrode is easy, fast and reproducible. Therefore, in the future, we want to convert this promising electrode modification to the screen printed technology which allow miniaturization and next development of specific detection systems. These systems then will be used for measurement in real conditions.

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PEPTIDE MODIFIED CARBON NANOTUBES FOR DRUG DELIVERY

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Abstract: Multi-walled-carbon nanotubes (MWCNTs) are widely explored as carriers for drug delivery, due to their facile transport through cellular membranes, and are reportedly found to be effective against cancer. In the present study, we have designed a MWCNTs nanocarrier for doxorubicin delivery to prostate cancer tissue. The cell penetrating peptide (SMSIALR) modified MWCNTs provide better penetration of nanocarrier into cancer cells. Using fluorescence measurement, the doxorubicin (DOXO) binding efficiency was estimated to 100%. MALDI analysis confirms the presence of doxorubicin and peptide on the MWCNTs structure. The stability behavior of the colloid suspension indicates the positive charge of carbon nanocarrier and due to this fact, the cancer cells membranes carry negative charge, the MWCNTs is established as suitable drug delivery system for doxorubicin.

Key Words: Multiwall carbon nanotubes, Cell penetrating peptides, Doxorubicin, Drug delivery

INTRODUCTION

Research aims of many pharmaceutic studies are orientated to delivery of drugs to specific intracellular targets and reduction of the drugs toxicity. This can be achieved by using various transporters for drug delivery (Zugazagoitia et al. 2016). The carbon nanotubes (CNTs) present materials which have great membrane penetration qualities and high drug loading capacities as a drug container, providing excellent opportunity for use of this material in drug delivery (Goenka et al. 2014). One form of CNT can be imaginatively produced by rolling up many layers to form a concentric cylinder which is called multi-walled CNT (MWCNTs). The possibility of their modification by various molecules has been reported (Eatemadi et al. 2014). MWCNTs can be oxidized using strong acids as oxidising agent, resulting in introduction of carboxyl groups at the ends of tubes, which increases their possibility for covalent binding (e.g. through amide linkages, hydrazone bond, carbamate or esterification) (Sherigara et al. 2003, Esplandiu 2009). However, delivery of drugs to the specific target still remains as the main problem. Nowadays, using peptides for specific target delivery can present a promising tool in drug delivery. Peptides, based on their chemical and biological properties, can provide easy delivery of various cell-impermeable covalently or non-covalently conjugated cargos due to their abilities for intracellular delivery of a wide range of molecules (Wang et al. 2014). The ability of MWCNTs and peptide to interact with various molecules, offers the potential of using MWCNTs as vehicles for the delivery of small drug molecules such as doxorubicin, elipticine, etoposide and others, while peptide enables the delivery of vehicles to specific targets. Although these MWCNTs conjugates with peptides displayed no cytotoxicity in vitro, for further development, it will be important to assess their metabolism, bio-distribution and clearance from the body (Wang et al. 2014). The aim of our experiment was to design the nano-vehicle composed of doxorubicin loaded multi-walled carbon nanotubes (MWCNTs) modified by cell homing peptide (SMSIALR) for treatment of prostate cancer. We characterized doxorubicin loaded MWCNTs by fluorescence analysis to determine the effectiveness of MWCNTs binding capacity for doxorubicin. Drug releasing from MWCNTs was observed under the

conditions corresponding to conditions in extracellular and intracellular environment. Finally the effect of conjugate was determined after treatment of androgen-sensitive human prostate adenocarcinoma cells (LNCaP).

MATERIAL AND METHODS

Chemicals

Chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, USA) in ACS purity unless noted otherwise. The deionised water was prepared using reverse osmosis equipment Aqual 25 (Czech Republic). The deionised water was further purified using apparatus MilliQ Direct QUV equipped with the UV lamp. The pH was measured using pH meter WTW inoLab (Weilheim, Germany).

Peptide synthesis

For synthesis of R9C peptide, Liberty Blue peptide synthesizer was used (CEM, Matthews, NC, USA). The sequence and monoisotopic molecular weight of synthesized peptide was as follows: SMSIALR and 776.9 g/L. Deblock of Fmoc protecting group was performed with 20% piperidine v/v in N,N-dimethylformamide (DMF). Coupling was achieved using N,N,N′,N′-tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU), N,N-diisopropylethylamine (DIEA) and DMF. Cleavage of side chain protecting groups was performed by treating the peptides resin with 95% trifluoroacetic acid (TFA) v/v, 2.5% H_2O v/v and 2.5% triisopropylsilane (TIPS) v/v for 30 min at 38 °C under microwave irradiation.

Oxidation of MWCNTs

1 mg of MWCNTs was taken in an Eppendorf tube and 1 mL concentrated HNO_3 was added to it for oxidation. Then it was heated using a Thermo-mixer (Eppendorf, Hamburg, Germany) for 20 min at 80 °C, 800 rpm. Subsequently it was sonicated using an ultrasonic bath (Bandelin, Berlin, Germany) for 15 min and centrifuged at 25000 rpm at 20 °C for 10 min using a table top centrifuge machine (Eppendorf, Hamburg, Germany). The supernatant was discarded and the product was washed 6–7 times by centrifugation (25000 rpm at 20 °C for 10 min) with MilliQ water until the pH became 7. Finally the volume was made up to 1 mL using MilliQ water.

Modification of MWCNTs

The stock solution of peptide (SMSIALR) was prepared in concentration of 1 mg/mL. Doxorubicin concentration was set at clinical trial doses of 54.3 μg/mL (Misset et al. 1999). After centrifugation and water removal from oxidized MWCNTs into Eppendorf tube was added 1 mL of Doxorubicin (54.3 μg/mL) solution which is mixed overnight at room temperature. Then prepared conjugate was washed three times with MilliQ water in order to remove unbound Doxorubicin. 1 mL of (1 mg/mL) peptide solution (SMSIALR) was added to it and mixed overnight in room temperature. Two control solutions were also prepared using the same procedure. All the samples and controls were washed three times by centrifugation (25000 rpm at 20 °C for 10 min) with MilliQ water to remove unbound chemicals.

Fluorescence measurement

Fluorescence spectra were acquired by a multifunctional microplate reader Tecan Infinite 200 PRO (TECAN, Switzerland). Excitation wavelength for doxorubicin was found at 480 nm. The fluorescence scan of doxorubicin was measured within the range 400–850 nm per 2-nm steps. The detector gain was set to 100%. The sample (100 μL) was placed in transparent 96 well microplates with flat bottom by Nunc (Thermo Scientific, USA). All measurements were performed at 30 °C controlled by the Tecan Infinite 200 PRO (TECAN, Switzerland).

Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS)

The mass spectrometry experiments were performed on a MALDI-TOF mass spectrometer Bruker ultrafleXtreme (Bruker Daltonik GmbH, Germany). The matrix used in the MALDI method was 2,5-dihydroxybenzoic acid (DHB) and/or α-cyano-4-hydroxycinnamic acid (HCCA). The saturated matrix solution was prepared in 30% acetonitrile and 0.1% TFA. All measurements were performed in reflectron positive mode in the m/z range 0–4 kDa. The mass spectra were typically acquired by

averaging 2000 sub spectra from a total of 2000 laser shots per spot. Laser power was set 5–10% above the threshold.

Electrochemical characterization of complexes

Electrochemical measurements were performed with the AUTOLAB Analyzer (EcoChemie, Netherlands) connected to VA-Stand 663 (Metrohm, Switzerland), equipped with a standard electrochemical cell with three electrode setup, as working electrode hanging mercury electrode was employed. Precursors and their conjugates were analyzed by differential pulse voltammetry (DPV). Acetate buffer (0.2 M CH_3COONa + CH_3COOH, pH 5) was used as background electrolyte.

Determination of complex cytotoxicity - MTT assay

The suspension of approximately 5000 cells (LNCaP) was added to each well of standard microtiter plates (E-plates 16). After addition of medium (200 µL), plates were incubated for 2 days at 37 °C to ensure cell growth. After that, medium was replaced by medium containing MWCNTs-DOXO-PEPTIDE complex (0–25 µg/mL), and medium without agents as a control. Plates were incubated for 24 h; then, medium was removed and replaced by a fresh one, three times a day. Further, medium was replaced by 200 µL of fresh medium containing 50 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT [5 mg/mL in PBS]) and incubated in a humidified atmosphere for 4 h at 37 °C, wrapped in aluminum foil. After the incubation, MTT-containing medium was replaced by 200 µL of 99.9% dimethyl sulfoxide to dissolve MTT-formazan crystals. Then, 25 µL of glycine buffer was added to all wells and absorbance was immediately determined at 570 nm (VersaMax microplate reader, Molecular Devices, Sunnyvale, CA, USA).

RESULTS AND DISCUSSION

Characterization of Complexes (MWCNTs-DOXO-PEPTIDE)

Different strategies have been develop, in order to load drug molecules onto CNTs through either covalent bonds or noncovalent adsorption. Mostly, molecules are bound to MWCNTs by physical adsorption thought aromatic ring surface of MWCNTs enabling hydrophobic interaction (including π–π stacking) between molecules and MWCNTs (Tsai et al. 2013).

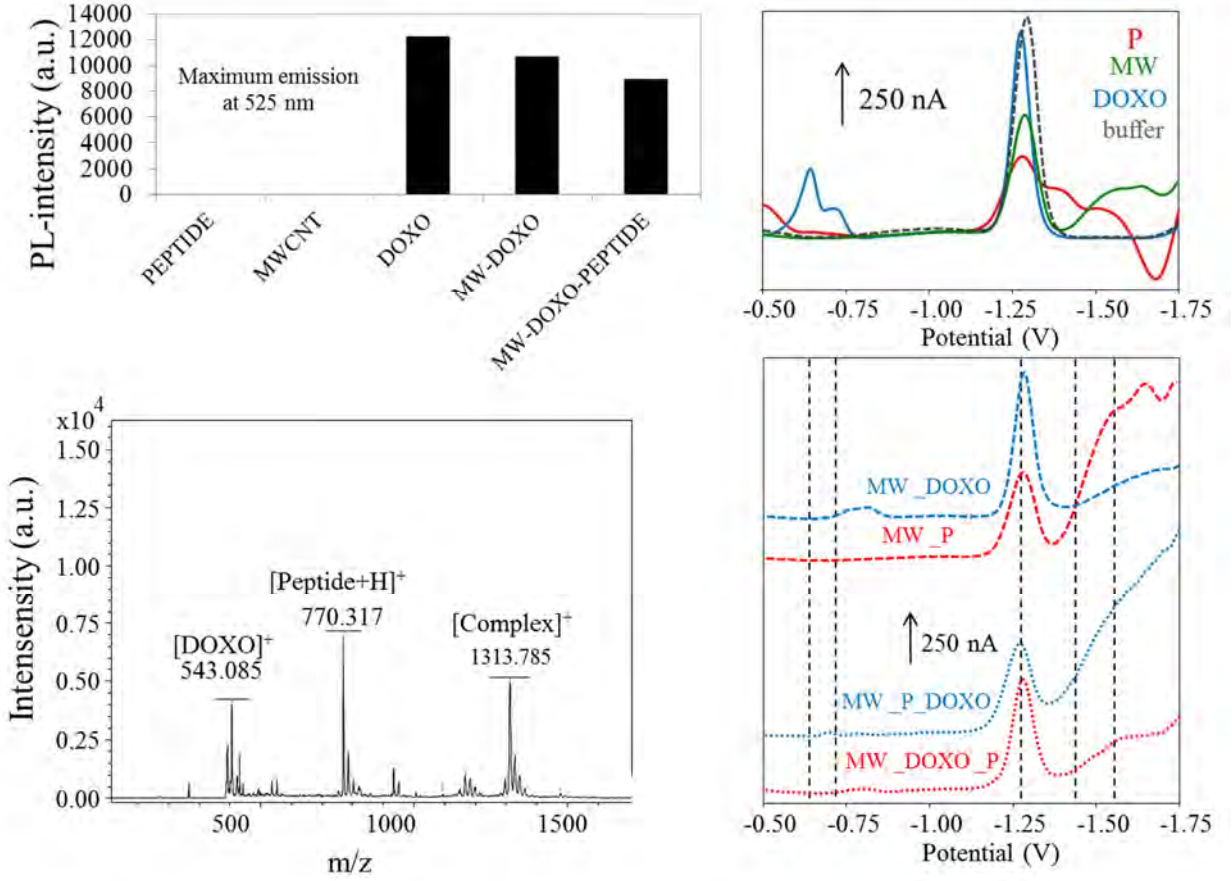
In this experiment surface activation of MWCNTs was conducted by carboxylation using concentrated nitric acid. This results in strong oxidation process, which leads to introduction of carboxyl groups at the ends of MWCNTs. Surface modification of MWCNTs with peptide and loading of doxorubicin was estimated by fluorescence study. The doxorubicin loading efficiency was calculated as 100%, after sample was washed three times and fluorescence intensity was measured in supernatant (data not shown). Fluorescence intensity was measured at excitation wavelength of 350 nm. From obtained results it is obvious that MWCNTs and peptide have no detected fluorescence at this excitation wavelength (Figure 1A). However, maximum emission fluorescence of doxorubicin was found at 525 nm and confirms its encapsulation into MWCNTs. Fluorescence intensity of doxorubicin was decreased after interaction with MWCNTs and peptide. This leads to conclusion that doxorubicin is not only loaded on MWCNTs surface but also some amount is encapsulated into MWCNTs-peptide structure (Wong et al. 2013).

Stability of the complexes and the presence of doxorubicin and peptide in complex was studied by MALDI-TOF-MS (Figure 1B). The peak obtained at *m/z* = 543 Da corresponds to the molecular weight of doxorubicin. In the spectrum, there is also peak at *m/z* = 770 Da which corresponds to peptide molecular weight. It is evident that suggested complex, MWCNTs-DOXO-PEPTIDE, was found at m/z = 1313 Da, showing lower intensity than peptide, while higher than doxorubicin. However, we cannot confirm which type of bonding has occurred in our complexes, but it is obvious that the complex is stable.

Further method that has been involved in study of MWCNTs complexes and their precursors was electrochemistry. Using DVP method, precursors were characterized first (Fig. 1C), followed by complexes. Binding interactions between precursors were confirmed by the presence of DOXO double peak (potential -0.68 ± 0.02 V and -0.73 ± 0.02 V) and by the potential shift and/or shape changes of this double peak in complexes with DOXO. On the other hand, complex MWCNTs-peptide was demonstrated by the absence of this double peak. The same was observed with a peak corresponding to

the peptide (potential -1.45 ± 0.05 V) and in the case of its complexes. In comparison to other complexes, no peak was observed in the complex MWCNTs-DOXO.

Figure 1 Characterization of MWCNTs-DOXO-PEPTIDE complexes



Legend: (A) Detection of fluorescence intensity at 525 nm maximum emission. (B) Mass spectrum of peptide, doxorubicin and MWCNTs-DOXO-PEPTIDE complexes detected by MALDI-TOF-MS. (C) Typical voltammograms of DOXO, MWCNTs and peptide and electrolyte (Brdicka buffer) measured by adsorptive transfer stripping technique (AdTS) coupled with DPV. (D) Comparison of typical volltammograms of three component complexes (MWCNTs-PEPTIDE-DOXO and MWCNTs-DOXO-PEPTIDE) with controls (PEPTIDE, MWCNTs, DOXO).

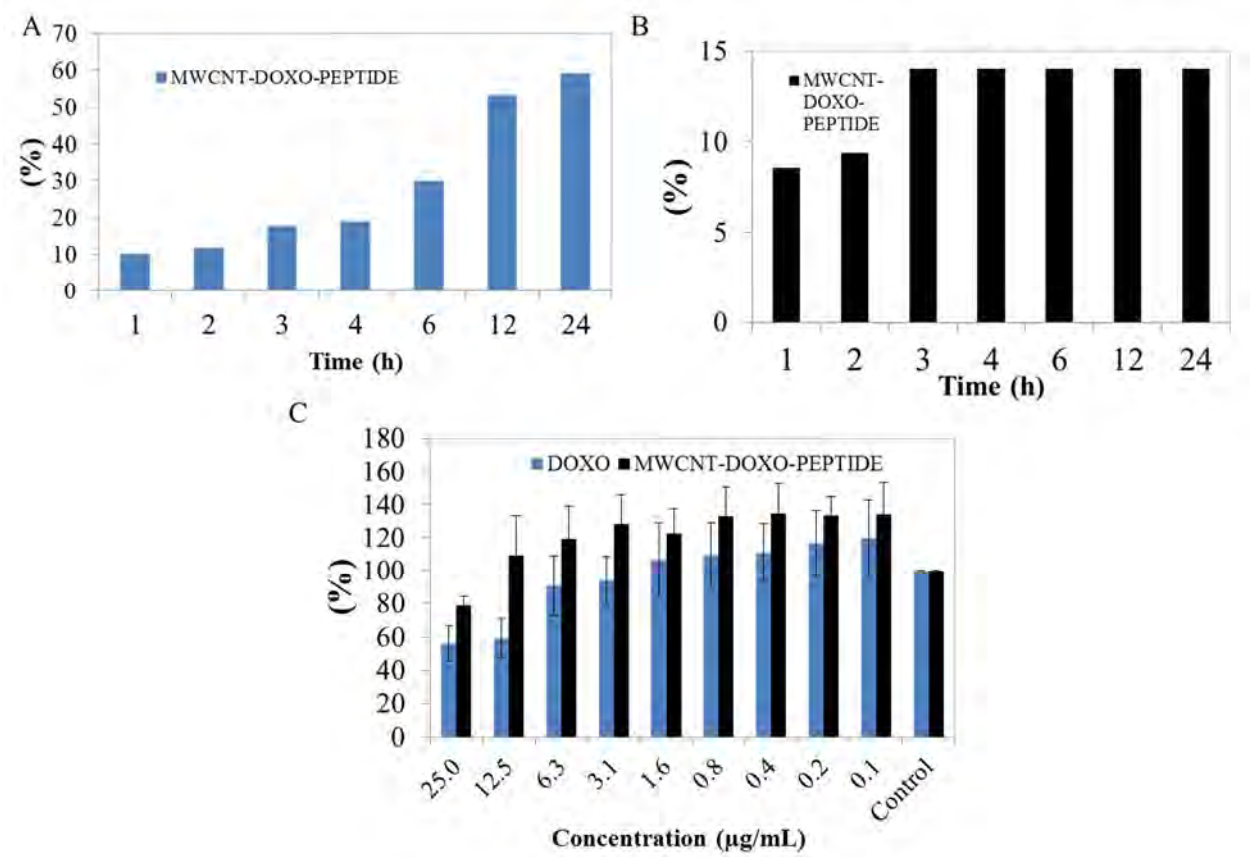
Cytotoxicity test and kinetics of drug release

The release of doxorubicin was evaluated in condition simulating environment in human plasma and intracellular fluid. Solution which simulates condition of intracellular fluid (Figure 2A) was based on the work of Corazzari et al. (Corazzari et al. 2013), and human plasma (Figure 2B) was prepared by the work of Williams et al. (Williams et al. 1999). It was found that doxorubicin release from complex in conditions simulating intracellular fluid was 60% in 24 hours. However, results obtained from human plasma environment conditions show 14% of drug release after 24 hours. These results can be explained by influence of different pH in applied solutions (human plasma pH 7.4, and intracellular fluid pH 4.6). It was reported (Heister et al. 2012), that doxorubicin release in acidic condition was more than 50% for 24 hours comparing with basic conditions where only 10% of doxorubicin is released. They suggest that doxorubicin was replaced by various proteins during the incubation of complexes.

We selected human prostate cancer cell line (LNCaP), commonly used in biomedical research to confirm toxicity effect of complexes. As shown in Figure 2C, doxorubicin induces high toxicity, reducing cells viability more than 50% at applied concentration of 25 μg/mL. However, after application of MWCNTs-DOXO-PEPTIDE complexes on cells, it was found that cell viability decreases only for 25% at applied concentration of 25 μg/mL. From obtained results it is clear that MWCNTs and peptide do not have any influence on cells viability. This leads to conclusion that lower toxicity of complex

comes from slow release of doxorubicin. This result is in correlation from results obtained in drug releasing experiment where in 24 hours only 60% of doxorubicin is released.

Figure 2 Kinetics of drug releasing and MTT assay of MWCNTs-DOXO-PEPTIDE complexes



Legend: (A) Kinetics of drug release from complexes in intracellular environment (A) and plasma (B) detected during 24 h at 37 °C. (C) Cells viability test after application of doxorubicin and complexes during 24 h at various concentrations.

CONCLUSION

Drug-delivery system based on peptide functionalized MWCNTs has been developed. In order to improve the delivery of doxorubicin to targeted position and its transfer through the cell membrane, the peptide SMSIALR was conjugated to MWCNTs surface. Accordingly, the MALDI technique was employed for observation of peptide and doxorubicin attachment to the MWCNTs structure. Complex formation was demonstrated by electrochemical characterization based primarily on potential shift and changes in shape of peak corresponding to the presence of doxorubicin and peptide in observed complexes. Doxorubicin is adhered to oxidized MWCNTSs through π–π stacking and electrostatic interactions which provide easy drug release in acidic conditions. MWCNTs modified by peptide show high potential in drug delivery due to the lack of toxicity and show great potential as carriers for drug-delivery systems.

ACKNOWLEDGEMENTS

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DRINKING WATER CONTAMINANTS ARISING FROM HOUSEHOLD WATER PIPES AND PIPEWORK MATERIALS

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Abstract: Water pipe materials can have significant effect on drinking water quality. Whereas heavy metals originate from metal pipes and couplings, new plastic polyethylene pipes can also leach different organic compounds that can effect olfactory and taste properties of water, and even be harmful to consumers. In this project the effect of crosslinked polyethylene (PEX) pipes on water quality was studied in laboratory and in residences with drinking water pipes made of different pipe materials. PEX pipes leached several organic volatile compounds, such as methyl and ethyl tert-butyl ethers (MTBE and ETBE) and tert-butyl alcohol (TBA) in the water in both laboratory tests and in the studied house. Water incubated in the pipes in laboratory was found to leach anti-androgenic and anti-retinoid X-like compounds. Water metal content was not affected by PEX pipes but rather the couplings and other network materials. Risks of leached compounds were low, and mostly affecting the taste and odour of water. Metal couplings and other metal network materials in household can potentially increase harmful metal exposure of residents.

Key Words: drinking water, water pipes, PEX, volatile compounds, MTBE, ETBE, TBA, heavy metals, anti-hormonal activity

INTRODUCTION

Clean drinking water is one of the most important factors in health worldwide. Contamination of drinking water by chemicals can occur at several stages of its journey from source to tap. Since water can reside in the distribution system for several days before reaching the user (Kekki et al. 2007), the materials in distribution system have a great impact on water chemical content.

Water pipe materials used in distribution system and households include metal pipes of steel and copper, but more and more commonly plastic pipes such as polyethylene (PE) and cross-linked polyethylene (PEX), or PVC. Plastic pipes can leach chemicals with harmful health effects and cause unwanted taste or odour into drinking water. PE and PEX pipes leach several additive compounds used in pipe manufacturing. These include, for example, oxygenate-compounds ethyl-tertbutyl ether (ETBE), methyl-tertbutyl ether (MTBE) and its degradation product tert-butyl alcohol (TBA); degradation products of antioxidants, such as 2,4-di-tert-butyl phenol (DTBP); and BTEX-solvents (Durand and Dietrich 2007; Kowalska et al. 2011, Lund et al. 2011, Skjevrak et al. 2003). ETBE, MTBE and TBA can cause unwanted taste and odour to the water (Kelley et al. 2014). So far 158 compounds are known to leach from PEX pipes into water (Whelton and Nguyen 2013). Many more chemicals can still remain unknown. Furthermore, mixture effect of the chemicals is not well understood. For this effects-directed cell-based assays can be a good solution (Escher et al. 2014).

Metals detected in water pipes can originate from the distribution system and household pipes, couplings and other components of the pipework (Gonzalez et al. 2013). As plastic pipe materials are currently replacing metal pipes in communal and private water distribution systems, it would be anticipated that levels of harmful heavy metals in drinking water will become lower, especially in countries with lead pipes. However, household metal couplings and tap materials can also have an effect to the concentration of metals in water.

MATERIAL AND METHODS

Water and pipe samples

Total 3 different PEX pipe samples (inner diameter 16 mm) were bought from local construction and renovation shops in Brno, Czech Republic. One PE pipe (inner diameter 26 mm) was received from local water pipe manufacturer. Pipes were flushed three times and filled with either de-ionized water or cold flowed tap water. Water was incubated in pipes for 24 h. Incubation was repeated 2-3 times. Water samples from residences were collected in Helsinki capital area, Finland, in five different locations (Table 5). Residents were asked not to flow the water for about 8-10 hours before sampling in order to get pipe-incubated water samples. Flowed water samples were collected after 2 min flowing. Water samples were also collected at the local drinking water treatment plant (DWTP).

Table 1 Overview of fungicide treatments

Sampling location	Building year	Pipe materials	Specific info
A	2005	Unknown/copper	
B	2015	PEX/copper	
C	1960	Galvanized steel/copper	Non-rehabilitaed
D	1950	Copper	Pipes rehabilitated in 2007
E	2014	PEX	

Analysis of volatile compounds

Samples were collected head space-free in 2 40 mL glass vials. Volatile organic compounds were analyzed in contracted accredited laboratory ALS Global in Brno, Czech Republic. The analytes were in total 71 and are listed in Certificate of Accreditation No. 819/2015 of 30/11/2015 using US EPA 8260 (GC-MS) method.

Analysis of metals

Inductively Coupled Plasma - Mass Spectrometry (Agilent 7700x ICP-MS, Agilent Technologies, Japan) was used for determination of metals (iron, copper, nickel, zinc, cadmium, and lead) in the 25 mL of acidified water samples.

Yeast-cell-based assays for anti-hormonal activity

Water samples (about 1 L) were extracted using solid phase extraction and method described in Rajasärkkä et al. (2016). 2 µL of DMSO extracts were tested as described in Rajasärkkä et al. (2016) using yeast-cell-based assays for estrogenic and androgenic (Leskinen et al. 2005) and retinoid-X-like (Kabiersch et al. 2013) activities.

RESULTS AND DISCUSSION

Volatile compounds in water samples

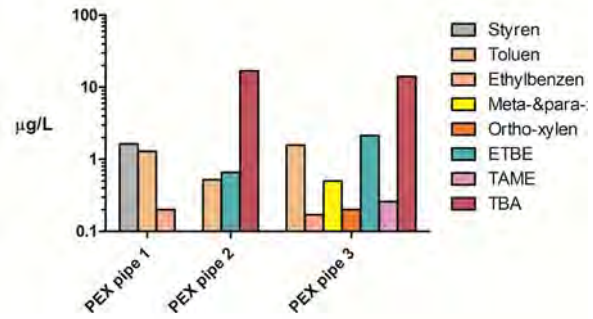
Several volatile compounds were detected in the PEX pipe samples (Figure 1 A) and B)). These included BTEX-compounds, such as styren, toluen, ethylbenzene and xylens, which are used as solvents in pipe manufacturing. Also several oxygenates such as methyl and ethyl tert-butyl ethers (MTBE and ETBE), tert-butyl alcohol (TBA), and tert-amyl methyl ether (TAME) were detected. Highest concentrations were those of TBA, which was 12–20 µg/L. Concentrations of other compounds were clearly lower. Even that of ETBE was at maximum 2.1 µg/L although in another study its concentration was higher than 23 µg/L (Durand and Dietrich 2007).

The PEX pipes had some differences. Pipe number 1 leached only low concentrations of BTEX-compounds while pipes 2 and 3 leached also oxygenate compounds (Figure 1 A) and B)). Pipe number 3 leached highest number of different compounds. It has been found out earlier that different PEX brand, and even different lots of a same brand can have great differences in leaching amounts of compounds (Whelton and Nguyen 2013).

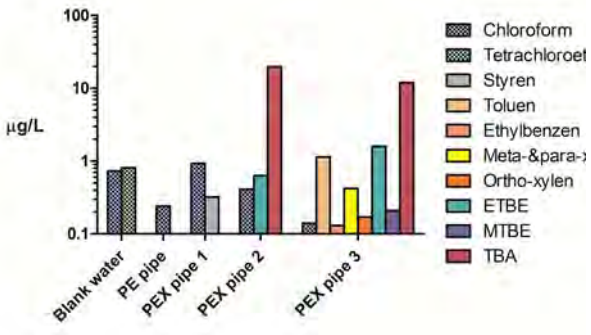
In case of PE pipe, no volatile compounds originating from the pipe itself could be detected. In addition, no significant differences were detected between compound concentrations in de-ionized (A)) and tap water (B)), except for chlorination by-products.

Figure 1 Volatile compounds detected in water incubated in sample pipes in laboratory using de-ionized (A) and tap water (B), and in recently built residence B with PEX pipes (C)

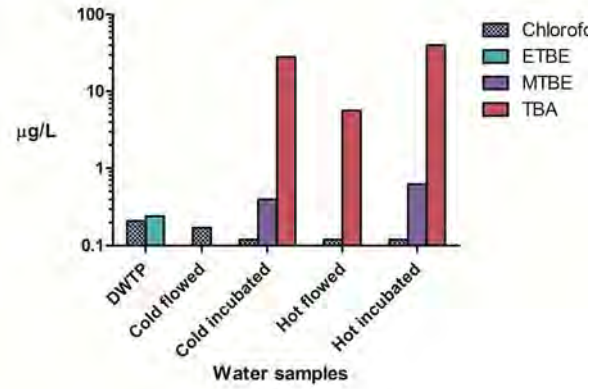
A) Sample pipes with de-ionized water



B) Sample pipes with tap water



C) Recently build residence B



In the studied residential buildings and DWTP, mainly only chlorination by-product chloroform was detected at an average level of 0.17 µg/L. Also ETBE was detected in DWTP sample at low concentration of 0.24 µg/L. Only on most recently built residence B several PEX-pipe-related volatile compounds were detected (Figure 1 C). Again TBA was the dominant detected leaching compound with concentrations of 5.6–40 µg/L in pipe-incubated and hot water samples. These concentrations are even higher than those in the laboratory study, which indicates that leaching of TBA can be long-term problem. Also MTBE concentrations were 2-3-fold higher in the incubated water samples in the residence compared to the laboratory incubation study. The resident was complaining that the water had had bad taste right after moving in to the apartment, but later had resolved.

Unfortunately antioxidant degradation products such as 2,4-di-tert-butyl phenol that have been previously documented to leach from PEX pipes (Skjevrak et al. 2003) could not be analysed by the methods used.

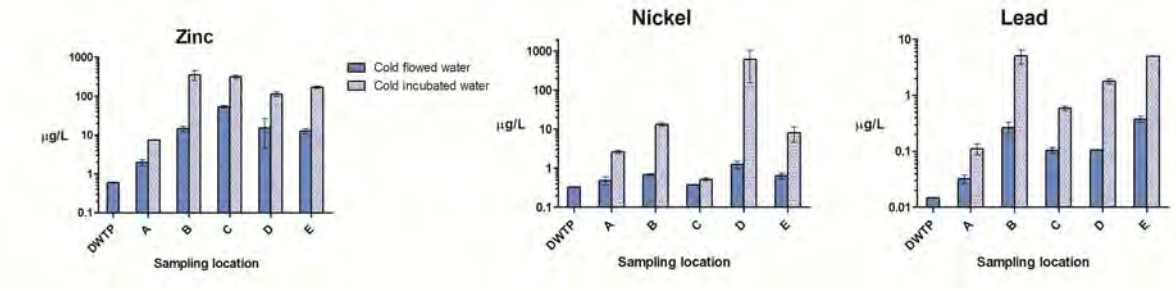
Metal results

In the pipe incubation studies it was noticed, that all metals detected in the water incubated in the pipes originated from the pipe caps and couplings, since the couplings alone leached similar levels of metals into the water when immersed in similar volume of water. The PEX pipe coupling leached nickel, copper, zinc and lead in maximal concentrations of 15.2; 7.4; 19.5; and 1.8 µg/L, respectively. The large brass cork used to seal the PE pipe leached nickel, copper, zinc, cadmium, and lead in maximal concentrations of 14.2; 102, 470, 0.3; and 66.5 µg/L, respectively. The concentration of lead exceeds the quality limit of The European Drinking Water Regulations (European Union 2014) of 10 µg/L. Thus,

although metal pipe replacement to plastic ones reduces the amount of metals, couplings can turn out to be a significant source of (heavy) metals in drinking water.

The effect of recent renovation or building to the metal concentrations was clearly seen in the sampled residences. Concentrations of nickel, zinc, and lead had the greatest variation between the sampled locations. Incubated water had generally higher concentrations of metals than flowed water (Figure 2). Iron was high only at the oldest non-renovate location C, in which galvanized steel pipes were used. These pipes leached high concentrations of iron and zinc (660 and 343 μg/L respectively). Surprisingly, the most recently built locations B and E and recently renovated location D had similar and even higher concentrations of zinc (Figure 2). The non-renovated location had lowest concentration of nickel, whereas the renovated location D had very high concentration of over 1 mg/L in the pipe-incubated water. Also lead concentrations were higher in the recently built or renovated locations than in the non-renovated location which could be anticipated to leach most lead due to old pipes (Figure 2). These results further indicate that metal couplings and other pipework materials used in renovation and reconstruction can be of poor quality and leach even more harmful metals than old non-renovated pipework.

Figure 2 Zinc, nickel and lead concentrations in tap water of residence buildings



Hormonal assay results

Effect-directed and qualitative analysis of water samples from laboratory pipe incubation studies and tap water samples from residential buildings were assayed with yeast-cell based luminescent bioreporters for hormonal and antihormonal activities. Yeast strains expressing human estrogen receptor, androgen receptor and retinoid X receptor were used. Yeast cells were exposed to DMSO extracts of water samples in either alone or in combination with approximately half-maximal effective concentration (EC50) of a reference hormone or compound.

Tap water samples did not show hormonal or antihormonal activity beyond the detection limits of any of the yeast assays. However, water samples incubated in laboratory in PEX pipes 2 and 3 did show both antiandrogenic and anti-retinoid-X-like activity, meaning they reduced the luminescent signal of EC50 concentration of the reference compound. PEX pipe 2 water sample (dilution factor 66) caused 77% inhibition in the EC50-level response of the androgenic yeast assay while PEX pipe 3 water sample (dilution factor 62) caused similar inhibitory effect of 74%. In retinoid-X-receptor the PEX pipe 2 water sample caused even higher inhibition of 85%, while those of PEX pipe 3 caused 66% inhibition. Thus, PEX pipes do leach chemicals that have antiandrogenic and anti-retinoid-X-like activity. Anti-androgenic chemicals have been suspected to be responsible for, for example, the reduction of male fertility, while retinoid-X-receptor-disrupting compounds can play role in the metabolic syndrome and obesity disorders (endocrine disrupting compounds reviewed by De Coster and van Larebeke (2012). The concentration of these compounds in the water studied water samples was low, and thus also exposure is low. However, since the identity of the compounds remains unknown, bioaccumulation and other properties of the compounds affecting their toxicity cannot be overruled, and true risk remains to be elucidated.

CONCLUSION

Plastic polyethylene pipes can leach several different compounds in tap water, potentially causing un-wanted taste and odour. PEX pipes in laboratory and residences studied in this research mainly

leached compounds such as TBA, ETBE, and MTBE, of which TBA was dominant compound. However, these compounds are not considered toxic and consequently do not pose a risk to residents.

Of laboratory-studied PEX pipes two were found to leach anti-androgenic and anti-retinoid-X-like compounds, but their concentrations were low. Furthermore, no (anti)hormonal activity was found in water samples taken directly from the residences. Thus, leaching of these compounds is probably limited to the beginning of use of the pipes.

Polyethylene pipes did also not leach metals into water, however, the couplings and caps were found to leach significant amounts of metals. Of the studied residences the recently renovated or constructed locations had relatively high concentrations of metals that were found to leach from the couplings. In this respect new pipework can even increase exposure to harmful metals such as lead.

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OPTIMIZATION OF THE PROCEDURE FOR A LIGNINOLYTIC ENZYMES ISOLATION FROM THE WHITE-ROT FUNGI

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Abstract: The white-rot fungi produce wide range of extracellular enzymes (especially ligninolytic enzymes) and they can degrade complicated and difficult degradable compounds. An immobilization of enzymes causes their stability, reusability and cheapness, so they could be effectively used in biotechnologies and other branches. In this work we focused on the optimization of isolation procedure namely precipitation the enzymes, which will be used for immobilization in the next step. 75% ammonium sulphate was used to salting the enzymes and experimentally set the optimum pH for two species of white-rot fungi – *Trametes versicolor* and *Fomes fomentarius*. Our results show that the optimum pH for precipitation by ammonium sulphate is different for used species. Consequently, pH optimum for *Trametes versicolor* was 6, for *Fomes fomentarius* was 8.

Key Words: white-rot fungi, *Trametes versicolor*, *Fomes fomentarius*, protein precipitation, pH

INTRODUCTION

The ligninolytic enzymes produced by the white-rot fungi – especially laccase, manganese peroxidase and lignin peroxidase belong to the compounds with the highest potential in green chemistry, biotechnologies and in various other applications. These enzymes are able to degrade dyes (Mielgo et al. 2003), polycyclic aromatic hydrocarbons (Eibes et al. 2006), estrogenic compounds (Suzuki et al. 2003) and other environmental pollutants, which are normally resistant to microbial degradation (Syed and Yadav 2012). Laccases from fungi create isoenzymes, which are oligomerized to form multimeric complexes (Claus 2004) and are sorted to the group of blue copper oxidases (d'Acunzo et al. 2002). Manganese peroxidase belongs due to their structure to the glycosylated heme proteins (multiple forms) (Hofrichter 2002) and lignin peroxidase to a heme glycoprotein (Mester et al. 2001, Tien and Kirk 1983).

In general, the enzymes belonging to macromolecules contain a chain of the amino acids linked together by the peptide bonds (Walsh 2002). The immobilization of enzymes increases their stability against thermal and chemical denaturation (Bornscheuer 2003) – against pH, temperature, inhibitors, denaturants and organic solvents (Sheldon, 2007). A lot of steps, which can be influence by many factors, are necessary to optimize in the way from the crude enzymes to the immobilization enzymes. A protein concentration and purification from a solution is one of the steps and it can be done with a precipitation.

The precipitation is carried out using the organic solvents, the organic polymers and other chemicals; an affinity precipitation; a selective denaturation (Scopes 1987); the neutral salts and an adjustment of solution pH (Walsh 2002). The most common type of protein´s precipitation is salt induced precipitation with ammonium sulphate (Scopes 1987), which is achieved by dehydration in the microenvironment of the protein molecule. A large number of water molecules in the protein solution are bound to a sulphate ion SO_4^{-2} and this causes a reducing the amount of water available to interact with the protein molecules (Rosenberg 2005) and increase protein-protein interactions, especially between hydrophobic patches on the surface of adjacent protein molecules (Walsh 2002).

Ammonium sulphate is also commonly used for precipitation and purification of the enzymes from white-rot fungi (Desa and Nityanand 2011) due to salting out effectiveness, high solubility and low price (Scopes 1987). It is important to set the right concentration of the ammonium sulphate, but it is

changeable for different sources: *Coriolus hirsutus* 90% of saturation (Koroljova-Skorobogat'ko et al. 1998), *Phlebia floridensis* 50–75% (Arora and Rampal 2002), fungus *Chaetomiaceae* 80% (Saito et al. 2003), *Loweporus lividus* 80% (Sahay et al. 2008). So it is very important to set the right conditions to precipitate the enzymes. Except the saturation setting optimum pH is also needed, due to protein´s solubility dependence on the pH of the solution. This pH mostly corresponds to the isoelectric point of the protein (Nakai and Modler 1996).

The aim of this study was determined the optimum pH for the precipitation of the enzymes from the white-rot fungi (the species *Trametes versicolor* and *Fomes fomentarius*) by 75% ammonium sulphate.

MATERIAL AND METHODS

Culture conditions

Two white-rot fungi *Trametes versicolor* and *Fomes fomentarius* were used. They were cultivated on the agar plates for 7days in the dark and at 20 °C using a Potato dextrose agar. After this time cultures were inoculated into Erlenmeyer flasks (250 ml) containing 90 ml of Potato dextrose broth and $CuSO_4$. The fungi were grown at 28 °C for 7 days with continuous shaking at 150 rpm. The experiment was performed in duplicate.

pH gradient

The content of the Erlenmeyer flasks were pureed trough a strainer and then centrifuged (4500 rpm/4 °C/15 min). The obtained supernatant was collected and divided into the beakers for pH gradient preparation with NaOH and HCl. Every beaker contained a fluid with different pH (3, 4, 5, 6, 7, 8, 9 and 10).

Precipitation of the enzymes

Ammonium sulphate was added to the crude culture supernatant into the beakers with different pH to 75% saturation at 4 °C with continuous stirring and left over for 90 minutes. After that, the precipitate was recovered by centrifugation (4500 rpm/4 °C/15 min). The precipitants were collected and dissolved in the buffer (pH 4.5) and centrifuged (10000 rpm/4 °C/5 min). In the supernatant a laccase activity and a protein concentration were assessed for a determination of the pH optimum for the precipitation procedure.

Laccase activity

The enzyme activity was determined spectrophotometrically using a UV/VIS Lambda 25 Spectrophotometer (Perkin-Elmer). Laccase activity was measured at 415 nm by detecting the oxidation of 2,2-azino-bis-[3-ethyltiazoline-6-sulfonate] (ABTS, Sigma Aldrich) at pH 4.5 in 0.1 M sodium acetate buffer (Bourbonnais and Paice 1990). One unit of enzyme activity was defined as 1 μmol of substrate oxidized per minute under the assay conditions. The enzyme activity assay was always performed in triplicate.

Protein concentration

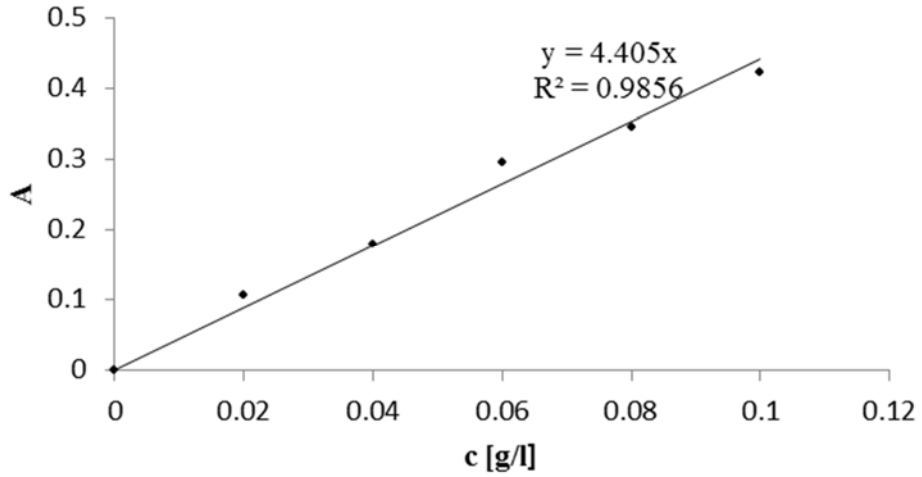
The protein concentration was determined using the Bradford method. A Bradford reagent (Coomassie G250, ethanol and H_3PO_4) was added to the sample and a degree of coloration was measured after 5 minutes incubation at 595 nm spectrophotometrically using a UV/VIS Lambda 25 Spectrophotometer (Perkin-Elmer). The protein concentration was always performed in triplicate.

RESULTS AND DISCUSSION

Calibration curve

First of all, protein assay standard curve was determined by Bradford method, as a standard bovine serum albumin in concentrations 0; 0.02; 0.04; 0.06; 0.08 and 0.1 g/l was used (Figure 1).

Figure 1 Protein assay calibration curve

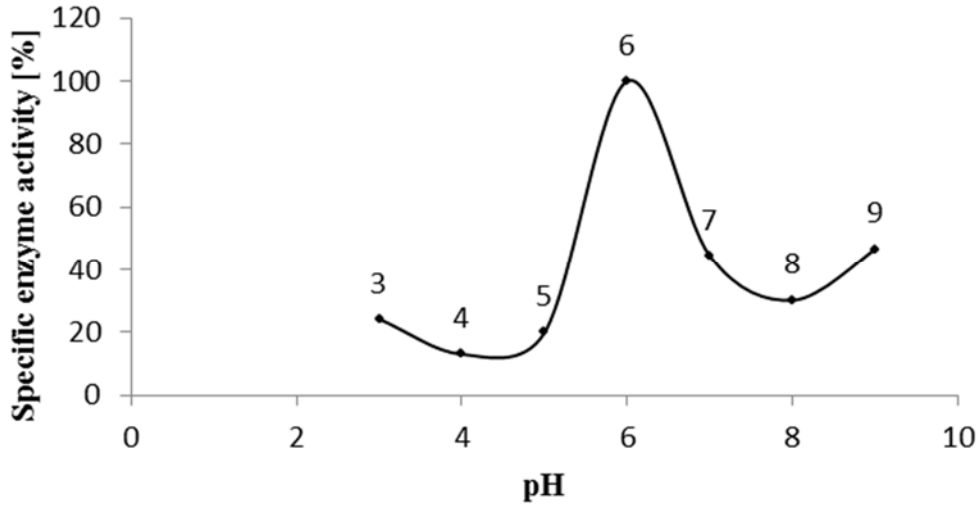


Specific enzyme activity

The specific enzymatic activity for each supernatant with different pH was calculated based the protein concentration [g/l] and enzymatic activity [U/l]. From the obtained results an optimal pH was determined for the precipitation of both white-rot fungi.

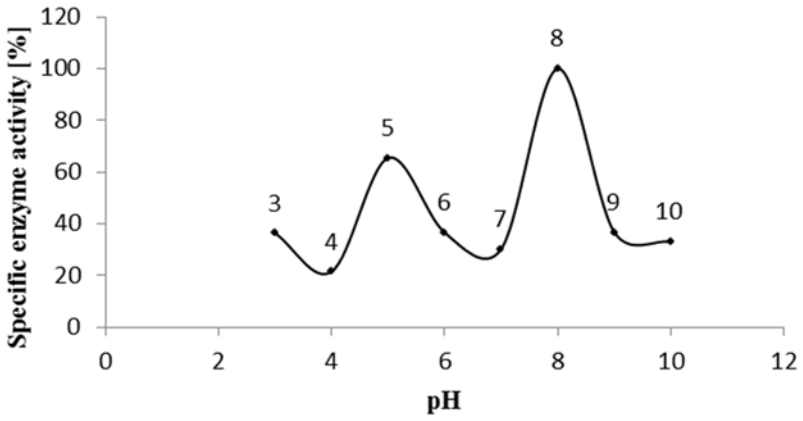
From the results of the specific enzymatic activity, which is related on mg protein in a solution, is evident that the best pH for precipitation of the enzymes from *Trametes versicolor* is pH 6. For a comparing of the results we used specific enzymatic activities expressed in percentages (Figure 2) (100% = the optimum pH for the precipitation).

Figure 2 Effects of different pH on the specific enzymatic activity for a fungus Trametes versicolor.



In the case of *Fomes fomentarius* the optimum pH for precipitation of the enzymes was pH 8. For an evaluation of the results we used specific enzymatic activities expressed in percentages (Figure 3) (100% = the optimum pH for the precipitation). Two local maxima of pH (5 and 8) can be probably connected with the possible formation of isoenzymes.

Figure 3 Effects of different pH on the specific enzymatic activity for a fungus Fomes fomentarius.



Precipitation is widely used for product recovery of biomolecules especially proteins. This process is usually induced by addition of a salt or an organic solvent or by changing the pH to alter the nature of the solution (Young 1994). A choice of a suitable precipitant and definition an optimum temperature and pH is necessary to obtain the best results. So in this study we focused on a variable pH value and our results prove that the pH optimum for the precipitation is unique for each different source. For *Trametes versicolor* the pH optimum for precipitation is documented about a value 4-7 (Matijosyte et al. 2010). The optimum pH for the precipitation was recorded for *Trametes versicolor* pH 6 and for *Fomes fomentarius* pH 8. Kumar et al. (2012) also showed that laccase is usually precipitated in the pH middle layer.

CONCLUSION

It is necessary to immobilize the enzymes due to their lack of reusability and stability beside the free enzymes. One of the most important steps during immobilization procedure is the optimization of concentration and purify of the enzymes. This step is commonly done with salting by using ammonium sulphate, but it is necessary to set the optimum conditions for the precipitation (optimum concentration of the precipitant, the optimum temperature and pH). In our study we focused on a setting the pH, which is the best for precipitation our two used species of the white-rot fungi – *Trametes versicolor* and *Fomes fomentarius*. Our results show, that for *Trametes versicolor* is the optimum pH value 6 and for *Fomes fomentarius* 8.

ACKNOWLEDGEMENTS

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EFFECT OF COPPER ON SECONDARY METABOLISM OF MICROALGAE SCENEDESMUS QUADRICAUDA

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Abstract: This study is focused on the effect of copper on the redox status and biosynthesis of antioxidants and simple phenolic compounds in microalgae *Scenedesmus quadricauda*. These compounds are constitutively expressed in higher plants. However, there is still lack of information about the activity of these compounds and their role within antioxidant system in the microalgae species. Our data indicate a strong effect of Cu^{2+} on the higher biosynthesis of simple phenolic acid (phenylpyruvic acid, phenylacetic acid 3,4 dihydroxyphenylacetic acid) and polyphenol (rosmarinic acid) in the microalgae *Scenedesmus quadricauda*. The similar effect we observed in biosynthesis of GSH and GSSG respectively. A decrease in the ratio GSH/GSSG was observed in the each concentrations of Cu^{2+} (1, 4 and 8 mg/l) already after 24 hours compared to the control sample. The biggest decrease in the ratio was observed at the concentration of 8 mg/l, which also negatively affected the viability and growth of microalgae cells.

Key Words: microalgae, heavy metals, simple phenolic compounds, glutathione, ascorbate

INTRODUCTION

Heavy metals are very toxic elements which are not biologically degraded like many organic pollutants. The presence of toxic metal ions in the environment poses a serious health threat to both animals and humans (Anastopoulos and Kyzas 2015). Heavy metals are naturally occurring elements. Natural resources include weathering process, volcanic eruptions, marine aerosols or forest fires, but most environmental contamination is caused by anthropogenic activities such as industries, domestic or agricultural activities. Although they are very toxic, some of them are also essential for both plants and algae (Nagajyoti et al. 2010).

Copper (Cu) is important for normal plant growth and development. Cu participates in numerous physiological processes, mainly in electron transport in photosynthesis, cell wall metabolism and mitochondrial respiration. (Yruela 2009). It is essential co-factor for several oxidative stress-related enzymes such as catalase, superoxide dismutase or peroxidase (Tchounwou et al. 2012). However it is also potentially toxic, when excess copper is present in cells (Li et al. 2006). It was found that copper has an inhibition effect on cell growth and photosynthesis in algae. Microalgae are unicellular photosynthetic, generally aquatic organisms, which have wide application. Recently, they are very popular as a biosorbents because of their sorption uptake and affinity to pollutants (Zhang et al. 2013). They can be used as feed, nutraceuticals, fertilizers, and as fuel source (Trentacoste et al. 2015). Many studies have focused on the uptake of heavy metals by microalgae, however the effects of heavy metals on the biosynthesis of simple phenolic compounds and their interplay in the antioxidant system within the microalgae kingdom are little known (Machu et al. 2015, Sasso et al. 2013).

Phenolic includes more than 8000 different compounds that can be divided up to 10 different groups depending on their structure and function. They are important natural antioxidants. It is presumed, that the concentration of phenolic in microalgae is on the low level compared to the terrestrial

plants. However, it was determined that simple phenolic compound such as phenolic acids and even some flavonoids can be found in several microalgae. Furthermore, there is a lack of information about interaction of these phenolic metabolites and their role within the antioxidant system in the green microalgae (Machu et al. 2015, Maruyama et al. 2014, Safafar et al. 2015, Sasso et al. 2012). This work is focused on the effect of copper on the content of glutathione and simple phenolic compounds and their interaction within antioxidant system in microalgae *Scenedesmus quadricauda*.

MATERIAL AND METHODS

Biological material

Green microalgae *Scenedesmus quadricauda* (Turp.) Breb. (Chlorophyta, Chlorophyceae) tribe UTEX 76 was obtained from the University of Texas, Austin.

Microalgae cultivation

Scenedesmus quadricauda was cultivated in BBM (Bolt Basal/Bristol Medium) (Nichols and Bold, 1965); 21 ± 1 °C, and illuminated at 2800 lux light intensity with a light/dark cycle of 12:12 h for a few weeks until a considerable percentage increase in biomass was obtained. Than microalgae were inoculated into the 200 ml glass test tubes containing various concentrations of $CuCl_2 .2H_2O$ (0, 1, 4 and 8 mg/l) in BBM. The cell number was determined in a Bürker chamber under light microscopy.

Determination of heavy metals content in algae

Atomic absorption spectrometer Agilent 200 Series AA Systems was used for this assay. Briefly, the samples were dried for 24 h at 80 °C and mineralized by using 300 µl 65% HNO_3 and 200 µl 30% H_2O_2 and 10 mg of dried samples in a microwave extractor Multiwave 3000 (Anton Paar, Austria). Then the samples were diluted to final volume of 5 mL and measured in air-acetylene flame (FA AAS).

Analysis of antioxidant compounds

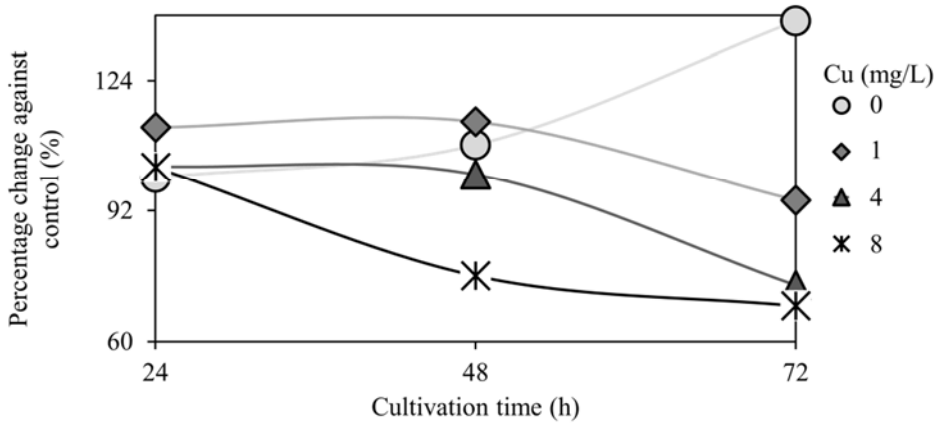
Glutathione (GSH/GSSG), ascorbate and simple phenolic compounds were determined by LC-MS/MS Agilent 1200 Series Rapid Resolution LC system coupled on-line to a detector Agilent 6460 Triple quadrupole with Agilent Jet Stream Technologies (Santa Clara, California, United States). Samples were extracted by homogenization in 500 µl of 0.1M HCl.

RESULTS AND DISCUSSION

Effect of heavy metals on the growth curve of microalgae

The effect of copper ions on the growth of *S. quardicauda* (UTEX-76) microalgae cells is shown in Figure 1. The cultures were cultivated with three concentrations of copper chloride solution (1, 4 and 8 mg/l). The increase in biomass was observed only in the control sample during the whole experiment (Figure 1, (line with circle symbols)). The accrual of biomass was also determined in the samples cultivated 24 h with 1, 4 and 8 mg/l Cu^{2+} compared to the control samples (line with square, triangle and star symbols respectively). However, after 48 h of cultivation a decrease was determined in the all of the treated samples except sample 8 mg/l where the biomass rapidly decreased (about 25%) already after 24 hours. The most growth-inhibiting effect (about 32% of decrease) on the cell was observed in the concentration 4 and 8 mg/l, at the time 72 h. This corresponded with the low viability of the cells (results not shown). Similarly, inhibition of the cell growth and consequent decrease in biomass of *Chlorella pyrenoidosa* and *Scenedesmus obliquus* after application of Cu^{2+} at concentration higher than 1 mg/l were observed in another study by Zhou et al. 2012. The copper treatment caused a significant concentration-dependent decrease of algal culture density even in the copper-tolerant microalgae but with lower effect (Backor et al. 2007, Magdaleno et al. 2014).

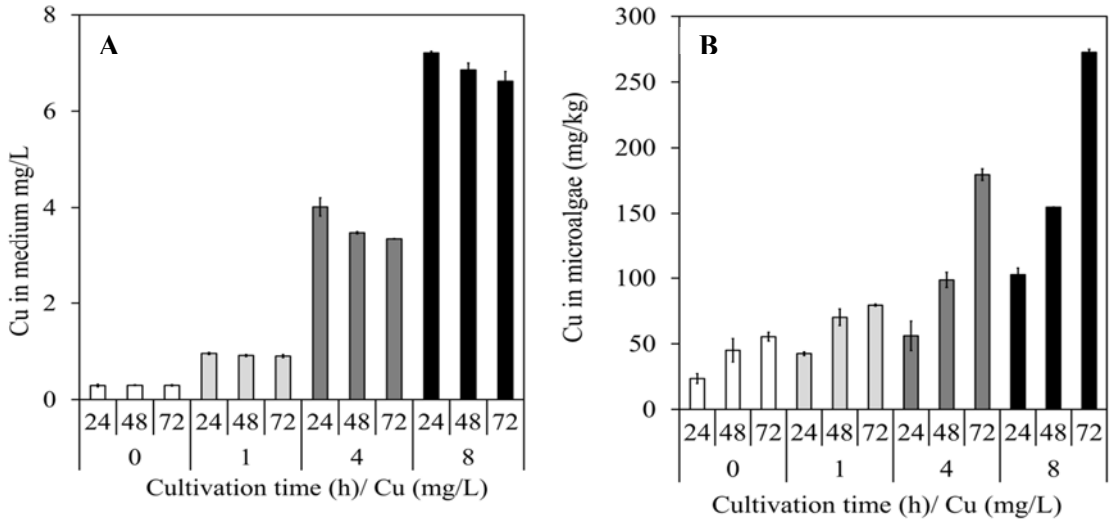
Figure 1 The percentage change of the Scenedesmus quadricauda biomass in the presence 0, 1, 4 and 8 mg/l $CuCl_2.2H_2O$.



Copper content in algae and culture medium

In the study by Zhou et al. 2012 the microalgae *Scenedesmus obliquus* was used to remove of the copper ($CuSO_4 \cdot 5H_2O$) from the cultivation media. The efficiency was increased gradually during the whole experimental period. After 8 days of culturing the microalgae *S. obliquus* removed 75.9–91.4% of copper with initial concentration of 0.2–2 mg/l. Our experiment was performed in the time range from 0 to 72 hours. The copper was detected using AAS technique every 24 hours in the dry and mineralized samples. The amount of copper in the medium declined with elevated time (Figure 2A). On the contrary the concentration of the copper in the microalga *S. quadricauda* has continually increased (Figure 2B). At the concentration of 1 mg/l the bioaccumulation of copper increased from 42.5 mg/kg after 24 hours to 79.4 mg/kg after 72 hours (Figure 2B, glaucous column). The result obtained at the concentration of 4 mg/l of copper shows (Figure 2B, dark grey column), when the increase was higher from 56.1 mg/kg after 24 hours to 180 mg/kg after 72 hours. The sharpest increase in the bioaccumulation of the copper was obtained at concentration of 8 mg/l from 103 mg/kg after 24 hours to 273 mg/kg after 72 hours. The results show that the copper was continuously accepted by microalgae *S. quadricauda* in the tested conditions.

Figure 2 The concentration of copper in A) medium and B) microalgae Scenedesmus quadricauda depending on the cultivation time 0, 24, 48 and 72 hours



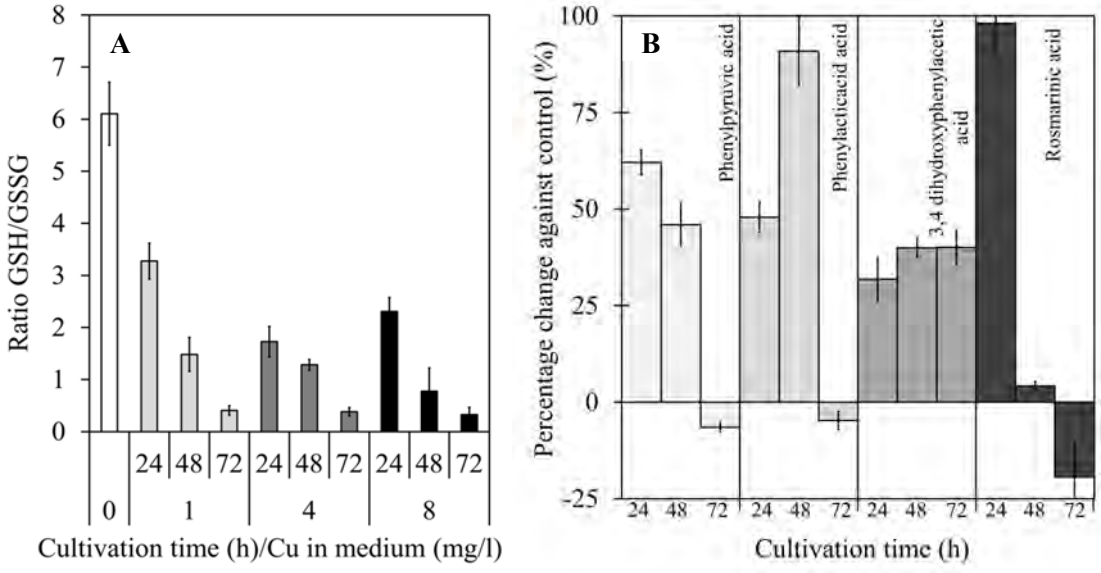
Reduced and oxidized glutathione

To maximize the potential application of the microalgae to bioremediation of heavy metals, it is necessary to understand how they defend against them and which mechanisms are involved during the bioaccumulation process (Zhang et al. 2013, Zhou et al. 2012). In this work, we have performed a quantitative analysis of the oxidized (GSSG) and reduced (GSH) intracellular glutathione. The ratio between GSH and GSSG content is considered as an indicator of antioxidant status that indicates stress rate in the organism. In the absence of stress, ratios can be at least 20:1 in the plant tissue. However, the ratio can be diverse according to the specific both subcellular compartment and species (Nagajyoti et al. 2010, Noctor et al. 2012, Singh et al. 2016). The lower ratio indicates the greater disruption of the cell metabolism. Figure 3A relates the redox state after metal treatment of the microalgae *S. quadricauda*. A decrease in the ratio of GSH/GSSG was observed in the each concentration of Cu^{2+} ions already after 24 hours compared to the control samples. The biggest decrease in the ratio has been observed at the concentration of 8 mg/l except concentration of 4 mg/l and time 24 hours Figure 3A. The increase of the amount of GSSG can be compensated by elevated biosynthesis of GSH or by reduction of GSSG using glutathione reductase enzyme (Noctor et al. 2012). In the *Trebouxia erici* Ahmadjian (UTEX 911, wild-type) that also belongs to the unicellular green algae and commonly occur as symbionts in lichens was treated with 10 μM $CuSO_4$ for 24 hours a significant decrease of reduced glutathione (GSH) was investigated that suggesting strong oxidative stress caused by Cu^{2+} (Backor et al. 2007).

Biosynthesis of simple phenolic compounds

In this work we show the presence of some phenolic acid such as phenylpyruvic acid, phenylacetic acid 3,4 dihydroxyphenylacetic acid and polyphenol rosmarinic acid in the microalgae *Scenedesmus quadricauda* and their reaction on the stress induced by Cu^{2+} ions. As our data indicate the amount of all above-mentioned compounds increased after 24 hours in the treatment pattern with 8 mg/l Cu^{2+} ions, it was even more than 95% in the case of rosmarinic acid (Figure 3B). It is interesting to note that the concentrations of rosmarinic acid (after 24h) markedly decreased and stabilize on the level corresponding to the control samples. The high content after 24 hours was found in the phenylacetic acid (90%) in compared to the control conditions. Interestingly, we have not investigated any rapidly decrease amount of these phenolic acids during the whole experiment.

Figure 3 A) The ratio of reduced (GSH) and oxidized (GSSG) glutathione depending on cultivation time B) Percentage changes of phenolic acid depend on cultivation time 24, 48 and 72 hours and concentration of Cu^{2+} 8 mg/l.



CONCLUSION

Microalgae belong to the model organisms that are used in a wide variety fields like ecology and ecotoxicology, biogeochemistry, systematics and evolution, cell biology. They could have answered some questions related with the biosynthesis of phenolic compounds in microalgae and higher plants. Our data indicate that presence of simple phenolic acid (phenylpyruvic; phenylacetic; 3,4 dihydroxyphenylacetic acid) and polyphenol (rosmarinic acid) in the microalgae *Scenedesmus quadricauda*. The concentrations of these compounds were changed by Cu^{2+} ions which indicate their important role in the antioxidant system. Owing to the technical progress in the instrumental methods we can expect that the analyses of these molecules become more precise and sensitive in the future. This could lead to the prediction of another phenolic compounds involved in the secondary metabolism of the microalgae.

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PROS AND CONS OF PLANT NUCLEAR PROTEIN ENRICHMENT

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Abstract: Nuclear proteome contains important regulatory proteins. To improve the detection of these proteins, Percoll gradient-based fractionation techniques have been developed and optimized. However, owing to the ever increasing sensitivity of identification methods based on liquid chromatography and mass spectrometry, the time and material consuming fractionation methods may no longer be necessary. Here, we show that a Percoll-based nuclear protein fractionation of tomato leaf proteome increased the number of detected proteins, but at least some nuclear proteins were lost or depleted in the process.

Key Words: nuclear proteome, LC-MS, proteome fractionation

INTRODUCTION

The nucleus is a complex and heterogeneous organelle that is composed of two main structural parts: nuclear envelope and nucleoplasm. The later contains most of the cell's genetic information but also other molecules, including proteins that play a central role in regulating gene expression (Petrovská et al. 2015). The identification and characterization of these regulatory proteins presents a challenge. They are usually in a low abundance and subjects of post-translational modifications. The enrichment of phosphorylated or acetylated proteins (e.g. Černý et al. 2013b) may increase the detection limits, but the most effective technique to isolate nuclear proteins seems to be a cell fractionation. The standard protocol consists of a homogenization of tissues, pelleting, elimination of contaminating organelles and separation on a density gradient. Plant extracts are more complicated and require an additional filtration step after a homogenization to remove large debris (e.g. Sikorskaite et al. 2013). The extraction of nuclei is a time consuming step and may have a negative impact on proteome quality. For instance, even the addition of protease inhibitors does not necessarily prevent a residual protease activity during filtration and pipetting steps. Further, the efficiency of the extraction protocol is not comparable to that of phenol extraction or acetone/TCA precipitation and thus the amount of starting material has to be considerably larger. Here, we compare results of an optimized protocol for nuclear protein extraction and standard protein precipitation protocol in the analysis of tomato leaf proteome.

MATERIAL AND METHODS

Plant material

Leaves of 4-week-old tomato (*Solanum lycopersicum*, cv. moneymaker) were collected, frozen in liquid nitrogen, homogenized (Retsch Mill MM400), aliquoted and stored at -80 °C.

Protein extraction

Total protein extracts were prepared by acetone/TCA/phenol extraction (Carpentier et al. 2005, Černý et al. 2014, Novák et al. 2015) from 200 mg of ground tissue. In brief, the homogenized tissue was washed with 1.5 ml acetone (4 °C, 30 min), clarified by centrifugation, washed with 10% (w/v)

TCA in acetone, 10% (w/v) TCA in distilled water then 80% (v/v) acetone, resuspended in 0.8 ml SDS buffer [2% (w/v) SDS, 30% (w/v) sucrose, 5% (v/v) β-mecraptoethanol, 5 mM EDTA, 100 mM Tris, pH 8.0], and protein was extracted by 0.4 ml buffer-saturated phenol. Phenolic phase was collected and protein was precipitated overnight in 1.6 ml ice-cold 100 mM ammonium acetate in methanol (-20 °C). Protein pellets were washed with 1.0 ml 80% (v/v) acetone in distilled water and dried. Nuclei were extracted on percoll gradient from 2 g of ground tissue as described previously (Sikorskaite et al. 2013). In brief, 1 g of homogenized tissue was extracted in 5 ml of NIB buffer [10 mM MES-KOH (pH 5.4), 10 mM NaCl, 10 mM KCl, 2.5 mM EDTA, 250 mM sucrose, 0.1 mM spermine, 0.5 mM spermidine, 1 mM DTT], decanted through two layers of pre-wetted cheesecloth, treated with Triton X-100 (dropwise to 0.5%), agitated for 20 min at 4°C and centrifuged at 1000 × g for 10 min. The pellet was resuspended in 10 ml of NIB and nuclei were purified using Percoll/sucrose density gradient (2.5 M sucrose, 60% Percoll for isolation; 35% Percoll for washing). Nuclear proteome was precipitated with 10% (w/v) TCA in acetone and washed 1.0 ml 80% (v/v) acetone in distilled water and dried. The resulting protein pellets were solubilized (100 mM ammonium bicarbonate, 8 M urea) and digested with an immobilized trypsin (Promega) overnight and desalted by C18 SPE (Černý et al. 2013a).

LC-MS proteome analysis

Analyses were performed using a gel-free shotgun protocol based on nano-HPLC and MS/MS (Baldrianová et al. 2015). Briefly, tryptic digests were dissolved in 0.5% (v/v) formic acid in 5% (v/v) acetonitrile, and then analyzed by nanoflow C18 reverse-phase liquid chromatography using a 40 cm column (0.075 mm inner diameter; NanoSeparations) and a Dionex Ultimate 3000 RSLC nano-UPLC system (Thermo) directly coupled to a CaptiveSpray nanoESI source (Bruker) and an UHR maXis impact q-TOF mass spectrometer (Bruker). Peptides were eluted with up to a 120-min, 4% to 40% acetonitrile gradient. Spectra were acquired at 2 Hz (MS) and 10 to 20 Hz (MS/MS) using an intensity-dependent mode with a total cycle time of 7 s.

Protein identification

The measured spectra were extracted by Bruker's Data Analysis 4.1 and processed as described previously (e.g. Cerna et al. 2016). In brief, recalibrated MGF files were searched against Tomato protein database (ITAG 2.4; 8/2014) by Sequest HT, MS Amanda and Mascot 2.4 with the following parameters: Enzyme - trypsin, max two missed cleavage sites; Mass tolerance - 35 ppm (MS) and 0.1 Da (MS/MS); Modifications - up to three dynamic modifications including Met oxidation, Asn/Gln deamidation, Lys methylation, N-terminal acetylation, Ser/Thr/Tyr phosphorylation.

RESULTS AND DISCUSSION

Identification of tomato proteins

Total protein extracts and nuclear enriched extracts were prepared in 14 replicates each. To increase the proteome coverage, MS spectra were processed by a combination of three complementary search algorithms and the resulting data obtained from all replicates were combined. Altogether, 1,711 and 1,199 protein groups were identified in total protein extracts and nuclear protein extracts, respectively (Figure 1).

Enrichment of nuclear proteins does not necessarily improve their detection

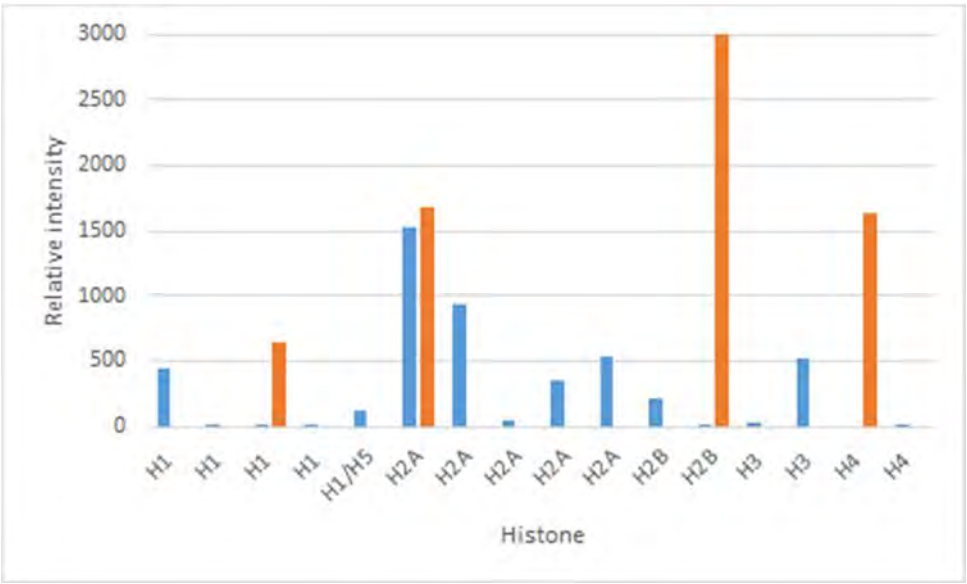
The overlap in identified proteins between total protein and nuclear extracts is high, representing 51% and 74% of all identified proteins, respectively (Figure 1A). A similar distribution is also reflected on a peptide level (45% and 65%; Figure 1B). Further, identification of 139 proteins unique to nuclear extracts is based only on a single identified peptide and these are thus not suitable for a quantitative analysis. In depth analysis of our data showed that at least some nuclear proteins are significantly enriched in nuclear extracts, including DNA-directed RNA polymerase II (not detected in total protein extracts) or three proteins of histone family H1 (200×), H4 (not detectable in total protein extracts) and H2B (200×). However, 12 histones and four histone-associated proteins were detected only in total protein extracts (Figure 2). Similarly, only four of eight detected 14-3-3 proteins were found in nuclear extracts. This shows that the nuclear protein enrichment may in fact have a negative impact on the detection of at least some of the nuclear proteins. To our knowledge, this is the first report of its kind in tomato proteomics and we can not exclude that this is a consequence of a tomato specific protease

activity. Standard protocols for nuclei extraction consists of several incubation steps at 4 °C and employ protease inhibitors. If the inhibition efficiency was lower (as is often the case) quality of the proteome would suffer.

Figure 1 Identification of proteins in total protein extracts (blue) and nuclear extracts (orange)

A) Identified proteins *B) Identified peptides*

Figure 2 Relative abundances of detected histones in total protein extracts (blue) and nuclear extracts (orange) based on sum of all assigned peptide spectral matches



CONCLUSION

Analysis of nuclear protein extracts increased the number of identified proteins and peptides in tomato leaves by ~16% and ~19%, respectively. Insufficient annotation of tomato proteome does not allow to easily assess the localization of all 316 proteins unique to nuclear extracts. However, based on the profiles of several well-known nuclear proteins we conclude that the established Percoll-based protocol shows disproportionate yields in nuclear proteins and may even decrease the detectability of some. This would imply that the acetone/TCA total protein extraction is superior in the qualitative analysis.

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IN VIVO FLUORESCENCE VISUALIZATION OF QUANTUM DOT NANOPARTICLES IN PLANTS

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Abstract: Visualization of nanoparticles can be exceedingly useful in tracking of targeted drug delivery systems. For this purpose, highly luminescent quantum dot nanoparticles may represent a suitable option due to their superior photophysical properties and versatile surface chemistry. This study was mainly focused on application of CdTe quantum dot nanoparticles for fluorescence imaging of their transport in plants. *In vivo* experiments were carried using leaves of sunflower plant (*Helianthus annuus*). Leaves, soaking water solution of CdTe-PVP and CdTe/ZnS quantum dots, were monitored (λ_{em} 535 nm and 600 nm respectively) for 8 hours at time intervals of 60 minutes using *In Vivo* Xtreme Imaging System (Bruker, MA, USA). Autofluorescence of biomolecules present in plants, including chlorophyll, carotene and xanthophylls, represents a crucial problem in fluorescence imaging of plants. However, by using adequate excitation and emission filters during fluorescence images acquisition, this phenomenon can be effectively suppressed. Moreover, multispectral imaging and spectral modelling can be performed in order to distinguish fluorescence of quantum dots. In this study, a comparison of two different modifications of CdTe quantum dots is provided together with recommendations on setting of appropriate excitation and emission range for image acquisition.

Key Words: CdTe quantum dots, fluorescence *in vivo* imaging, *Helianthus annuus*, nanoparticles transport monitoring

INTRODUCTION

Nanoparticles have found their applications in diverse fields, including therapy, diagnostics and targeted drug delivery (Michalet et al. 2005). In this regard, quantum dot nanoparticles (QDs) have been in the centre of a great interest of research community (Medintz et al. 2005). These semiconductor nanocrystals belong to a class of fluorophores that allow combination of excellent fluorescence properties with versatile surface chemistry for directing their bioactivity (Deerinck 2008). Considering these properties, QDs may outperform traditional organic dyes in many *in vivo* applications. However, only small body of literature focuses on nanoparticles transport monitoring in plants.

Several studies have been proposed concerning plant uptake of nanoparticles and their examination in plants, including gold nanoparticles in rice and tomato (Li et al. 2016); upconversion nanoparticles in *Phalaenopsis* and *Arabidopsis* plants (Hischemöller et al. 2009); QDs nanoparticles in plant chromosomes visualization (Müller et al. 2006); or potential toxicity of heavy metals and metal-containing nanoparticles on plants (Mustafa and Komatsu 2016). However, the uptake of nanoparticles by plants is not well investigated as well as the way how they affect their biochemistry. Generally, experimental design consists of plant soaking of the nanoparticles from soil or solution. As a next step, roots or stems are cut in order to examine whether the nanoparticles can be detected in different parts of the plant. Usually, confocal laser scanning microscopy is used. However, to our best knowledge, fluorescence i*n vivo* imaging had not been used for monitoring of nanoparticle uptake by plants.

Nevertheless, thanks to its noninvasiveness and possibility of long term monitoring and examination, we assume that whole leaf fluorescence imaging of nanoparticles represents a great potential.

On the other hand, biomolecules present in leaf tissues, such as chlorophyll, xynthophyll and carotene, as well as stressed or photodamaged cells, are the main source of autofluorescence (Gupta and Ibaraki 2014), which represents a special challenge for fluorescence imaging. Chlorophyll fluorescence of chloroplasts mainly occupies the red/far-red region of the spectrum (650–750 nm) and fluorescence of lignin present in cell walls is obtained in a wide range of visible spectrum (490–620 nm) (Chapman et al. 2005). Therefore, strong chlorophyll fluorescence limits the use of red fluorescent markers in green leaves. Quantum dot nanoparticles appear to be beneficial platform for *in vivo* fluorescence monitoring in plants. Due to their size-tunable characteristics, their emission spectra can be shifted toward region with minor autofluorescence.

In this study, the uptake of CdTe quantum dot nanoparticles was examined in *Helianthus annuus* L. plant. Two kinds of CdTe quantum dots were used, specifically CdTe/ZnS and CdTe-PVP. Whole leaf fluorescence images have been captured by fluorescence scanner at periodic time intervals for 8 hours. The uptake of QDs nanoparticles to the petiole and main venation can be confirmed.

MATERIAL AND METHODS

Plant cultivation

Sunflower (*Helianthus annuus* L.) Kongo hybrid was used as an experimental plant. The achenes were sterilized (20 minutes in 20% SAVO solution) and planted in perlite substrate. Then the achenes were germinated for seven days at 22 °C with photoperiod day/night 16/8 hours. As a next step, the grown seedling plants were transplanted into the hydroponic container containing Murashige-Skoog medium including vitamins (Duchefa Biochemie, Netherlands). Finally, seedlings were grown for 6 weeks under standard conditions at 22 °C, day/night 16/8 photoperiod and humidity of 55%.

Quantum dots synthesis

CdTe QDs have been prepared following the procedure published in (Guszpit et al. 2015, Krizkova et al. 2015). A solution of CdTe QDs was prepared by mixing of cadmium acetate dihydrate (0.053 g in 76 mL of water) with mercaptosuccinic acid (MSA) (60 mg/mL) followed by addition of 1M NH_3 (1.8 mL). Then, a solution of sodium tellurite (0.0066 g/mL) was added and sodium borohydride (40 mg) was poured into the stirred solution. Volume was adjusted with water to 100 mL. Vials were heated at 60 °C in microwave oven Multiwave 300 (Anton Paar, Graz, Austria) (300 W, 10 min) to obtain green QDs.

Spare solution of ZnS was prepared by mixing solutions of zinc acetate (0.022 g), MSA (30 mg), 1 M NH_3 (0.9 mL) and 2.5 mL Na2S (0.24 g/50 ml) (Krejcova et al. 2013). Water was added to 50 mL.

Core shell CdTe/ZnS were obtained by mixing CdTe QDs with spare solution of ZnS in 1:1 ratio and heating in microwave oven at 60 °C.

CdTe-PVP QDs were obtained by adding 100 mg polyvinylpyrrolidone (PVP 40 kDa) to 50 mL of green CdTe QDs, shaking overnight and filtering of solution through a frit.

QDs were further characterized using fluorimeter Infinite M200 Microplate reader (Tecan, Switzerland) and dynamic light scattering (Zetasizer Nano ZS90, Malvern instruments, Malvern, UK).

In certain cases, quantum dots were precipitated by isopropanol (1:1 ratio) to remove the excessive reagents according to published methods (Duan et al. 2009; Stanisavljevic et al. 2013).

Fluorescence bioimaging of QDs distribution

QDs distribution in sunflower leaves has been observed using *In Vivo* Xtreme Imaging System (Bruker, MA, USA). Image acquisition parameters were set as follows: exposition time - 2 s, binning - 4x4 pixels, fStop - 1.1, field of view - 15x15 cm.

CdTe/ZnS quantum dots uptake have been monitored using excitation filter λ_{ex} 550 nm and λ_{em} 600 nm. Monitoring of CdTe-PVP QDs has been examined using filters of wavelength λ_{ex} 480 nm and λ_{em} 535 nm.

RESULTS AND DISCUSSION

Series of *in vivo* experiments has been done in order to evaluate the potential of CdTe quantum dots in nanoparticle transport monitoring. Two nanoparticle modifications have been examined.

As a first step, leaves of 7 weeks old sunflower plant were cut and the petiole was immersed in a tube filled with quantum dot solution. At time 0, the first image was captured. Next, nanoparticle distribution monitoring has been performed at 60 minute intervals for 8 hour period. Additional image (in case of confirmed plant uptake during 8-hour period) was acquired after 24 hours from the start of the experiment.

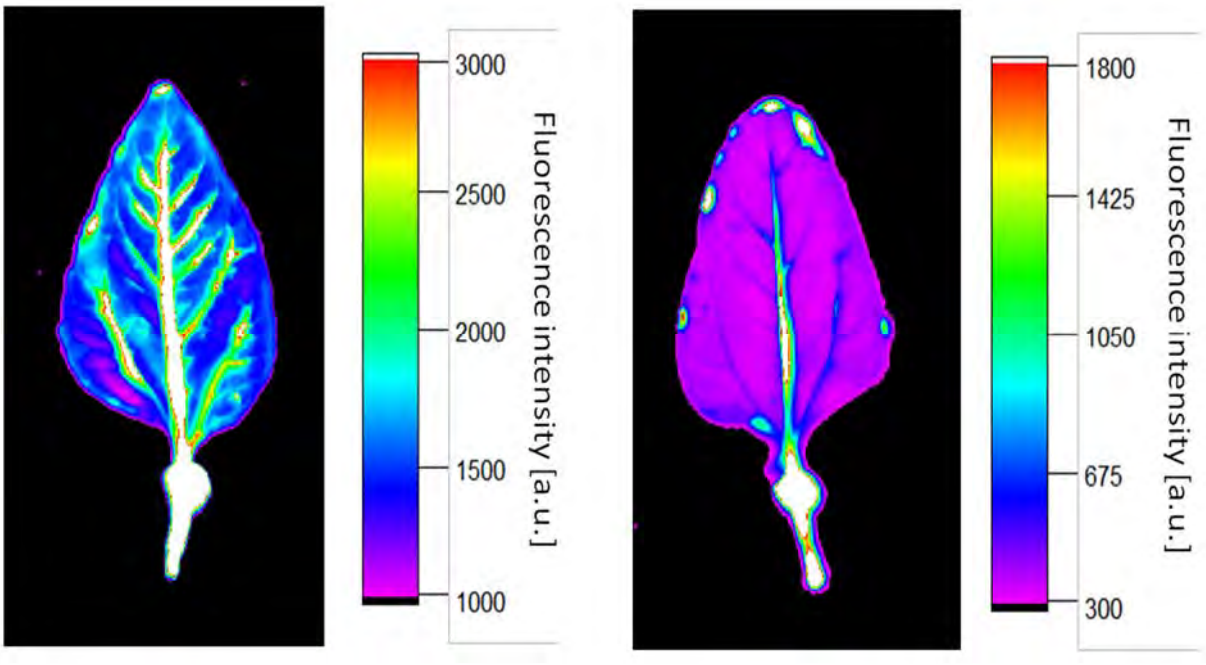
When using CdTe QDs solution without any added substances, only a little uptake of the fluorophore through petiole and main venation could be seen for first 3 hours. After that the soaking of QDs solution was interrupted probably due to an obstruction in vascular bundle.

On top of that, CdTe QDs solutions diluted 10 times with Milli-Q water have been examined. Fluorescence of CdTe-PVP QDs was clearly visible after 2 hours from the beginning of the experiment and the QDs uptake to the petiole and main venation was increasing in time.

Similarly, CdTe/ZnS QDs solution with no added components was tested. The uptake was visible only for approximately 3 hours from the start of the experiment. Nevertheless, the Milli-Q water diluted CdTe/ZnS QDs solution appeared to be accumulated in the main venation when examined after 24 hours from the start of the experiment.

As can be seen in the pictures below captured after 24 hours from the beginning of the experiments, CdTe/ZnS (Figure 1, right) provide lower quantum yield in comparison with CdTe-PVP (Figure 1, left). Moreover, it appears that CdTe-PVP can better penetrate through main and also side venation, whereas CdTe/ZnS quantum dots could be confirmed only in the main venation of the leaf.

Figure 1 Comparison of CdTe-PVP QDs (λ_{ex} 480 nm, λ_{em} 535 nm) (left) and CdTe/ZnS QDs (λ_{ex} 550 nm, λ_{em} 600 nm) (right)



As can be seen in Figure 2, the fluorescence of CdTe/ZnS QDs is clearly visible after 2 hours from the beginning of the experiment and the QDs uptake to the main venation is increasing in time. False colouring 300-1800 counts was used in this case.

Furthermore, precipitation of quantum dots by isopropanol has been done and Milli-Q water solution of precipitated QDs has been examined in this set of experiments. Nonetheless, this procedure has not led to any visible plant uptake.

Figure 2 CdTe/ZnS QDs. Whole-leaf fluorescence images taken at time intervals as proposed above pictures. Pseudocoloring 300-1800 counts was applied.

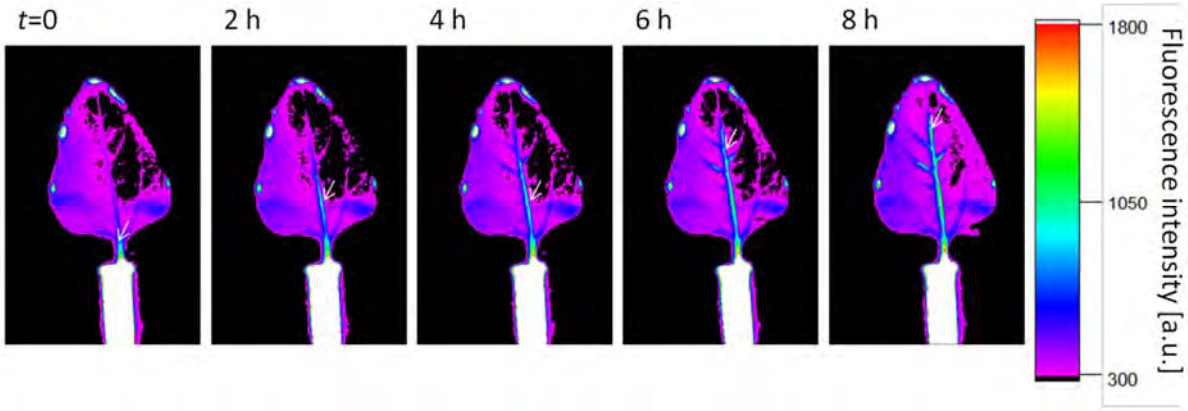
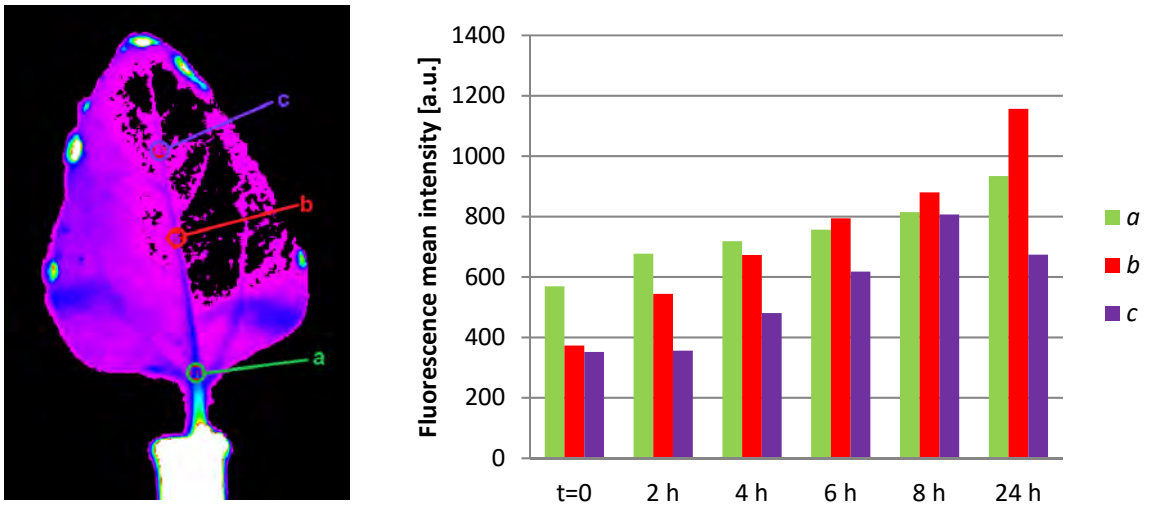


Image analysis was performed using Bruker Molecular Imaging Software. The fluorescence intensity was evaluated in three regions of the main venation (Figure 3, left). Growing mean fluorescence intensity in regions *a* and *b* confirms the uptake of QDs (Figure 3, right). On the other hand, during first 8 hours of the experiment, the mean fluorescence in region *c* was growing, but examination after 24 hours showed decrease of fluorescence, which indicates that QDs tend to cumulate in the central part of the leaf.

Figure 3 CdTe/ZnS QDs. Image analysis in regions a-c of the leaf (as shown left). Graph of the mean fluorescence intensity in these regions (right).



CONCLUSION

Aim of this study was to provide evaluation of CdTe QDs potential for *in vivo* fluorescence monitoring in plants. In this regard, core shell CdTe/ZnS and CdTe-PVP quantum dots have been examined in leaves of sunflower plant. The most promising results were obtained by examination of CdTe PVP QDs diluted with Milli-Q water as after 24 hours from the beginning of the experiment QDs could be seen not only in the petiole and main venation, but also in the side venation of the leaf. These particular quantum dot nanoparticles benefit from the size of 5.6 nm and their emission spectrum located in the green region of visible spectrum, so the chlorophyll autofluorescence of the leaf can be sufficiently suppressed.

Our pilot experiments on young whole plants of sunflower show promising results; particularly indicate that QDs can enter the pores of root system. However, further examination of proposed nanoparticles and *in vivo* QDs distribution monitoring need to be done especially in terms of their toxicity on plants and impact on biochemistry. In future, quantum dots may become promising tool to be used in agriculture for targeted drug delivery and its visualization.

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GEOCHEMICAL CHARACTERIZATION OF SOILS FROM EXPECTED CONTAMINATED SITES IN THE ODRA HILLS AND DRAHANY UPLAND

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Abstract: The aim of the study paper was to evaluate a rate of soil contamination by heavy metals from the expected contaminated site 1 (Odra Hills) and expected contaminated site 2 (Drahany Upland) using Coefficient of Industrial Pollution (CIP). In the total, 37 topsoil samples were collected. The pH and ORP parameters were used to evaluate changes in environment. Among the study elements are arsenic, copper, zinc and lead and to evaluate them the X-ray spectroscopy was used. The Coefficient of Industrial Pollution (CIP) reported medium soil contamination at the Shooting Range areas from ECS1 as well as from ECS2. The main source of copper and lead probably come locally from the munitions. The heavy metal content in the other anthropogenic-changed landscapes was compared to the natural background.

Key Words: heavy metals, soil contamination, pH, redox potential, X-ray spectroscopy

INTRODUCTION

The Drahany Upland and Odra Hills are chosen to evaluate heavy metal contamination in their topsoils. Human activity (practice shooting, traffic) may contribute to enhance heavy metal levels in the soil environment (Alloway 2015, Ash 2013). The Drahany Upland considers being a geomorphologic unit, falling down into the Brno Highlands. The area is 1 178.68 km² and it extends among the cities Brno, Vyskov, Prostejov, Boskovice and Konice. The second site is receded from the first site approximately 60 km towards to south-east, falling down into a geomorphologic unit the Odra Hill and a geomorphologic complex the Nizky Jesenik. The area of Odra Hills is 580 km^2 and it extends among the cities Olomouc, Lipnik nad Becvou, Hranice, Potstat and Sternberk (Demek et al. 2006). Shale, greywacke and siltstone are the dominant rock types in both areas. These rocks were deposited during Flysch Culm Sedimentation in Carboniferous. The Moravice and Hradec-Kyjovice Formation represents the Nizky Jesenik area while the Drahany Upland by Protivanov, Myslejovice and Rozstani Formation (Kalvoda and Melichar 1999). Galenites with silver content and shales have been mining for centuries in the Odra Hills thus we expect higher natural lead content (Gottvald 1980). This anthropogenic activity had been performed at the Willibald mine and the Franz and Moritz mine in the Barnov Town (Losert 1957). This paper gives an idea about heavy metal contamination in soils from potential contaminated sites.

MATERIAL AND METHODS

The soils from the expected contaminated site 1 (Odra Hills) and the expected contaminated site 2 (Drahany Upland) were investigated. The totals of 37 soil samples were collected in both potentially-contaminated sites. Solid matrices were homogenized and sieved less than 0.063 mm. The X-ray fluorescence was used to accurate analyse of elements in the samples. The tool Innov-X Systems, Inc., Delta was used with following settings. The power of first X-ray was 1–40 kV with exposure time 40

seconds. The power of second X-ray was 2–10 kV with exposure time 40 seconds. The device was calibrated using metal standards supplied by the manufacturer and the mode Geochem-Vanad was used. Each measurement was carried out twice and average of the measurments is presented in results. Total measure time took 260 seconds. The Limits of Detection (LODs) in ppm for appropriate elements are following (As: 1–3; Pb: 2–4; Zn: 3–5; Cu: 5–7). The reproducibility, measurement error and applitcation of this spectroscopic method is reviewed in (Geršl and Knésl 2009).

The chemical parameters, such as soil active reaction (pH/H_2O) and redox potential (ORP by another name Eh) were measured as well. The pH/H_2O was measured by the all samples according to well-known method ISO 10390 (UNMZ 2011). Solid matrices were undergone to an infusion by the distilled water and resulting suspension were shaken and prepared to measuring by the tool WTW InoLab Multi 720 with SenTix 81 electrod. Soil contamination by heavy metals has been demonstrated using Coefficient of Industrial Pollution (CIP).

RESULTS AND DISCUSSION

Heavy metal content

Soil contents of arsenic, lead, zinc and copper were evaluated. The results show the highest lead (214 ± 2.61 mg/kg and 409 ± 2.32 mg/kg), zinc (431 ± 3.77 mg/kg and 380 ± 2.69 mg/kg), copper (1768 ± 9.08 mg/kg and 578 ± 4.17 mg/kg) and arsenic (33 ± 1.76 mg/kg) levels at the Shooting range area Daskabaty from ECS1 (Table 1).

Table 1 Overview of heavy metals content in soils of various human areas

Territory	Locality	ECS	Sample	Element (mg/kg)			
				As	Pb	Zn	Cu
Shooting range area for fighting vehicles	Praslavice	1	Bar11	16 ± 0.69	34 ± 0.90	93 ± 1.46	150 ± 2.53
	Praslavice	1	Bar16	17 ± 0.71	32 ± 0.92	97 ± 1.52	153 ± 2.61
	Smilov	1	Bar15	21 ± 0.93	68 ± 1.22	125 ± 1.81	186 ± 2.99
	Smilov	1	Bar09	22 ± 0.81	51 ± 1.03	152 ± 1.81	171 ± 2.69
Shooting range area	Daskabaty	1	Bar18	25 ± 0.89	55 ± 1.16	137 ± 1.86	278 ± 3.36
	Daskabaty	1	Bar19	33 ± 1.76	214 ± 2.61	431 ± 3.77	1768 ± 9.08
	Daskabaty	1	Bar20	18 ± 1.66	409 ± 2.32	380 ± 2.69	578 ± 4.17
	Daskabaty	1	Bar21	17 ± 0.86	71 ± 1.13	109 ± 1.59	230 ± 2.95
	Daskabaty	1	Bar22	19 ± 0.77	48 ± 0.99	106 ± 1.56	166 ± 2.65
	Smilov	1	Bar14	22 ± 0.76	32 ± 0.96	112 ± 1.65	167 ± 2.76
	Smilov	1	Bar17	25 ± 0.82	36 ± 1.04	130 ± 1.82	185 ± 2.96
	Smilov	1	Bar10	23 ± 0.81	48 ± 1.03	124 ± 1.69	165 ± 2.70
	Smilov	1	Bar07	22 ± 0.76	35 ± 0.97	118 ± 1.69	192 ± 2.88
	Smilov	1	Bar08	19 ± 0.80	52 ± 1.03	113 ± 1.61	169 ± 2.69
	Ferdinandsko	2	DV35	14 ± 1.20	184 ±1.66	106 ± 1.55	74 ± 2.22
	Ferdinandsko	2	DV36	17 ± 1.51	344 ± 2.11	99 ± 1.46	52 ± 2.00
	Brezina	2	DV37	11 ± 0.62	28 ± 0.82	60 ± 1.19	24 ± 1.78
	Brezina	2	DV38	17 ± 0.71	44 ± 0.92	120 ± 1.53	34 ± 1.83
Water training ground	Barnov	1	Bar04	24 ± 1.02	97 ± 1.34	150 ± 1.88	195 ± 2.92
	Barnov	1	Bar05	10 ± 0.55	25 ± 0.71	106 ± 1.36	123 ± 2.12
Hand-Thrown area	Brezina	2	DV33	4 ± 1.27	234 ± 1.77	121 ± 1.58	39 ± 1.94
	Brezina	2	DV34	6 ± 1.21	206 ± 1.69	174 ± 1.86	62 ± 2.10
Blasting pit	Hanacka louka	2	DV39	18 ± 0.77	50 ± 1.00	126 ± 1.61	43 ± 1.96
	Hanacká louka	2	DV40	21 ± 0.73	33 ± 0.94	124 ± 1.64	51 ± 2.07
	ZMC sv. Anna	2	DV42	21 ± 0.70	33 ± 0.88	75 ± 1.30	23 ± 1.80

Compared to results from ECS1, the highest lead (184 ± 1.66 mg/kg and 344 ± 2.11 mg/kg) concentrations were also reported at Shooting range area as well as at Hand-Thrown area (234 ± 1.77 mg/kg and 206 ± 1.69 mg/kg) from ECS2. Moreover, copper contents are more than three times higher in all anthropogenic-changed areas from ECS1.

Territories, such as the Olovensky Hill, a woodland and a meadow represent natural background in both sites. In the Barnov I the highest lead (143 ± 1.48), zinc (217 ± 2.11 mg/kg) and arsenic (33 ± 1.14 mg/kg) contents were observed while reporting the highest copper concentrations (186 mg/kg) in the woodland at Barnov Town (Table 2). We became conscious of being not significant difference in content of studied elements between natural background and anthropogenic-changed landscape unless it expects elevated lead and copper contents at the Shooting Ranges and Hand-Thrown. Eventually, there are evident higher copper contents in the Odra Hills while naturally lowering in the soils from south-eastern part of the Drahany Upland.

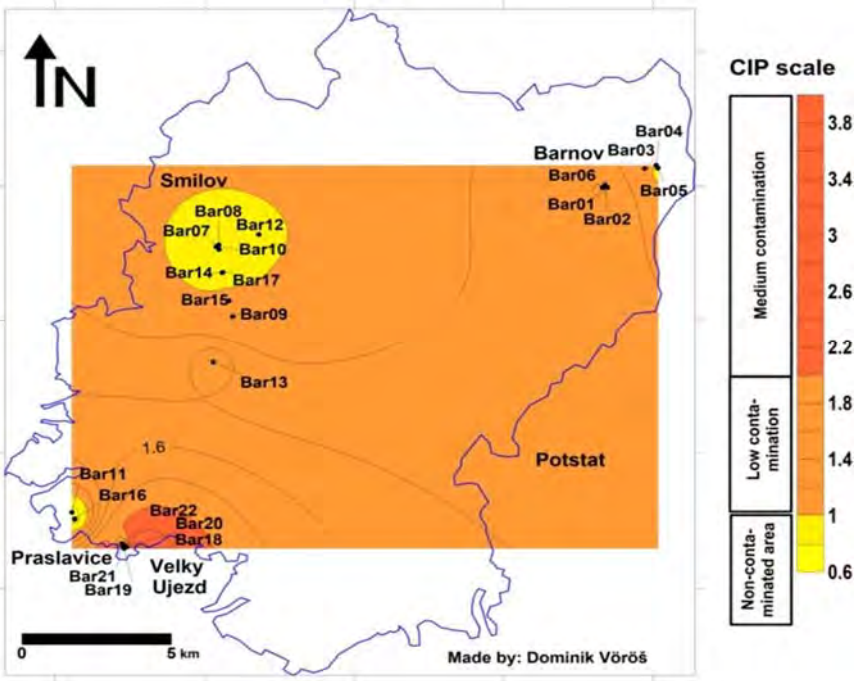
Table 2 Territories related to natural background and heavy metal content in soils

Territory	Locality	ECS	Sample	Element (mg/kg)			
				As	Pb	Zn	Cu
Olovensky Hill	Barnov	1	Bar01	33 ± 1.14	143 ± 1.48	217 ± 2.11	183 ± 2.74
	Barnov	1	Bar02	25 ± 0.79	43 ± 0.98	139 ± 1.76	176 ± 2.73
	Barnov	1	Bar06	26 ± 0.88	66 ± 1.12	181 ± 1.94	176 ± 2.71
Woodland	Barnov	1	Bar03	22 ± 0.90	73 ± 1.17	153 ± 1.85	186 ± 2.81
	Bores castle	1	Bar13	44 ± 1.10	135 ± 1.36	99 ± 1.45	127 ± 2.31
	Brezina	2	DV32	23 ± 0.82	71 ± 1,05	95 ± 1,38	21 ± 1.72
Meadow	Brezina	2	DV30	16 ± 0,72	46 ± 0,93	79 ± 1.31	21 ± 1.75
	Brezina	2	DV31	24 ± 0,75	42 ± 0,93	116 ± 1.54	29 ± 1.85
	Brezina	2	DV41	12 ± 0.63	30 ± 0,83	67 ± 1.23	21 ± 1.76
	Smilov	1	Bar12	24 ± 0.78	44 ± 0.98	145 ± 1.76	153 ± 2.58

Heavy metal contamination

Using the CIP, the soils at the Shooting range area from ECS1 (Figure 1) as well as from ECS2 (Figure 2) are medium contaminated by studied elements. Especially elevated lead and copper content from ECS1 were investigated.

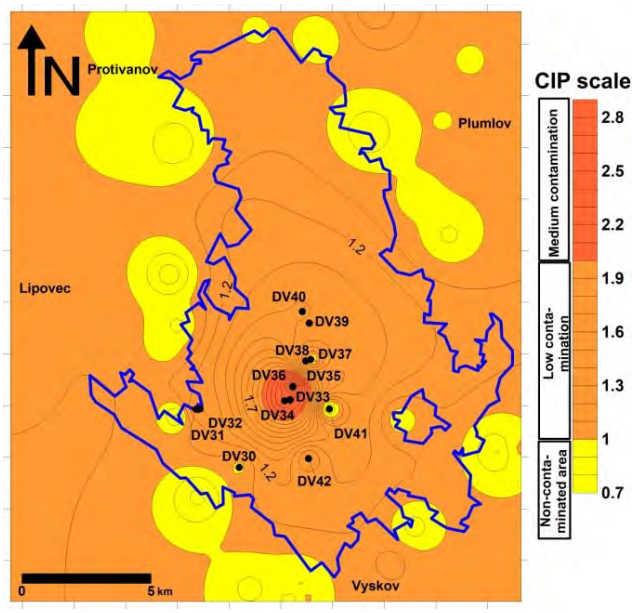
Figure 1 Rate of soil contamination by As, Pb, Zn and Cu from ECS1



According to heavy metal contents (especially copper and lead), human activity locally influences even more to metal composition in the ECS1 soils unlike those from ECS2. These contents might come from ammunition because copper is used to the munitions production. It was investigated in some study pits that bullet core is mostly composed of lead 94.5%, while bulk mantle of copper 83.2% (Ash et al. 2013). Also, a cartridge casing production supports to have enhanced zinc and copper in the environment (Plíhal 2010).

On the contrary, non-contaminated soils were evaluated at the Praslavice village on the south-west as well as at the Smilov ex-village on the north-west where the Shooting range area and the Shooting range areas for fighting vehicles are placed. Adjacent to the Barnov Town there are soils which are non-contaminated by studied elements. As we mentioned above the copper content is significantly distributed in soils from the Odra Hills. It might have caused the last ore mining in the Barnov Town (Losert 1957) thus nowadays copper content is much higher unlike the soils from ECS2 where none ore was mined although the cerusite and galena veins are found (Posmourny 2000).

Figure 2 Rate of soil contamination by As, Pb, Zn and Cu from ECS2



Environmental characterization of soil environment

The pH values in majority of soil samples from ECS1 ranging mostly between 5 and 6 while the soils from ECS2 tend to being a little bit more acidic (4.5–5.0). Moreover, the alkaline pH was observed at soils in the Hand -Thrown area (Figure 3A).

There is a variability in redox potential (ORP) of soils from ECS1 and ECS2 (Figure 3B). Basically, we observed decreasing in Eh values from 275 mV to 100 mV at the soils from ECS1 while achieving the soils from ECS2 higher values (225–375 mV). Two samples DV39 (−41.11 mV) and DV36 (−450.51 mV) are not included in the table, because extremely reduction conditions were observed.

The soil process, denitrification (1) where nitrates are reduced using bacteria to elemental state of nitrogen which is going on especially in the poor-drained and low aerated soils at ORP around 200 to 400 mV (Brady 1984). According to Alloway (2013), this process typically occurs when the Eh achieves values between 200–400 mV. Although, at lower Eh values around 100–200 mV, a Mn^{IV} oxide reduction and dissolution (2) are thought to be the common process in the soil environment (Alloway 2013). Also, Alloway (2013) reported that at pH 6 some cationic and anionic forms are probably bound onto Fe and Mn oxides and metals and metalloids are firstly released unless they are bound onto Fe and Mn oxides.

$$C_3H_6O_3 + 2NO_3^- \rightarrow 3CO_2 + 3H_2O + N_2 \quad (1)$$

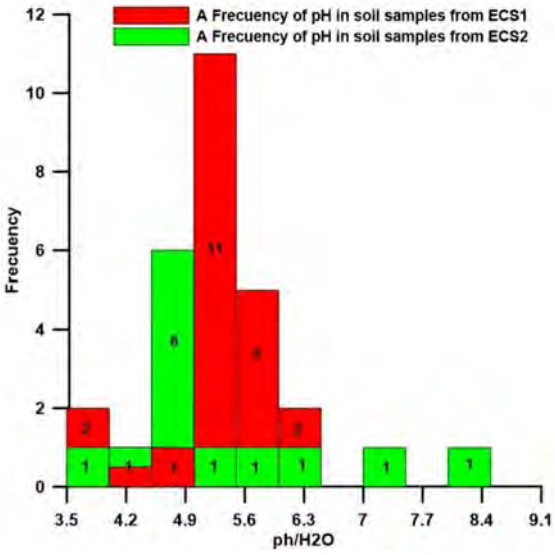
$$MnO_2(s) + 4H^+ + 2e^- = Mn^{2+} + 2H_2O \quad (2)$$

In principle, we expect to occur soil process denitrification especially in the Odra Hill's soils where natural conditions are probably different unlike the Drahany Upland where is expected to be going

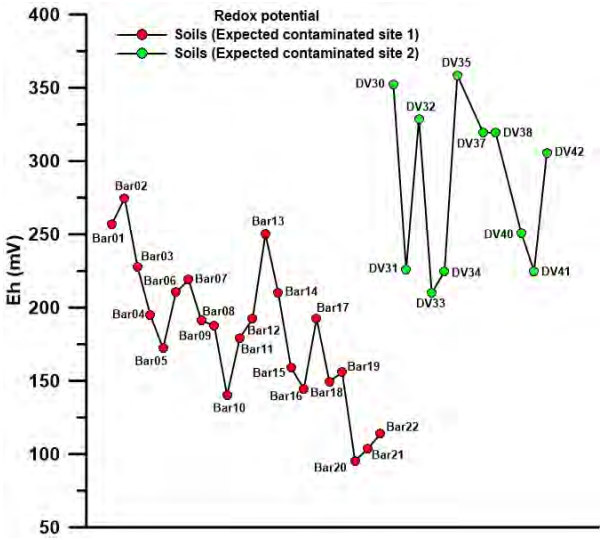
on the Mn^{IV} oxide reduction and dissolution. These interpretations are requiring a caution because soils are generally porous media and biological activity and decay of organic matter highly influence on the ORP (Sposito 2008, Kabata-Pendias 2001).

Figure 3 Changes in the soil environment from ECS1 and ECS2

A) Soil active reaction



B) Redox potential



Heavy metal mobility is patterned on definite environmental conditions (pH and ORP). When pH is more acidic and ORP more oxidative, we could expect increasing in zinc, lead and copper mobilization (Siegel 2002) even though copper is accumulated mostly in the top soil horizons and is also rather immobile (Kabata-Pendias 2009). There is a need to pay attention to heavy metals bounding because, for example copper tends to accumulate itself onto suspended organic matter (SOM), clay minerals and Mn-Fe oxohydroxides.

According to results we could expect moderate zinc and lead mobilization in soils from the Odra Hills. Previous study showed quite good zinc and lead mobility in soils from the Drahany Upland. We became conscious of lead and zinc bounding onto Fe and Mn oxo-hydroxides, representing reducible fraction. An experiment showed also significant arsenic and copper immobility (Voros et al. 2015).

According to pH/H_2O and ORP we can claim the zinc, copper and lead would appear to be more mobile in soils from the Drahany Upland while the arsenic would seem to be more mobile in soils from the Odra Hills because arsenic mobility increases when ORP becomes more reducing and pH more alkaline (Hooda 2010). Arsenic content was higher in soils from the Odra Hills and it might also be considered as an anthropological source.

CONCLUSION

Rate of soil contamination has been evaluated. We conclude that soils from the Shooting range areas in ECS1 and ECS2 and Hand-Thrown area are medium contaminated by arsenic, lead, zinc and copper. The probably source of copper and lead in the anthropogenic-changed landscapes come from ammunition. Except of the Shooting range areas and the Hand-Thrown areas, human activity like a shooting does not significantly distribute studied elements into soils from the others anthropogenic territories which are low contaminated or non-contaminated and their contents is rather comparable with natural background in the Drahany Upland.

Expect high lead content in the Odra Hills has not been proved but higher copper content is related to the natural background because these elevated contents are distributed through the whole area. We suppose to not being mobilized copper in the soil environment from both sites although the arsenic could under specific conditions (low pH and low ORP) happen to mobilize. In the soil media is difficult to understand all processes which are going on however we supposed to have an effect of denitrification

process on soils from the Odra Hills while manganese oxides are reducing in soils from the Drahany Upland. A periodic monitoring in these areas can more reveal geochemical processes in soils.

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