MendelNet 2017

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Proceedings of International PhD Students Conference

8–9 November 2017, Brno, Czech Republic

Faculty of AgriSciences

Mendel University in Brno

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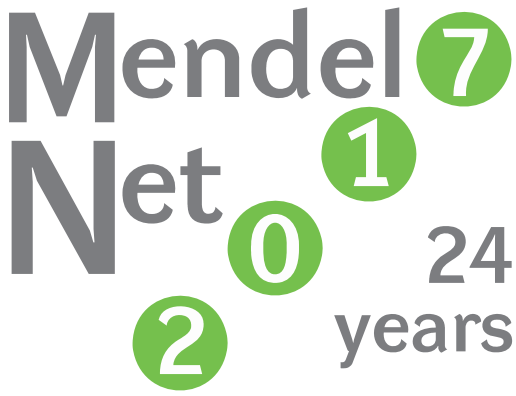

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Proceedings of 24th International PhD Students Conference
November 8 and 9, 2017, Brno, Czech Republic

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The MendelNet 2017 conference would not have been possible without the generous support of The Special Fund for a Specific University Research according to the Act on the Support of Research, Experimental Development and Innovations and the **support of**:

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Profi Press s.r.o.

Research Institute of Brewing and Malting, Plc.

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All contributions of the present volume were peer-reviewed by two independent reviewers. Acceptance was granted when both reviewers' recommendations were positive.

ISBN 978-80-7509-529-9

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PREFACE

The 24th International PhD Students Conference for undergraduate and postgraduate students was hosted by the Faculty of AgriSciences, Mendel University in Brno, the Czech Republic, on November 8–9, 2017. It provided a relevant 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, and beyond with participants arriving both from the Czech and European educational and research institutions.

The success of the event is reflected in the papers received, with participants coming from diverse backgrounds – stimulating a substantial international and multicultural exchange and mutual share of experience and ideas. The accepted papers are published in full in these proceedings after being admitted to Conference Proceedings Citation Index (Clarivate Analytics).

The conference of this calibre can succeed only as a team effort, so the editors express their thanks and gratitude to all committees and reviewers both for their outstanding work and invaluable comments and advice.

The Editors

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PLANT PRODUCTION

THE YIELD OF POTATOES AND SPELT IN TERMS OF ORGANIC FARMING

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Abstract: Nitrogen fertilization cannot be used by actual needs of plants during vegetation in organic farming. The proper crop rotation and harmonic nutrition are necessary for good and quality products. The methods of treatment are mainly realized by cultivation of green manure crop and fertilizing by organic fertilizers. The aim of the long-term experiment was to evaluate the effect of different localities and different organic fertilizers on crop yield in organic farming. Variants of fertilization included in the experiment are: 1. Unfertilized control, 2. Green manure, 3. Green manure + renewable external sources, 4. Green manure + renewable external sources + auxiliary substances, 5. Green manure + farm fertilizers, 6. Green manure + farm fertilizers + auxiliary substances. Potatoes were fertilized and planted in experimental years 2015–2016. Winter wheat spelt was sown in the experimental year 2016–2017 and there were no fertilization with organic fertilizers in this year. Average yield of potatoes was the highest after combination with green manure + renewable external sources (compost + digestate) + auxiliary substances. This variant achieved yield about 34.1 t/ha, which is increased by 9.4 t/ha compared to the control variant. The highest yield of spelt was observed on the variant with green manure + farm fertilizers. This variant achieved yield about 5.5 t/ha, which is increased by 0.7 t/ha compared to the unfertilized variant. The result from this experiment indicated that farming without livestock may be similar to the production with livestock. However, these results are obtained only from two experimental years. Statistical difference of achieved yields was observed between each experimental station in both experimental years.

Key Words: potatoes, spelt, organic farming, yield

INTRODUCTION

Organic agriculture is currently 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 such as 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 the content of organic matter and quality of humus in the soil (Martin and MacRae 2014).

The basis of nutrition in organic farming should be a proper crop rotation (Urban et al. 2003). The supply of nitrogen from external environment is primarily achieved by growing legumes and plants suitable for green manure. Another invaluable sources of nutrients are organic fertilizers, especially manure and slurry but also organic compost and increasing use of digestate. The combination of well-chosen crop rotation with adequate dose of properly selected organic fertilizer is very important and proves irreplaceable role for organic farming (Barker 2010).

This work is a part of a long-term experiment established in 2014 by the Central Institute for Supervising and Testing in Agriculture. 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 yield of potatoes and spelt from years 2016 and 2017 will be evaluated.

MATERIAL AND METHODS

Small plot field experiment was established as a precise and long-term research. The experiment took place at 5 different experimental stations representing different production areas (Table 1). The experiment tried to compare different organic fertilizers simulating systems with or without breeding livestock in organic farming. Each variant had three repetitions. The yields potatoes were evaluated in experimental year 2016. The yields of spelt were evaluated in the experimental year 2017.

Table 1 Characteristics of experimental stations

Experimental station	MASL	Characteristics				
		Crop area	Soil type	Soil texture	Average annual precipitation (mm)	Average annual temperature (°C)
Věrovany	207	Sugar beet	Black Soil	Clay	502	8.7
Čáslav	260	Sugar beet	Black Soil	Clay	555	8.9
Jaroměřice nad Rokytnou	425	Cereals	Brown Soil	Clay Loam	481	8.0
Horažďovice	475	Potatoes	Cambisol	Sandy Loam	585	7.8
Lípa	505	Potatoes	Cambisol	Sandy Loam	594	7.5

The application of compost and manure (for experimental year of 2016) was performed in August of 2015. Green manure crop (*Pisum sativum var. arvense*) was sown immediately after the incorporation of the organic fertilizers. The average yield of green manure ranged between 0.5 to 4.7 t/ha (depending on experimental station) in dry matter. Green manure was incorporated into the soil by mulching before winter. The fertilization of potatoes is described in Table 2. Planting of potatoes was carried out approximately 14 days after the incorporation of digestate and fermented urine to the soil in early April of 2016. The auxiliary substance for potatoes was applied two times in May. Auxiliary substance was based only on mixture of natural, water soluble oligopeptide, amino acids, magnesium, potassium and trace elements. Harvest of the potatoes was performed at the first half of September.

Table 2 Variants of fertilization of potatoes used in the experiment (same for all locations, 2015–2016)

Variants of fertilization	Application of organic fertilizers				Auxiliary substance (AS)
	Dose of fertilizer	Period	Dose of fertilizer	Period	Dose and period
1. Unfertilized	-	-	-	-	-
2. Green manure (GM)	-	-	-	-	-
3. GM + renewable external sources	27 t/ha of compost	Autumn 2015	14 t/ha of digestate	April 2016	-
4. GM + renewable external sources + AS	27 t/ha of compost	Autumn 2015	14 t/ha of digestate	April 2016	5 l/ha 2x in May
5. GM + farm fertilizers	27 t/ha of manure	Autumn 2015	14 t/ha of fermented urine	April 2016	-
6. GM + farm fertilizers + AS	27 t/ha of manure	Autumn 2015	14 t/ha of fermented urine	April 2016	5 l/ha 2x in May

Legend: AS - Auxiliary substances: magnesium as MgO – min 4.0%, potassium as K_2O – min 1.0%, boron as B – 0.04%, manganese as Mn – 0.1%, copper as Cu – 0.05%, molybdenum as Mo – 0.001% , zinc as Z – 0.2% , iron as Fe – 0.04%.

Spelt was not fertilized by organic fertilizers in experimental year 2017. Only the application of auxiliary substance was performed in this year. The idea behind this is to simulate common praxis. If there is a good forecrop fertilized by organic fertilizers (potatoes in 2016), there is usually not necessary to fertilize in second year. The second reason for omitting fertilization of spelt was its low

level of resistance to lodging. The application of auxiliary substance to the soil on variants 4 and 6 was performed before sowing of spelt. Bacteria fertilizer was used as an auxiliary substance. Harvest of the spelt was performed at the end of July. The obtained results were evaluated by two factors analysis of variance (ANOVA) with subsequent verification based on Tukey test ($P < 0.05$). The data were processed using the STATISTICA CZ 12. Results are expressed as a mean ± standard deviation.

RESULTS AND DISCUSSION

Yield of potatoes in 2016

The impact of locality was evaluated as a statistically significant. The average estimated yield of potatoes in the Czech Republic in 2016 was about 29.4 t/ha (CZSO 2016). It is evident from Figure 1 that only two experimental stations (Jaroměřice nad Rokytnou and Lípa) achieved lower yields. However, it is a good result achieved in organic farming. For example, results obtained from experiment performed by El-Sayed et al. (2015) and Plaza et al. (2013) proved that organic production of potatoes could be an alternative method to conventional production without significant reduction of yield.

Each locality had different prerequisites for achieving the optimal yields. The experimental station in Věrovany was determined as a reference locality (100%) due to the best soil and climate conditions. The achieved yield on this locality was very high due to the optimal course of weather during the year. Potatoes yields achieved from every other station were detected lower compared to reference station (Figure 1). This fact was caused mostly by drought in these stations during the experimental year of 2016, especially in germination and after emergence of potatoes.

Figure 1 Average yield of potatoes in the experimental stations (2016)

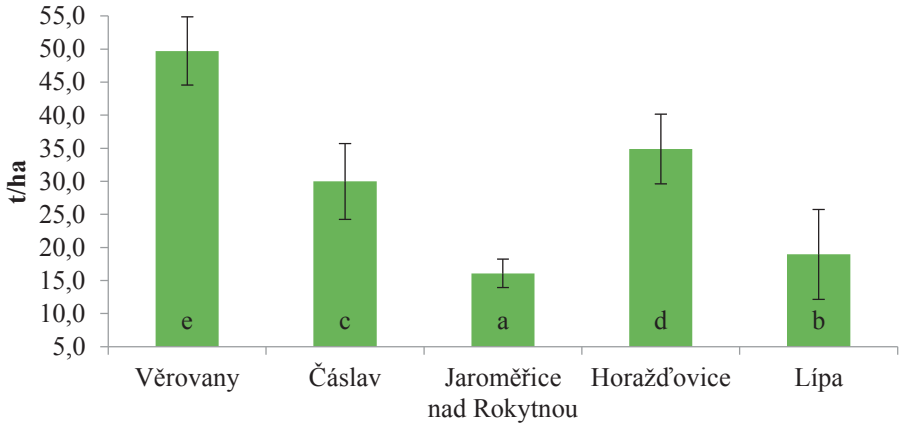
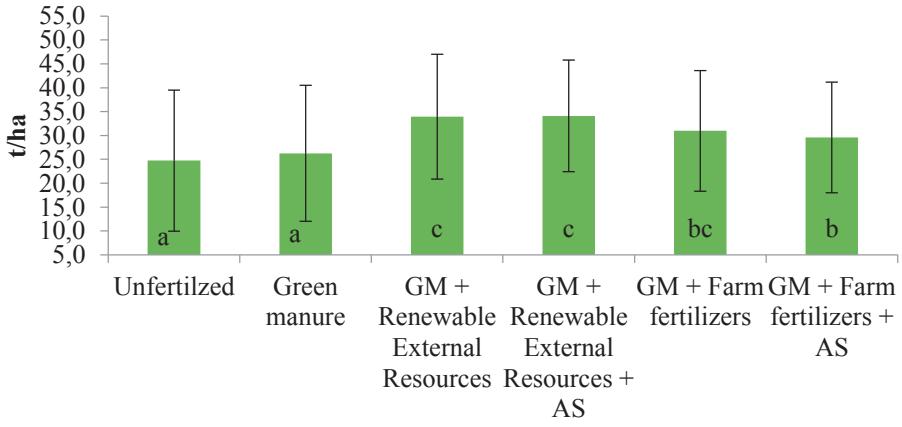


Figure 2 Average yields of potatoes (2016)



There is also statistical difference between examined variants of fertilization (Figure 2). Unfertilized control and variant with only green manure provided similar yield. Green manure itself could not fully substitute the incorporation of organic fertilizers. Variants with organic fertilization provided better crop yield. This is a different result compared to the experiment performed by Makarewicz et al. (2015). Their results showed, that fertilization with green manure itself fully substituted manure in the production system of potato cultivation. Similar yield were achieved on variants 3 and 4 with GM + renewable external sources and then on the variants 5 and 6 with GM + farm fertilizers. The variants 3 and 4 with GM + renewable external sources provided the highest yield. The yield of these variants was increased by about 9.2 t/ha (37%) compared to the control variant. Most importantly, there was also an increase of yield compared to the variants with GM + farm fertilizers by 3.0 t/ha and 4.5 t/ha. These results were probably caused by higher content of nitrogen in organic fertilizers (compost + digestate) used in this variant compared to other variants.

Yield of spelt in 2017

The results show statistically significant difference between experimental localities as can be seen from Figure 3. Konvalina (2013) is describing average yield of spelt in terms of organic farming about 2.8 t/ha in Czech Republic and 2.2 t/ha in Austria. Average yield of spelt achieved in this experiment was 5.2 t/ha. This result was probably caused by good forecrop (potatoes) fertilized two times by organic fertilizers (Table 2). The concerns about lodging of spelt have not been confirmed, spelt at each experimental station endured at upright position during whole vegetation. This fact has also contributed to the reduction of harvest losses.

Figure 3 Average yields of spelt in the experimental stations (2017)

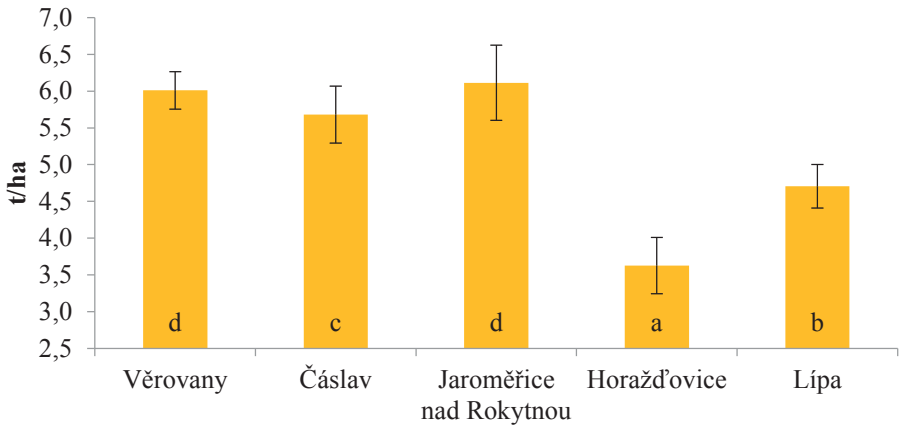


Figure 4 Average yields of spelt (2017)

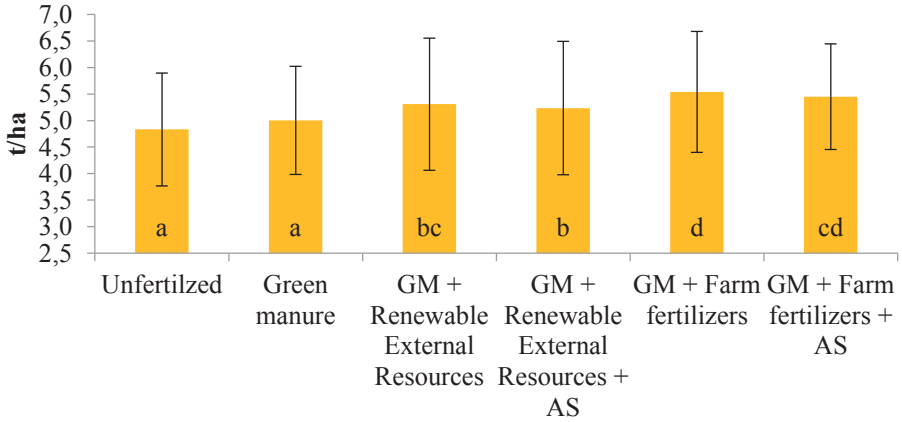


Figure 3 shows average yields of grain and their statistical significance for each experimental station in year 2017. The lowest yields were observed at stations Horažďovice and Lípa. This result

was caused by drought during experimental year in combination with worse soil conditions at these localities. The highest yield, 6.1 t/ha, was achieved at station Jaroměřice nad Rokytnou. Experimental station Věrovany, which is determined as a reference station due the best soil and climate conditions, achieved slightly lower average yield (6.0 t/ha), probably due to the high occurrence of diseases (*Blumeria graminis*) and pests (*Oulema melanopus, Oulema lichenis*).

Figure 4 is describing average yields of grain and their statistical significance for each variant of fertilization in year 2017. The lowest yield with no statistical difference was observed on Unfertilized variant and variant with Green manure alone. This result is similar to experimental year 2016. However, the highest yields about 5.5 t/ha were provided by both variants with GM + farm fertilizers. This result is an interesting change in comparison with the result in previous year. Highest yield of potatoes provided by variants with GM + renewable external resources in year 2016 were caused by higher content of quickly available nitrogen in digestate and compost. Fertilization with organic matter was not performed in year 2017, so the spelt was only taking up rest of the nutrients from previous year. The result from this year indicates that fertilization with farm fertilizers (manure + fermented urine) provided more nutrients in second year after fertilization in comparison with renewable external resources (digestate + compost). Similar results were observed in experiment performed by Rieux et al. (2013), Miller et al. (2010), Hradil et al. (2007) and Gale et al. (2006). They have also described application of manure as preferable variant in comparison with compost. On the other hand, there are also results supporting fertilizing with compost as a superior choice (Miller et al. 2009, Larney et al. 2006, Sanchez et al. 2004).

CONCLUSION

The results obtained from the experimental year 2016 showed that any application of organic fertilizers either from green manure itself, renewable external resources or farm fertilizers proved the increasing yield compared to unfertilized variant. This result was not obviously surprising. However, in organic farming, it is not possible to rely on crop fertilizing during vegetation according to the current needs. The application of any organic fertilizers played therefore a crucial role for plants and yields. The results from the year 2017 confirmed that the variant with green manure itself provided better yields compared to unfertilized variant but the difference was not detected as statistically significant. Highest yields were observed on variants fertilized by organic fertilizers before forecrop.

A statistical difference between the examined variants of fertilization was found out in both experimental years. The obtained results showed, that the highest yields of potatoes were provided by the combination of compost and digestate. Organic fertilizers used in these variant contained more quickly available nitrogen in comparison to other variants of fertilization. The idea came out that compost provided more nutrients for plants in the first year after the incorporation compared to manure which was supported by the result of this experiment. The result obtained from the year 2017 showed, that the highest yield of spelt were provided by the combination of manure and fermented urine. This result supports the idea, that manure provides more nutrients for longer time after incorporation to the soil in comparison with compost.

A lot of auxiliary substances are allowed to be used in organic farming. The results obtained from this experiment showed that the application of auxiliary substances in organic farming did not provide any statistically different yield compared to the same variant of fertilization without AS.

Statistical difference of achieved yields was observed between each experimental station in both experimental years. This result was only a confirmation that the production of yields is heavily dependent on the content of nutrients in soil, good soil condition and optimal course of weather (mostly precipitation) during vegetation.

The result from this experiment indicated that farming without livestock may be similar to the production with livestock. However, these results are obtained only from two experimental years. It is going to be interesting to watch the difference between renewable external resources and farm fertilizers in the future of this long-term experiment.

ACKNOWLEDGEMENTS

The research was financially supported by IGA grant, no. IP_058/2017. Special thanks for the opportunity to be a part of this experiment belong to people from Central Institute for Supervising and Testing in Agriculture.

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THE FERTILIZATION OF SOYBEAN WITH SULPHUR

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Abstract: Different sulphur fertilizers and influence of their foliar application on biomass yield of soybean were determined in vegetation pot experiment. Variants of fertilization included in the experiment were: 1. Control variant, 2. Thiosulphate sulphur, 3. Elemental sulphur and 4. Polysulphide sulphur. The significant highest average yield of biomass, 198 g per pot, was obtained after fertilization with thiosulphate sulphur. Thiosulphate sulphur has increased yield of biomass by 48 g per pot (almost 32%) compared to unfertilized control variant. Content of nitrogen in plants detected 20 days after fertilization was also highest on thiosulphate variant. Content of nitrogen detected in plants fertilized by thiosulphate variant was 0.92%, which is by 0.06% more compared to nitrogen content in unfertilized plants. The effect of elemental sulphur on yield of soybean biomass was not statistically different in comparison with control variant.

Key Words: soybean, sulphur, yield

INTRODUCTION

The situation surrounding genetically modified food and genetically modified materials for industrial processing has been increasingly solved in recent years. Currently, the costumers are more interested in GMO and they are often pushing for GMO-free and "healthy" products (Loureiro and Hine 2001, Evanson and Santiello 2004, Kolodinsky 2008). Therefore, the pressure on production of GMO-free raw materials is increasing. For example, the producers in Germany or Switzerland can only sell GMO-free milk from dairy cows (Thomas and Venus 2015, Bickel et al. 2009). Naturally, these dairy cows have to be fed only with GMO-free feed. One of the most commonly used source of protein for livestock is extracted grit from soybean. Soybean grit contains high content of proteins and essential amino acids and it is characterized by good digestibility. The production of soybean in European Union is relatively insignificant in comparison with the import of soybean from the world (FEFAC 2017). The European Union is dependent on import of soybean from other countries almost from 80%. The highest production and export of soybean is coming from Brazil, Argentina and USA (COCERAL 2015). However, the majority of this production is GMO soybean. This genetically modified product may have a higher nutritional value or contain more vitamins or amino acids. The positive or negative long term effect of GMO products on human or animal organism is yet to be discovered and a lot of customers are looking for GMO-free product, as mentioned before. Therefore, it is important to find a possible way out. One of the possible options is to examine alternative sources of proteins. Another idea is focusing on cultivation of GMO-free soybean. The aim of this idea with fertilization of soybean is to produce GMO-free soybean products with similar quality as their genetically modified opposite.

This work is a part of a two year experiment. This experiment contains a several interconnected partial goals. The aim of the experiment is to secure the whole process of protein feed production for livestock. The partial goals are focusing on plant demands on soil and nutrition, microbiological purity of cultivated material (plants) or usability of nutrients, especially nitrogenous substances from individual feeds. Three possible sources of protein are examined in the experiment – soybean (*Glycine max*), buckwheat (*Fagopyrum esculentum*) and fenugreek (*Trigonella foenum-graecum*). The aim of this part of the experiment is to examine fertilization of soybean with sulphur and its effect to the yield of biomass. The obtained biomass is going to be used as a feed for animals in another part of the experiment.

MATERIAL AND METHODS

The study was established as a pot experiment in growth hall of the Department of Agrochemistry, Soil Science, Microbiology and Plant Nutrition, Faculty of AgriSciences, Mendel University in Brno in 2017. The experiment tried to compare the effect of different source of sulphur (thiosulphate, elemental and polysulphide sulphur) on yield of soybean biomass. The variants of fertilization are described in Table 1. Each variant had three repetitions.

Table 1 Variants of fertilization

Variants of fertilization	Fertilizer	Dose of fertilizers	Dose of sulphur per pot
1. Control variant	-	0	0
2. Thiosulphate sulphur	FOLIT®ThioSulf	3 l/ha	8.3 mg
3. Elemental sulphur	FERTI MK - S 800 SC	5 l/ha	36.4 mg
4. Polysulphide sulphur	SULKA - K	3 l/ha	6.7 mg

Soybean (variety Bohemians) was sown in the pot contains 10 kg of soil to a depth of 2–3 cm. Eight seeds were sown in each pot (19th May 2017). Foliar application of sulphur fertilizers was performed in stage of 10th leaf (23rd June 2017). Each pot contained 5 selected plants of soybean in this vegetative stage. Fertilizers had a recommended dose specified by the producer. This recommended dose per hectare was recalculated for use in the pot experiment (calculated with 550,000 plants per ha). The nitrogen content in plant was detected 20 days after fertilization (10th July 2017). One whole plant from each pot was taken for detection of nitrogen content. The content of nitrogen was determined by Kjeldahl method. Harvest of rest of the plants was performed at 2nd August 2017.

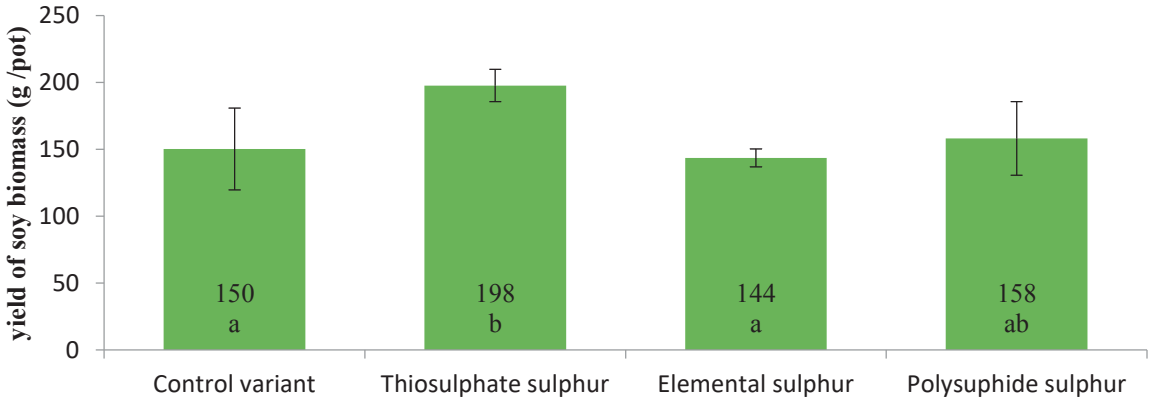
The obtained results were evaluated by single factor analysis of variance (ANOVA) followed by testing at a 95% ($P < 0.05$) level of significance using the Fisher (LSD test). The data were processed using the STATISTICA CZ 12. Results are expressed as a mean ± standard deviation (SD).

RESULTS AND DISCUSSION

The average yields of soybean biomass and their statistical difference among observed variants are described in Figure 1. The significant highest yield of biomass was achieved on variant with thiosulphate sulphur application. The production of plant biomass in this variant reached 198 g per pot, which represented an increase of 32% compared to the control variant. Thiosulphate sulphur is the most commonly used source of sulphur in fluid fertilizer with great effectiveness in supplying available S to the crop (King 2017). It is an effective source of sulphur and it also contains ammonium nitrogen. Riley et al. (2000) were examining the availability of different forms of sulphur to wheat and oilseed rape and sulphate fertilizer was the most available source of S in their experiment. Ammonium thiosulphate was also a more efficient sulphur source in the experiments performed by Islam (2012) and Subrahmanyam et al. (1992). On the other hand, there was no increase of meadow forage yield after application of various sulphur forms in the experiment performed by Šenkyříková and Ryant (2007).

The average yield of biomass on unfertilized variant was 150 g per pot. Elemental and polysulphide sulphur fertilization has not significantly increased soybean yield compared to unfertilized variant. The biomass yield obtained on these variant were 143 g and 158 g per pot, respectively (Figure 1). Polysulphide sulphur can be used as fertilizer, but it is commonly used more as a plant protection against diseases and insects, especially as a fungicide. Elemental sulphur is the most concentrated form of sulphur. This form of the sulphur has to be also oxidized to the sulphate before plants can use it. The effectiveness of elemental sulphur depends on several factors, including particle size, dose and method of application and environmental conditions.

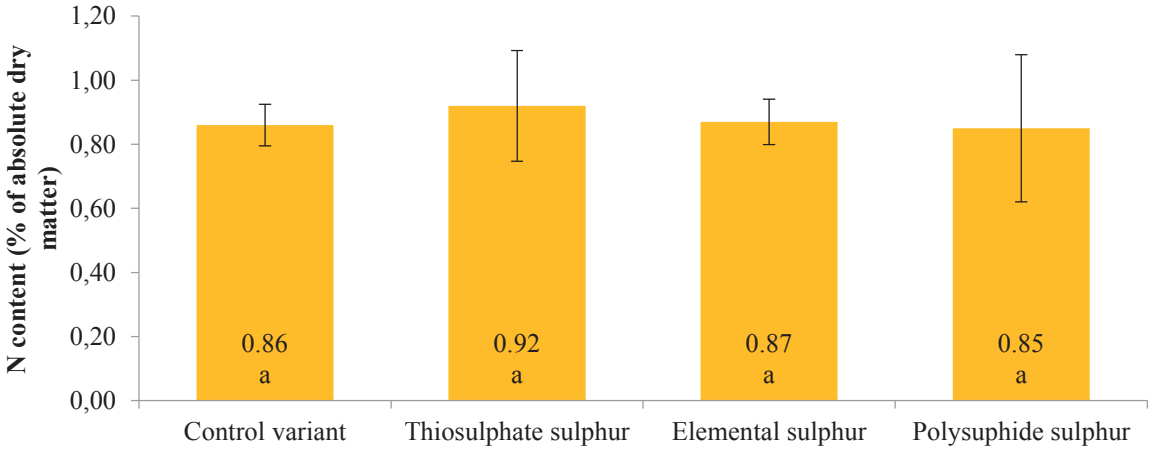
Figure 1 Average yield of soybean biomass (2017)



Legend: Means with same letter are not significantly different (P<0.05), results are expressed as a mean ± standard deviation

The highest yield of soybean biomass on variant fertilized by thiosulphate sulphur can be explained: 1) thiosulphate is very good effective source of sulphur, which has significant effect on nitrogen utilization (Klikocka et al. 2016, Olivoto et al. 2016, Channabasamma et al. 2013, Zhou et al. 2012, Ryant and Hřivna 2004) and 2) FOLIT®ThioSulf fertilizer contains more nitrogen (200 g N/l) in comparison with other fertilizers in the experiment. As it is evident from Figure 2, content of nitrogen in plants was also highest after thiosulphate sulphur application, despite no statistical differences between variants.

Figure 2 Average content of nitrogen in plants (2017)



Legend: Means with same letter are not significantly different (P<0.05), results are expressed as a mean ± standard deviation

Increased yield after fertilization with S and N was also observed by Sharma and Sharma (2014). According to some authors (Sharma et al. 2016, Sexton et al. 2002, Krishan et al. 2005), fertilization with sulphur alone or again in combination with small dose of nitrogen can also lower the content of amino acid of soybean, but increase its quality by raising the proportion of palmitic and linoleic acids at the expense of oleic acid.

CONCLUSION

The results obtained from the pot experiment with soybean performed at Mendel University in 2017 showed, that the highest yield of soybean biomass was achieved on variant fertilized by thiosulphate sulphur. This variant of fertilization proved statistically different compared to unfertilized control. The thiosulphate application also increased content of nitrogen in plants, although the difference between examined variants was not statistically significant. This result is

supporting synergy effect between sulphur fertilization and usage of nitrogen by plants. Variant of fertilization with elemental sulphur had no statistical effect on yield of biomass compared to unfertilized control.

ACKNOWLEDGEMENTS
The research was financially supported by IGA grant, no. TP_4/2017.

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SPECIES COMPOSITION OF VEGETATION IN WINE VILLAGES ŽABČICE AND UNKOVICE

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Abstract: Vineyards create a very interesting ecosystem with very specific conditions for vegetation. The purpose of this work is to compile a list of species growing in vineyards of the wine village Žabčice and Unkovice. During the monitoring of the vineyards of the Žabčice and Unkovice wine villages, 102 plant species were found. The most occuring species were: *Lolium perenne*, *Amaranthus retroflexus*, *Chenopodium album*, *Arrhenatherum elatius*, *Achillea millefolium*, *Setaria pumila*, *Convolvulus arvensis*, *Portulaca oleracea*, *Plantago lanceolata*, *Calamagrostis epigejos*, *Potentilla erecta* a *Cirsium arvense.*

Key Words: vegetation, vineyards, plant species, Žabčice, Unkovice

INTRODUCTION

Important branches of agriculture in the Czech Republic are the cultivation of wine and wine production. After joining Czech Republic to the European Union, are these sectors on the rise (Hlušek et al. 2015). The species-rich landscape is valuable cultural heritage, especially the landscape within wine regions. The landscape with vineyards increases an esthetical value and improve the space for recreation (Kraus 1999).

The wine region Moravia includes 96% of all registered wine areas in the Czech Republic. It consists of 4 wine sub-regions: Mikulov, Slovácko, Velkopavlovická and Znojmo within 312 wine villages (Wine of Czech Republic 2015).

For greening in vineyards is very important to choose the plant species, which are not competing with the wine plant. Some of these species have a positive effect on wine plant grow (Pavloušek 2014a, 2014b). Vineyard weed communities are formed of species adapted to the life cycle of the grapevine and to human interventions in the crop. Botanical researches in vineyard areas reveal species structure of weed flora, distribution and quantitative occurrence of individual species in several vineyards regions. They help to distinguish common species, most important species as well as rare or scarce species in vineyard areas (Eliáš 1983).

Decreasing of potential demands for plant protection is affected by the plant species (supporting species), which support the nature predators of wine parasites (or other wine harmful organisms); (Landis et al. 2000). E.g.: vegetation can increase the predators and parasitoids activity by nectar production (Winkler et al. 2006). This increased activity can have a positive effect on decreasing damages of the crop. Planting supporting species increase the total costs, however it is compensated by lower costs for crop protection (Bianchi et al. 2006). Nevertheless the effect of vegetation, which increase the predators and parasitoids activity, decreasing number of parasites and pests is not always clearly effective (Gurr et al. 2003).

The purpose of this work is to compile a list of species growing in vineyards of the wine village Žabčice and Unkovice. Other purpose of this work is to evaluate the importance of plant species in terms of vine growing and ecosystem.

MATERIAL AND METHODS

Clima characteristics area of interest is following: average year temperature is 9.42 °C, average year precipitation is 510 mm, and average year sunlight is 2 244 hour (Wine of Czech Republic 2015).

Characteristics of the interest territory Žabčice

The cadastral area of Žabčice is located in the South Moravian Region, about 20 km south of Brno. The altitude is in the range of 177–220 m. The area falls into a very warm and dry climatic region. The total area of the Žabčice cadastral area is 817.9 ha, of which the agricultural land is 673.9 ha. Within agricultural land, arable land is 479.4 ha, meadows and pastures 32.3 ha, orchards 25.4 ha and vineyards 117.5 ha.

The Žabčice village is governed by the wine law as a wine village belonging to the wine region of Moravia and the Velkopavlovická wine subregion. Within the wine village there are 5 vine lines of the Staré vinohrady, Horní díly, Koválov, Čtvrtky a Zahrádky. Čtvrtky and Zahrádky are not currently planted with vineyards and therefore have not been evaluated.

Characteristics of the interest territory Unkovice

Cadastral area of Unkovice is located in the South Moravian Region, about 20 km south of Brno. The altitude is in the range of 177–220 m. The area falls into a very warm and dry climatic region. The total area of the Unkovice cadastral area is 372.5 ha, of which the agricultural land is 276.5 ha. Within agricultural land, arable land is 206.8 ha, meadows and pastures 16.5 ha, orchards 13.7 ha and vineyards 28.5 ha.

The Unkovice village is governed by the wine law as a wine village belonging to the wine region of Moravia and the Velkopavlovická wine subregion. Within the wine village there are 2 vine lines of the Přední trať Unkovická, Díly.

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. Inspection routes were determined on the selected territories within the wine lines. Scientific names of individual plant species were used according to Kubát (2002), categories of plant rarity and endangerment follow redlist of Grulich (2012). 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 evaluated vineyards

The first evaluated area was the vine lines Staré vinohrady (wine village Žabčice). A similar cultivation method is applied to the entire vineyard. Alternating cultivation and grassed inter-rows are used here. During the monitoring, 65 plant species were found in total.

The second evaluated area was the vine lines Koválov (wine village Žabčice). The line area consist of vineyards or arable land. Alternating cultivated and grassed inter-rows are used here. During the monitoring, 54 plant species were found in total.

The third evaluated area was the vine lines Horní díly (wine village Žabčice). Most of the line area consist of vineyards. Alternating cultivated and grassed inter-rows are used here. During the monitoring, 71 plant species were found in total.

The fourth evaluated area was the vine lines Díly (wine village Žabčice). Most of the line area consist of arable land, however small parts of the area are consist of vineyards. On this plot is used grassed inter-rows. During the monitoring, 34 plant species were found in total.

The fifth evaluated area was the vine lines Přední trať Unkovská (wine village Unkovice). The line area consist of vineyards or arable land. Alternating cultivated and grassed inter-rows are used here. During the monitoring, 40 plant species were found in total.

During the monitoring of vegetation in vineyards was found 102 plant species. The plant species occurence within the vine lines and it´s intensity can be found in Table 1, Table 2 and Table 3.

Table 1 The plant species occurence within the vine lines and specified by wine village, Žabčice and Unkovice

Plant species	Žabčice			Unkovice	
	Staré vinohrady	Koválov	Horní díly	Díly	Přední trať Unkovská
Achillea millefolium	3	2	3	2	3
Amaranthus retroflexus	3	3	3	2	3
Anagallis arvensis	1	1	1	1	
Anthoxanthum odoratum	-	1	1	-	-
Arenaria serpyllifolia	1	1	1		1
Arrhenatherum elatius	3	3	3	2	2
Artemisia vulgaris	1	1	1		1
Atriplex patula	-	-	1	-	-
Berteroa incana	-	-	-	1	-
Bromus hordeaceus	1	-	-	-	-
Bromus sterilis	1	-	1	-	-
Bromus tectorum	1	1	2		1
Calamagrostis epigejos	2	3	3	1	2
Capsella bursa-pastoris	1	1	1	1	1
Carduus acanthoides	2	2	2	2	1
Carlina vulgaris	-	-	1	-	-
Cichorium intybus	-	-	1	-	-
Cirsium arvense	2	2	2	2	2
Convolvulus arvensis	3	3	2	2	2
Conyza canadensis	2	1	1	1	1
Cornus sanguinea	-	1	-	-	-
Crepis tectorum	-	1	1	-	1
Cynoglossum montanum	1	-	-	-	-
Dactylis glomerata	-	1	-	1	-
Daucus carota	2		1	2	
Digitaria sanguinalis	3		3		3
Echinochloa crus-galli		3	2	2	2
Echium vulgare	2	1	2	2	2
Elytrigia repens	1	-	-	2	-
Epilobium ciliatum	1	-	1	1	-
Erigeron annuus	1	1	1	-	-
Erodium cicutarium	2	1	2		1
Erophila verna	1	-	-	-	-
Eryngium campestre	1		2		
Euphorbia esula	1	-	-	-	-
Falcaria vulgaris	3	1	1	2	
Festuca pratensis	-	2	-	-	-
Festuca rubra	2	2	3		
Filago vulgaris	1	1	-	-	-
Galinsoga parviflora	-	-	1	-	-

Table 2 Continue of table 1

Plant species	Žabčice			Unkovice	
	Staré vinohrady	Koválov	Horní díly	Díly	Přední trať Unkovská
Galium mollugo	-	1	1	1	-
Galium verum	-	-	-	1	-
Geranium pusillum	1	1	1		1
Geum urbanum	1	-	1	-	-
Hieracium glomeratum	1	-	-	-	-
Hordeum murinum	1	1	3		1
Humulus lupulus	1	-	-	-	-
Hypericum perforatum	-	1	-	-	-
Chenopodium album	2	2	3	3	3
Chenopodium hybridum	1	1	-	-	-
Chenopodium pedunculare	1	-	-	-	-
Chenopodium pumilio	-	-	1	-	-
Chenopodium strictum	1	-	-	-	-
Lactuca serriola	-	1	-	-	-
Lamium amplexicaule	-	1	1	-	-
Lamium purpureum	-	-	1	-	-
Lappula squarrosa	1	1	1		1
Lathyrus tuberosus	-	-	-	1	-
Linaria vulgaris	-	-	-	-	1
Lolium perenne	3	3	3	2	3
Lycopsis arvensis	1	-	-	1	1
Malva neglecta	1	-	1	-	-
Matricaria discoides	-	-	1	-	-
Medicago lupulina	-	-	1	-	2
Melica transsilvanica	1	-	1	-	-
Papaver rhoeas	-	-	-	1	-
Parhenocissus quinquefolia	-	1	-	-	-
Petrorhagia prolifera	1	-	-	-	1
Plantago lanceolata	2	1	2	3	3
Plantago major	-	-	2	-	-
Poa annua	-	1	1	-	-
Poa bulbosa	1	-	-	-	-
Poa pratensis	2	-	-	-	-
Polygonum aviculare	1		2		1
Portulaca oleracea	3	3	3		3
Potentilla erecta	2	2	2	1	3
Prunella vulgaris	-	-	-	1	-
Robinia pseudacacia	2		2		
Rosa canina	2	1	1		1
Rubus sp.	-	-	1	-	-
Rumex acetosa	1	-	-	-	-
Rumex crispus	-	1	-	1	-
Sambucus nigra	-	-	1	-	-
Securigera varia	1	1		1	

Table 3 Continue of table 1

Plant species	Žabčice			Unkovice	
	Staré vinohrady	Koválov	Horní díly	Díly	Přední trať Unkovská
Senecio vulgaris	1	-	1	-	1
Setaria pumila	3	1	3	3	2
Setaria viridis	1	-	1	-	-
Silene latifolia	2	1		1	2
Solanum decipiens	-	-	1	-	-
Solanum nigrum	1	1	2		1
Solidago canadensis	-	-	1	-	-
Stachys palustris	-	-	1	-	-
Taraxacum sect. Ruderalia		1	1		1
Thlaspi arvense	-	1	1	1	-
Tragopogon orientalis	1	1	1		
Trifolium arvense	1	1	2		1
Trifolium pratense	-	-	1	-	-
Trifolium repens	2	1	2		2
Tripleurospermum inodorum	1	2	1	2	1
Urtica dioica		2	1		
Veronica persica	1	-	1	-	-
Viola arvensis	2	1	2		1

Evaluation of plant species occurrence in monitored vine lines

Most species were found on the Horní díly vine line (Žabčice wine village). On the contrary, the least species was found on the Díly vine line (Unkovice wine village).

Of the found plant species that are able to compete directly with the grape-vine, we can say above all: *Securigera varia, Humulus lupulus, Parhenocissus quinquefolia, Rubus sp., Arrhenatherum elatius, Artemisia vulgaris, Cirsium arvense, Euphorbia esula, Elytrigia repens, Rosa canina, Cornus sanguinea, Convolvulus arvensis, Rumex crispus, Rumex acetosa, Robinia pseudacacia, Calamagrostis epigejos*.

Of the found plant species that are classified as invassive species or expansive species, we can name above all: *Amaranthus retroflexus, Solanum decipiens, Lactuca serriola, Chenopodium pumilio, Arrhenatherum elatius, Galinsoga parviflora, Bromus sterilis, Bromus hordeaceus, Bromus tectorum, Portulaca oleracea, Robinia pseudacacia, Calamagrostis epigejos, Erigeron annuus, Conyza canadensis, Epilobium ciliatum* and *Solidago canadensis.*

In addition, several rare species of plants have been found among them: *Filago vulgaris* (C3), *Petrorhagia prolifera* (C4a), *Poa bulbosa*, *Melica transsilvanica* (C4a), *Lappula squarrosa* (C3) and *Cynoglossum montanum* (C2b). Of rare plant species, *Filago vulgaris* and *Melica transsilvanica* were found more frequently. The occurrence of other rare plant species was very small.

The occurrence of the plant species was influenced by the habitat in the vineyard. The most frequent plant species in around of trunks were *Convolvulus arvensis*, *Amaranthus retroflexus*, *Solanum decipiens* and other. Plant species *Amaranthus retroflexus*, *Chenopodium album*, *Setaria pumila* and *Portulaca oleracea* were dominant in cultivated inter-row. Perenial species *Lolium perenne*, *Arrhenatherum elatius*, *Achillea millefolium*, *Plantago lanceolata*, *Calamagrostis epigejos*, *Potentilla erecta* and *Cirsium arvense* were found especially in grassy inter-row.

CONCLUSION

During the monitoring of the vineyards of the Žabčice and Unkovice wine villages, 102 plant species were found. The most frequently occurring species belonged: *Lolium perenne, Amaranthus*

retroflexus, Chenopodium album, Arrhenatherum elatius, Achillea millefolium, Setaria pumila, Convolvulus arvensis, Portulaca oleracea, Plantago lanceolata, Calamagrostis epigejos, Potentilla erecta and *Cirsium arvense.*

Vineyards create a very interesting ecosystem with very specific conditions. As consequence, they are creating a place for a variety of plant species. Some rare plant species find survival sites here. However, the vineyards also contain invasive species, which pose a danger not only for the vegetation of the vineyards, but also for the surrounding ecosystems.

ACKNOWLEDGEMENTS

This work was supported by a Programme of applied research and development of national and cultural identity, project DG16P02R017 "Viticulture and wine for preservation and restoration of cultural identity of wine regions in Moravia ".

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SPECIES COMPOSITION OF VEGETATION IN THE ACTIVE PART OF THE MUNICIPAL WASTE LANDFILL IN NĚTČICE

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Abstract: The aim of the paper is to establish and determine the species composition of plants that are able to sustain themselves in an active landfill in Nětčice cadastral area. Two areas were selected within the land with the actively used part of the landfill. Municipal waste is deposited in the first area, and biowaste is deposited in the second area. The evaluation of the vegetation was carried out using the phytosociological methods. The evaluation of the coverage of the species found in the selected habitats was performed using a redundancy analysis (RDA). Altogether 77 plant species were found. The plants in landfills can have an affect even on the surrounding ecosystems. Deep-rooting species, species whose seeds are spread by the wind and entomophilous plants can be problematic. The most commonly found species were *Digitaria sanguinalis*, *Artemisia vulgaris*, *Medicago lupulina* and *Trifolium hybridum.*

Key Words: vegetation, landfill, municipal waste, biowaste

INTRODUCTION

The changes in the countryside are evident, and they are accompanied by a significant transformation of the biodiversity in Central Europe. In terms of prevention, research on ruderal species is therefore essential (Sukopp and Werner 1983).

According to Wania et al. (2006), habitats influenced by human activity are characterized by colonization by non-native species in the form of neophytes or archeophytes. Non-native species were found mainly in trampled habitats, annual ruderal vegetation sites, herbaceous anthropogenically-based vegetation sites or on weed-infested arable land. On the one hand, the current flora is enriched with new species, but on the other hand, many species are disappearing as well.

The presence and spread of species is affected not only by abiotic factors, but also by human activity. These factors then have the effect of influencing the so-called species diversity (Čepelová and Munzbergova 2012).

Plant species are influenced by human regulatory intervention. This process of disturbance, an event that results in plant species being suppressed, opens up space for colonization by non-native species. Such a change in the regime results in the disturbance of the competitive relationships between domestic species, and the whole community with increased susceptibility to invasive species is destabilized (Hobbs and Humphries 1995).

Municipal waste landfills are a typical habitat of ruderal vegetation. The supply of new diaspores (fruits, seeds, and vegetative propagation organs), the sufficiency of nutrients and the constant disturbance of the habitat create conditions for sustaining only certain plant species. The aim of the study is to determine the species composition of plants that are able to sustain themselves in an active landfill and to divide them into functional groups according to their ability to spread to the surrounding environment.

MATERIAL AND METHODS

Characterization of growing locality

The work was conducted in the cadastral area Nětčice. The area is located in a triangular space delimited by main roads connecting the villages of Zdounky, Nětčice and Troubky-Zdislavice 450 m SW of Nětčice. It is a sanitary landfill incorporated with multilayer composite bottom liner, leachate and landfill gas collection system, and a final cover system. In terms of maintenance, the landfill is classified in the S-category - other waste, sub-category S-OO3. Up to now, Stage I of 19 200 m^2 has been constructed together with parts of Stage II (5 500 m^2) and Stage III (7 500 m^3). The facility receives waste (category of other waste) from a catchments area with the population of ca. 75 000 residents. The approved landfill sector for waste of sub-category S-OO1 has not been opened yet. The sector will be intended for the disposal of waste (category of other waste) with the low content of organic biologically degradable substances. A sector of the landfill will be intended largely for the disposal of asbestos-containing wastes, gypsum-based waste, stabilized waste, waste with the high sulphur content and waste with the increased content of metals. Waste with the substantial content of organic biologically degradable substances must not be stored in that sector (Vaverková et al. 2012).

The area belongs in the Kojetín bioregion (Culek 1996) situated in central Moravia and occupying the geomorphological subunit of Central Moravia Floodplain. The bioregion is formed by a broad alluvial plain with regulated rivers. Biota is of azonal character and dominated by agrocoenoses, preserved floodplain forests, remainders of meadows and ponds with abundant fauna (Vaverková et al. 2012).

According to Quitt (1971), the entire region lies in the warm zone T2. Weather is warm, with low rainfall.

Methodology for vegetation evaluation and statistical processing

Two areas were selected within the land with the actively used part of the landfill. Municipal waste is deposited in the first habitat, and biowaste is deposited in the second habitat before it is composted.

The evaluation of the vegetation was carried out using the phytosociological method. The size of the phytosociological plots was 20 m^2. The coverage was estimated as a percentage. The monitoring took place in July 2017. Five phytosociological plots were recorded at each habitat (together ten). The scientific names of each weed species were used according to Kubát (Kubát et al. 2002).

The evaluation of the coverage of the species found at the selected habitats with different waste was carried out by means of multidimensional analyses of ecological data. A redundancy analysis (RDA) based on the linear response model was used.

RESULTS AND DISCUSSION

Altogether 77 plant species were found. The average coverage of species found in the monitored habitats with different wastes deposited is specified in Table 1.

The results of the RDA analysis, which evaluated the relationship of the habitat to the different types of waste and plant species, are significant for all canonical axes at the significance level $\alpha = 0.064$ and are therefore statistically inconclusive. The ordination diagram (Figure 1) represents the graphical results. However, based on the results, the species can be divided into four groups.

The first group of species was more common in the habitat with biowaste, and according to the composition of the species, we can postulate that they are probably species that were brought to the habitat with the biowaste. The group included the following species: *Amaranthus retroflexus*, *Anethum graveolens*, *Atriplex patula*, *Cannabis ruderalis*, *Capsella bursa-pastoris*, *Cucurbita maxima*, *Fallopia convolvulus*, *Helianthus tuberosus*, *Hordeum vulgare*, *Chenopodium album*, *Lactuca serriola*, *Malva neglecta*, *Sisymbrium officinale*, *Solanum tuberosum*, *Tagetes patula* and *Triticum aestivum.*

The second group of species was more common in the habitat with biowaste, and according to the composition of the species, we can postulate that they are probably species that were already present in the habitat and were able to thrive in the landfill. The group included the following species: *Acer negundo*, *Anagallis arvensis*, *Atriplex hortensis*, *Cirsium arvense*, *Conyza canadensis*, *Crepis*

biennis, *Dipsacus fullonum*, *Erigeron annuus*, *Euphorbia helioscopia*, *Galinsoga parviflora*, *Juglans regia*, *Medicago lupulina*, *Papaver somniferum*, *Persicaria lapathifolia*, *Picris hieracioides*, *Polygonum aviculare*, *Portulaca oleracea*, *Solanum lycopersicum*, *Sonchus asper*, *Trifolium hybridum*, *Trifolium repens*, *Tripleurospermum inodorum*, *Urtica dioica* and *Veronica polita.*

The third group of species was more common in the habitat with the municipal waste, and according to the composition of the species, they are native species and do not have the tendency to populate new habitats. The group included the following species: *Achillea millefolium*, *Arrhenatherum elatius*, *Artemisia vulgaris*, *Ballota nigra*, *Bromus sterilis*, *Carduus acanthoides*, *Dactylis glomerata*, *Elytrigia repens*, *Chelidonium majus*, *Lolium perenne*, *Melilotus albus, Papaver rhoeas, Phragmites australis*, *Plantago lanceolata* and *Verbascum thapsus.*

The fourth group of species was more common in the habitat with the municipal waste, and according to the composition of the species, they are native species and have the tendency to populate new habitats or have a high tolerance to a disruption of their habitat. The group included the following species: *Apera spica-venti*, *Atriplex prostrata*, *Atriplex sagittata*, *Avena fatua*, *Calamagrostis epigejos*, *Convolvulus arvensis*, *Daucus carota*, *Digitaria sanguinalis*, *Echinochloa crus-galli*, *Ligustrum vulgare*, *Malus domestica*, *Plantago major*, *Prunus avium*, *Reseda lutea*, *Robinia pseudacacia*, *Rosa canina*, *Rubus sp.*, *Setaria pumila*, *Silene latifolia*, *Sisymbrium loeselii*, *Solanum nigrum* and *Tanacetum vulgare.*

Table 1 The average coverage of species in the observed habitats with different waste

Species	Abbreviations	Habitat (average coverage in %)	
		Municipal waste (Waste)	Biowaste (Bio_waste)
Acer negundo	*Ace negu*		1.0
Achillea millefolium	*Ach mill*	3.2	
Amaranthus retroflexus	*Ama retr*	0.6	
Anagallis arvensis	*Ana arve*		2.4
Anethum graveolens	*Ane grav*	0.6	
Apera spica-venti	*Ape spic*	0.2	
Arrhenatherum elatius	*Arr elat*		5.0
Artemisia vulgaris	*Art vulg*	8.6	6.4
Atriplex hortensis	*Atr hort*	0.2	
Atriplex patula	*Atr patu*	1.2	1.4
Atriplex prostrata	*Atr pros*	0.6	
Atriplex sagittata	*Atr sagi*	2.0	
Avena fatua	*Ave fatu*	0.6	
Ballota nigra	*Bal nigr*		0.4
Bromus sterilis	*Bro steri*	0.2	
Calamagrostis epigejos	*Cal epig*	0.2	
Cannabis ruderalis	*Can rude*	6.2	1.8
Capsella bursa-pastoris	*Cap burs*	3.0	
Carduus acanthoides	*Car acan*	1.0	
Cirsium arvense	*Cir arve*	0.2	
Convolvulus arvensis	*Con arve*	0.2	0.6
Conyza canadensis	*Con cana*	1.6	
Crepis biennis	*Cre bien*		0.2
Cucurbita maxima	*Cuc maxi*		0.2

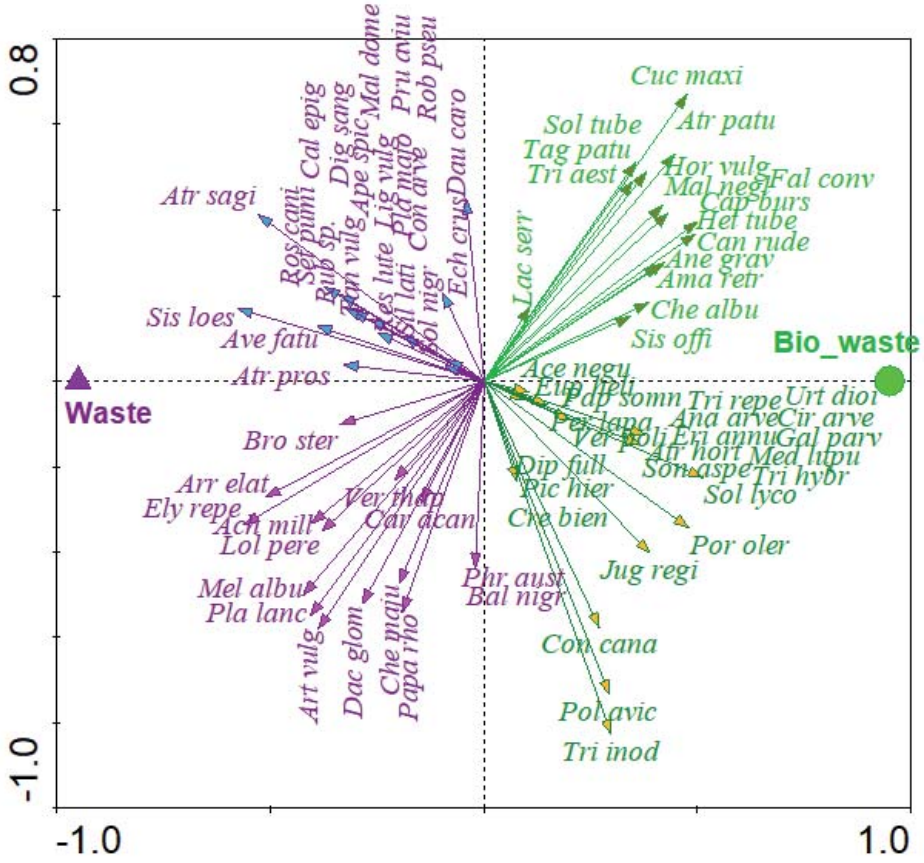
Table 2 The continue of Table 1

Species	Abbreviations	Habitat (average coverage in %)	
		Municipal waste (Waste)	Biowaste (Bio_waste)
Dactylis glomerata	*Dac glom*	0.2	
Daucus carota	*Dau caro*	5.0	3.0
Digitaria sanguinalis	*Dig sang*	17.0	3.2
Dipsacus fullonum	*Dip full*	0.8	
Echinochloa crus-galli	*Ech crus*	0.2	1.4
Elytrigia repens	*Ely repe*		0.2
Erigeron annuus	*Eri annu*	0.4	
Euphorbia helioscopia	*Eup heli*	0.6	1.4
Fallopia convolvulus	*Fal conv*	1.8	0.6
Galinsoga parviflora	*Gal parv*	0.2	
Helianthus tuberosus	*Hel tube*	0.4	
Hordeum vulgare	*Hor vulg*	6.6	5.2
Chelidonium majus	*Che maju*	0.2	
Chenopodium album	*Che albu*	1.4	1.4
Juglans regia	*Jug regi*	1.6	0.6
Lactuca serriola	*Lac serr*	6.0	
Ligustrum vulgare	*Lig vulg*		0.4
Lolium perenne	*Lol pere*		0.2
Malus domestica	*Mal dome*	1.8	
Malva neglecta	*Mal negl*	1.0	
Medicago lupulina	*Med lupu*	10.0	2.0
Melilotus albus	*Mel albu*	0.2	
Papaver rhoeas	*Papa rhoe*	0.2	
Papaver somniferum	*Pap somn*	0.6	
Persicaria lapathifolia	*Per lapa*	0.2	1.0
Phragmites australis	*Phr aust*	2.0	
Picris hieracioides	*Pic hier*	5.2	
Plantago lanceolata	*Pla lanc*	0.2	
Plantago major	*Pla majo*	0.4	
Polygonum aviculare	*Pol avic*	2.0	
Portulaca oleracea	*Por olera*	0.2	
Prunus avium	*Pru aviu*	11.6	2.0
Reseda lutea	*Res lute*	1.0	
Robinia pseudacacia	*Rob pseu*	0.6	
Rosa canina	*Ros cani*	0.8	
Rubus sp.	*Rub sp.*	0.2	
Setaria pumila	*Set pumi*		0.4
Silene latifolia	*Sil lati*	0.6	

Table 3 The continue of Table 1

Species	Abbreviations	Habitat (average coverage in %)	
		Municipal waste (Waste)	Biowaste (Bio_waste)
Sisymbrium loeselii	*Sis loes*	5.0	0.6
Sisymbrium officinale	*Sis offi*	0.4	
Solanum lycopersicum	*Sol lyco*	0.2	
Solanum nigrum	*Sol nigr*	0.2	
Solanum tuberosum	*Sol tube*		2.0
Sonchus asper	*Son aspe*	0.2	
Tagetes patula	*Tag patu*		0.2
Tanacetum vulgare	*Tan vulg*	3.0	
Trifolium hybridum	*Tri hybr*	9.6	6.0
Trifolium repens	*Tri repe*	4.0	
Tripleurospermum inodorum	*Tri inod*	1.0	
Triticum aestivum	*Tri aest*	0.2	
Urtica dioica	*Urt dioi*	1.2	1.8
Verbascum thapsus	*Ver thap*	0.4	
Veronica polita	*Ver poli*	1.0	

Figure 1 Ordination diagram (RDA) expressing the relationship of the plant species found and different habitats with different types of waste



Legend: A "waste" habitat with municipal waste. A "bio_waste" habitat with biowaste. Explanations of species abbreviations are mentioned in Table 1.

CONCLUSION

During the monitoring period, altogether 77 plant species were found. This is only a one-year observation, and further monitoring dates are necessary to found statistically conclusive. Nevertheless, the results indicate interesting behaviour in a many of the plant species found. Many plant species are contaminated with the waste in the habitat, and some of the species have been resisting the disturbances associated with the landfill process very well.

The plants in landfills can have an affect even on the surrounding ecosystems. Deep-rooting species (*Prunus avium*, *Convolvulus arvensis*), species whose seeds are spread by the wind (*Lactuca serriola*) and entomophilous plants (*Medicago lupulina*, *Trifolium hybridum*) can be problematic. Landfills can serve as a source of fruit and seeds, and these species can spread to the surrounding environment. The roots of some species can grow all the way to the waste and receive dangerous substances into their bodies from the waste. These substances can be contained not only in the fruits or seeds but also in the nectar, and they can spread to the surrounding environment by the wind and insects.

ACKNOWLEDGEMENTS

This work was created with the financial support of project no. TP 5/2017 of the Internal Grant Agency of the Faculty of AgriSciences at the Mendel University in Brno.

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SEASONAL GROWTH DYNAMIC OF NORWAY SPRUCE AT THE STUDY SITE OF RÁJEC (DRAHANSKÁ VRCHOVINA HIGHLAND)

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Abstract: The circumference increment assessment of Norway spruce focused on the effect of inter tree competition in the mature spruce stand was made at the study site of Rájec (Drahanská vrchovina Highland) over a 5-year period. Data were collected from 49 trees, which were monitored continuously with mechanical band dendrometers from 2010 to 2014. The dependency of the circumference increment on competition index, diameter at breast height, Lang's rain factor, mean temperature of various periods and sum of precipitation of various periods was evaluated. Climatic conditions of the study site are characterised with warm and wet summers and cold-dry winters. In 5 years average around 61% of the annual precipitation falls during growing season. There was highly significant correlation between relative increment and temperature ($p=2.324^{e-13}$) and significant correlation between relative increment and precipitation ($p=0.0439$). These results confirmed that inter–tree competition and diameter at breast height are sufficient variables for circumference increment estimation of unmeasured trees in the particular year. Coefficient of determination reached 0.25–0.63 for competition and 0.40–0.84 for tree diameter at breast height. The present investigation brings important results about tree growth and seasonal growth dynamics and its relation with competition and microclimatic conditions in mature spruce stand.

Key Words: dendrometers, seasonality, *Picea abies*, stem girth increment, competition

INTRODUCTION

Norway spruce (*Picea abies* (L.) Karst.) is one of the most important European tree species and also a tree species with the highest number of various health and growth problems which have appeared in the last decades (Rybníček et al. 2010). It is amongst the trees most strongly affected by forest dieback in Central Europe, which is generally attributed to industrial and automobile pollution (Eckenwalder 2009).

The presence of distinct seasonal changes is the main prerequisite for trees forming growth rings. However, our knowledge concerning the timing of the various phases and the rate of wood formation is still far complete (Savidge et al. 2000, Chaffey 2002). The seasonality of an organism's growth should be tuned to the annual cycle of resource availability (Muir et al. 1997). For many regions, the period of wood cells formation remains unknown, or the variation of growth rate during that period. The main reason for the gaps in our knowledge is the difficulty in measuring xylem formation at short intervals (Chaffey 2002).

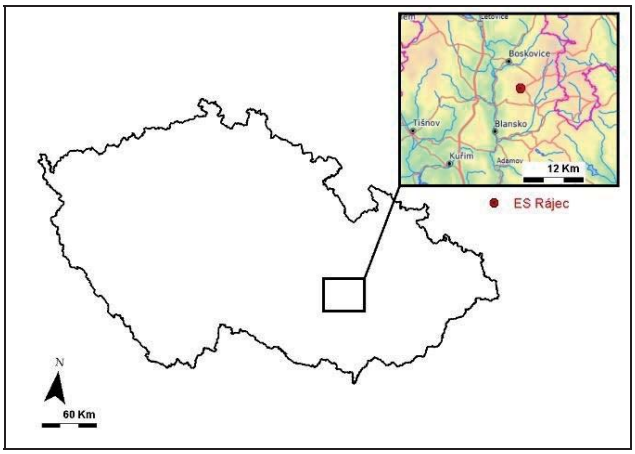
In this study, we presented the investigation of seasonal growth dynamic of Norway spruce at the research site Rájec. Our objective was then to assess the progress of the stem radial increment focused on the effect of diameter, climate and inter-tree competition in a mature spruce forest located in an intensive research plot situated in Drahanská vrchovina Highland, which plays important role in international ecological monitoring infrastructure.

MATERIAL AND METHODS

The samples for the study were obtained at the study site of Rájec (Figure 1), about 30 km to the north of Brno (geographic coordinates N49°26'37", E16°41'48"). The study site is located in the natural forest area 30 Drahanská vrchovina Highland, forest vegetation zone 5 (fir–beech), representing about 2.7% of the Czech Republic area. This study site was established for long–term detailed experiments for various scientific issues. The bedrock consists of intrusive rock acid granodiorite of Brno Massive (Hruška 1980). The soil type was determined as unsaturated acidic brown forest soil (Klimo 1992), and it is modal oligotrophic Cambisol (Němeček et al. 2001). The site is situated at an altitude ranging between 620–630 m a.s.l. (Klimo 1992) and in a moderate climatic region (Quitt 1971). Mean annual air temperature at the study site is 7.1 °C and mean annual sum of precipitation 673 mm (Marková et al. 2015).

Increment as a dependent variable, and competition index, diameter at breast height, Lang's rain factor (LRF), monthly temperatures and precipitations as independent variables for the study area were used to calculate the correlations of values of girth or circumference increments with climatic factors. Pearson's correlation analysis, Regression analysis and Lang's rain factor were performed to compare competition and stem increment. Meteorological measurements and dendrometer and circumference measurements were also performed. The seasonal variability was created in STATISTICA 10 application. Hegyi's single tree competition index model was proposed to calculate the competition index.

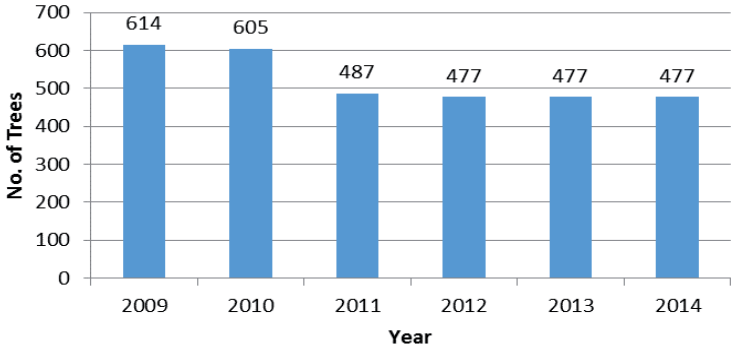
Figure 1 Location of the study site of Rájec (the Drahanská vrchovina Highland)



RESULTS AND DISCUSSION

In this research a dendroclimatic investigation on Norway spruce from 2010 to 2014 (2009 was additionally included) was conducted. The number of trees (Figure 2) at the studied stand has decreased between 2010 and 2011 due to an intense cutting, as most trees have felt down because of the silvicultural management and/or severe climatic conditions.

Figure 2 Number of trees per year at the study site of Rájec (Drahanská vrchovina Highland) in 2009–2014



The relative increment of the girth of the portion of stem in individual years (5-year increment is 100%) is shown on Figure 3. To see the effect of climatic conditions on stem girth increment there are shown only trees with recorded stem increment in whole 5-years' period. Figure 4 describes the relative increment of the stem girth in the studied years 2010–2014; the confidence interval was very wide (for comparison year of 2011 with the highest value of confidence interval and year of 2013 with the lowest confidence interval). The year 2012 showed the second highest confidence interval. Trees increment in 2013 had significantly lower than in 2012 and 2014.

At each of the development stages, climatic factors manifest different degrees of impact.

Figure 3 The relative stem increment in spruce stand at the study site of Rájec (Drahanská vrchovina Highland) in 2010–2014

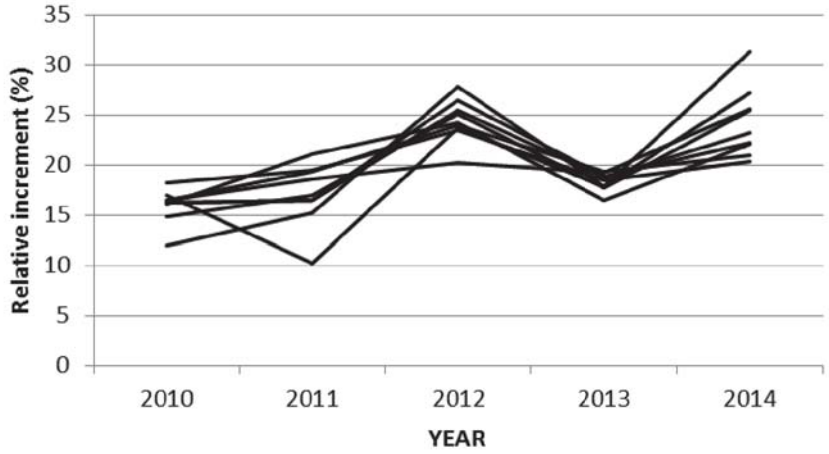
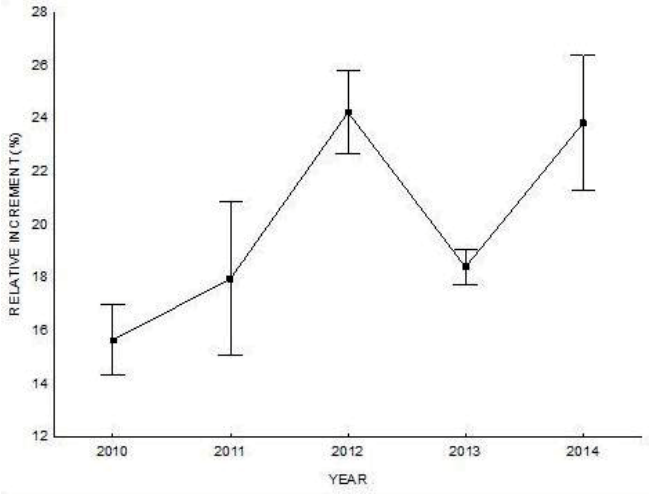


Figure 4 The relative stem increment in spruce stand at the study site of Rájec (Drahanská vrchovina Highland) in 2010–2014



The relative increment for any given year often integrates the effects of the previous and current's year's climate. There were tested 300 periods and combined all possible complex periods of the mean monthly air temperatures, and their correlations with stem increments, with the duration from one month up to January of the previous year to September of the current year, among which 25 best correlations of the girth or circumference increment with mean monthly air temperatures had positive highly statistical significant values. The period with the highest correlation of increment and mean monthly air temperature was from September of the previous year till September of the current year, i.e. the period of 13 months.

The growth of Norway spruce was statistically significantly affected only by precipitation in May of the previous year. In our case, the correlation was not good enough with precipitation to estimate the stem increment, as we had one result between significant and non-significant

($p = 0.0439$). The second best correlation of the girth or circumference increment with precipitation is from July to September, either of the previous year was not statistically significant. The growth of Norway spruce was less statistically significantly affected by precipitation in September of the previous year and the precipitation in September of the current year.

CONCLUSION

The effect of climate, tree size (characterized with diameter at the breast height) and competition on variations in annual circumference increment of Norway spruce (*Picea abies* (L.) Karst.) trees were investigated in a mature spruce stand located at the study site of Rájec (Drahanská vrchovina Highland, the Czech Republic).

There were tested 300 periods of the mean monthly air temperatures, and their correlations with stem increments, with the duration from one month up to January of the previous year to September of the current year. All possible complex periods were combined. 25 best correlations of the girth or circumference increment with mean monthly air temperatures had positive highly statistical significant values. Variability of circumference increment differed according the size of the trees, competition index and the number of days in a given period. The high variability of circumference increment during the season might be due to physiological process resulting in stem saturation of water dynamics.

The microclimate at the study site is characterized by warm-wet summers and cold–dry winters. This study revealed that competition index and stem diameter at the breast height were good parameters for tree growth prediction; correlation was very good with air temperature [$p < 0.01$ (highly significant)], and it is possible to say that it is a good estimator in this case. The growth of Norway spruce was less statistically significantly affected only by sum of precipitation. Overall, there was highly significant correlation between air temperature and relative stem increment, and significant correlation between sum of precipitation and relative stem increment was confirmed.

It is obvious that unsuitable climatic conditions for spruce can lead to stem shrinkage during growing season. Here we assume that these responses are caused mostly by water storage deficit in stem and this leads to decreasing of the tree vitality.

This study provides new data revealing the basic growth processes of Norway spruce trees, and provides significant information to quantify the responses of tree growth to expected global warming. This approach provided a great opportunity to deepen our understanding and knowledge about the interactions of different environmental factors with the short-, medium- and long-term growth dynamics of one of the most important forest tree species.

Dendrometer traces should be compared with dynamics of xylem cell development to date onset of cambial activity and girth or circumference stem growth (i.e., extracted daily girth or circumference increments, cambial activity and enlargement of first tracheids). A comparison with cellular analyses can be useful to determine crucial phenological events such as cambial growth onset and ending and stem radius increment on the basis of dendrometer data, as both techniques will enable direct observation of the periodic process of cambial activity and tracheid differentiation (girth or circumference cell enlargement, secondary wall thickening, lignification and cell death) as suggested in some recent studies.

ACKNOWLEDGEMENTS

This research was performed within the Institute of Forest Ecology (Faculty of Forestry and Wood Technology–FFWT) at Mendel University in Brno (Czech Republic).

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SPECIES SPECTRUM OF WEEDS IN BIOBELTS FOUNDED IN THE CADASTRAL TERRITORY SOBŮLKY

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Abstract: The article evaluates the weed species composition in selected agri-environmental measures - bio-belts. In order to determine the weed species spectrum, vegetation plots were recorded on selected land blocks located in the cadastral area of Sobůlky in the South Moravian Region. Bio-belts were founded in April 2017. The evaluation of vegetation was carried out in June 2017. Crops of winter wheat (*Triticum aestivum*) and maize (*Zea mays*) were sown on the land blocks. *Chenopodium album*, *Cirsium arvense*, *Papaver rhoeas* and *Helianthus tuberosus* were found as the most common weeds or dominants in biobelts. The differences in weed species between plots in bio-belts and in close or distant arable land are presented.

Key Words: agricultural landscape, agri-environmental measure Rural development program

INTRODUCTION

Within the agricultural landscape, we can find natural habitats or semi-natural habitats such as ecologically important landscape segments as wetlands, forests, shrubs or meadows (Rumanovská et al. 2011).

Farming systems that support the recovery and management of valuable habitats are expensive and therefore less sustainable. The need to protect and restore nature and environmental components has led to the introduction of programs and projects in developed European countries where farmers are paid for increased protection or environmental rehabilitation (Rumanovská et al. 2011).

Under rural development policy, a wide range of measures are being funded by the Member States or regions in the European Union to promote the sustainable development of their rural areas. Member States shall draw up their rural development programs (RDPs) at national or regional level according to their needs and in accordance with their national strategic plans. Rural development programs are co-funded by the EU and the Member States (European soil data center 2009). In the Czech Republic, agri-environment measures (agri-environment and climate measures) are supported by the Czech Republic's Rural Development Program 2014–2020, which was approved by the government on 9 July 2014 (SZIF 2014). Agri-environment measures are designed to strengthen the prevention of soil degradation, to strengthen the soil and landscape retention capacity. Furthermore, they have the task of preserving and restoring valuable habitats on agricultural and forest land in terms of species diversity, increasing the stability and aesthetic value of the landscape, and also strengthening the functional interconnection of the landscape (EAGRI 2014).

One of the financed agri-evnironmental measures are bio-belts. This measure supports the establishment of non-productive areas on arable land. The main objective is to increase the food supply for bird communities, as well as other animal species linked to agricultural landscape.

Bio-belts also form a significant contribution to the promotion of crop diversity. They have a varied species composition compared to the edges of the convection fields. The reason is a different way of farming, especially the restriction of pesticides and the application of fertilizers (Walker et al. 2007).

Bio-belts are considered as non-producing areas. Should they be treated the same way as field crops, it could result in a negative influence on the species composition of plants of bio-belts. Therefore, it is forbidden in the whole range of bio-belts to use fertilizers and plant protection products. These conditions can discourage farmers from the establishment of bio-belts. If there is a risk of spreading weeds from the bio-belts, the point use of herbicides is authorized with the written approval of the phytosanitary measure by the Central Control and Testing Institute of Agriculture. The occurrence of weeds, however, depends on many factors, one of which is, for example, the supply of seeds in the soil. However, the composition of the mixtures for sowing bio-belts should be designed to avoid excessive spreading of weeds outside the bio-belts.

Bio-belts create space for expansion of plant and animal species. However, weed also belongs between plant species. Goal of our work is to evaluate the participation of plant species, which can raise growing number of weed on land block with bio-belt and also on the neighbour land blocks.

MATERIAL AND METHODS

Characterization of selected area

The area of interest is located in the cadastral area of Sobůlky (Hodonín district) in the South Moravian Region (Czech Republic). The arable land is managed in this area by the agricultural cooperative Zemagro Strážovice Inc. and by private farmers. Arable land is managed under the conventional farming regime.

The geological bedrock is made up mainly of loess and loess clay, to a lesser extent the geological bedrock is made of clay, sand and gravel. Main types of soils are cambisols, chernozems, and leptosols (Inspire 2017). The territory is situated in warm, slightly dry region characterized by long, warm and dry summer, very short transition period with warm to moderately warm spring and autumn and short, slightly warm, dry to very dry winter with very short duration of the snow cover. The warmest month of the year is July with an average temperature from 18 °C to 19 °C; on the other hand, the coldest month is January with average temperatures from -2 °C to -3 °C (the climatic zone T2 according to Quitt 1971.

Characterization of bio-belts

Bio-belts can be characterized as stripes, 6–24 m wide and at least 30 m in length. It must be based on the edge of the land block or inside the land block. The distance between two bio-belts should be min. 50 m and it is also necessary to keep the same distance of 50 m from main roads (motorways, roads of 1st and 2nd class). Land blocks with bio-belts were searched in LPIS (Public land register). Bio-belts must be sown by the specified mixture of crops, with the minimal amount of seed to the given date (SZIF 2014). Crops of selected bio-belts were sown of following crop mixture: *Hordeum vulgare*, *Panicum miliaceum*, *Phacelia tanacetifolia*, *Fagopyrum esculentum*, *Brassica oleracea* and *Lupinus albus* in the period from 1. 4. 2017 to 15. 6. 2017. The crops of land blocks outside the bio-belts were winter wheat (*Triticum aestivum*; land block IDs 5503/4 and 5402/3) and maize (*Zea mays*; ID 5301/15).

Evaluation of vegetation

Four or five vegetation plots were recorded in each bio-belt, depending on its size. The vegetation plots were squared of an area 4 m^2. Apart from the vegetation plots in bio-belts, other plots were placed on the land block in crop in distance circa 3 meters from bio-belts. Furthermore, the third subset of vegetation plots was recorded on opposite site of the land blocks (at a distance of 80 to 500 meters from bio-belts, depending of the size of land block). Simplified scheme of location of vegetation plots is shown on Figure 1. List of plant species and their mean covers were recorded in each of the vegetation plot. Cover was estimated in percentage scale.

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 more species occurring in similar habitats, we present here only *Amaranthus* sp. on a genus level.

Figure 1 Simplified scheme of vegetations plots on land block.

Legend: red squares – vegetation plots in bio-belt; light blue squares – vegetation plots in crop in distance circa 3 meters from bio-belts, dark blue squares - vegetation plots on opposite site of the land block

RESULTS AND DISCUSSION

Following tables (Table 1, Table 2 and Table 3) show the average coverage of individual species found on land blocks.

Land block with winter wheat (5503/4 - ID from LPIS)

A total of 18 plant species were found on this land block, including 11 species of weeds. Of the weed species, the highest average coverage had *Cirsium arvense* and *Helianthus tuberosus*. *Cirsium arvense*, *Consolida regalis*, *Papaver rhoeas* and *Apera spica-venti* are the most common species which were found.

Table 1 Average coverage of species found on the land block with winter wheat

Land block ID 5503/4	In bio-belt n=4	In wheat (plots close to bio-belt) n=4	In wheat (plots distant from bio-belt) n=4
Species name	Average coverage (%)		
Hordeum vulgare *	3.50	0.00	0.00
Panicum miliaceum *	5.75	0.00	0.00
Phacelia tanacetifolia *	39.00	0.00	0.00
Fagopyrum esculentum *	10.75	0.00	0.00
Lupinus albus *	0.25	0.00	0.00
Brassica oleracea *	0.17	0.00	0.00
Triticum aestivum (crop, winter	0.50	100.00	95.00
Reseda lutea	1.25	0.00	0.00
Cirsium arvense	5.00	0.75	0.00
Arctium lappa	2.50	0.00	0.00
Capsella bursa-pastoris	0.50	0.00	0.00
Chenopodium album	0.75	0.00	0.00
Helianthus tuberosus	4.75	0.00	0.00
Descurainia sophia	0.75	0.00	0.00
Consolida regalis	0.25	0.75	0.00
Papaver rhoeas	0.00	1.25	1.67
Apera spica-venti	0.00	3.50	2.00
Galium aparine	0.00	0.00	2.33

*Legend: the sign * indicates the crops sown in bio-belts, LPIS - the public register of land, n= number of vegetation plots (replications) for calculation the mean coverage*

Land block with winter wheat (5402/3 - ID from LPIS)

A total of 9 plant species were found on this land block, including 6 weed species. *Helianthus tuberosus* and *Chenopodium album* had the greatest cover of the weed species.

Table 2 Average coverage of species found on the land block with winter wheat

Land block ID 5503/4	In bio-belt n=4	In wheat (plots close to bio-belt) n=4	In wheat (plots distant from bio-belt) n=4
Species name	Average coverage (%)		
Fagopyrum esculentum *	3.25	0.00	0.00
Phacelia tanacetifolia *	4.50	0.00	0.00
Triticum aestivum (crop, winter	0.00	100.00	100.00
Helianthus tuberosus	83.75	0.00	0.00
Papaver rhoeas	0.00	0.00	3.00
Anthemis arvensis	0.00	0.00	2.00
Chenopodium album	12.50	0.00	0.00
Cirsium arvense	1.25	0.00	0.00
Galium aparine	0.00	2.00	6.75

Land block with maize (5301/15 - ID from LPIS)

On this land block 10 plant species were found, including 5 species of weeds. Weed species *Chenopodium album* reached the highest ground cover.

Table 3 Average coverage of species found on the land block with maize

Land block ID 5301/15	In bio-belt n=5	In maize (plots close to bio-belt) n=5	In maize (plots distant from bio-belt) n=5
Species name	Average coverage (%)		
Hordeum vulgare *	4.00	0.00	0.00
Panicum miliaceum *	8.20	0.00	0.00
Phacelia tanacetifolia *	42.00	0.00	0.00
Fagopyrum esculentum *	12.00	0.00	0.00
Zea mays (crop)	0.00	21.00	27.00
Echinochloa crus-galli	0.00	2.00	5.60
Chenopodium album	24.40	35.00	17.40
Fallopia convolvulus	0.00	0.80	0.00
Cirsium arvense	0.80	0.00	0.00
Convolvulus arvensis	0.40	0.00	0.00
Polygonum aviculare	0.00	11.00	4.00

During the field observation, the most varied spectrum of species of plants was found in bio-belts. Crops from bio-belts did not spread further on the land block. Crops on the land block was already well connected, so crops from bio-belts could not be spreading into the crops on the land block. On the other hand, crops from the land block in the near of bio-belt can be spread to bio-belt (Table 1). Winter wheat was found in bio-belt (probably volunteer winter wheat from year 2016).

The weed species *Helianthus tuberosus* was found in bio-belts on land blocks, where winter wheat was grown (Table 1 and Table 2). Mean cover of 83.75% was found in bio-belt on one of the land blocks with wheat (ID 5503/4). According to Török et al. (2003), *Helianthus tuberosus* is easily propagated by tubers and rhizomes and is considered as invasive plant in numerous environments and also as a significant weed of field crops. The origin of *Helianthus tuberosus* in bio-belts can be due to previous cultivation near the field or spreading by animals.

Some of the found weed species are able to enrich the soil seed bank and it can cause a weed infestation of the area in the coming years (*Chenopodium album, Capsella bursa-pastoris*). Other

species of weeds can spread from bio-belts by anemochoria (*Cirsium arvense*). This may be the reason why bio-belts pose a certain risk of an increase weed infestation.

Chenopodium album was found on all land blocks. This species is characterized by high seed production and long seed life in the soil. The occurrence of *Chenopodium album* depends largely on the seed supply in the soil in locality. The fact that *Chenopodium album* did not appear in winter wheat could be due to the full involvement growth of winter wheat.

Echinochloa crus-galli and *Polygonum aviculare* are typical weeds of root crops, maize and vegetables, our records from land block with maize confirm this assumption. Whereas *Galium aparine* infests all crops, especially winter cereals and *Papaver rhoeas* winter cereals or winter rape.

Convolvulus arvensis can be included among species that can negatively affect crops and plants in bio-belts. It has a deep root system and is resistant to most of herbicides.

Typical weeds of arable land (*Papaver rhoeas, Galium aparine, Apera spica-venti, Echinochloa crus-galli*) did not grow in bio-belts and were present only in the cultivated crop, probably due to the high cover and density of crops and weeds in bio-belts.

CONCLUSION

During the field observation, a total of 15 weed species were recorded. The species composition of vegetation in bio-belts and adjacent plots differed significantly. The higher number of weed plants was found in bio-belts. The most common weeds were *Chenopodium album*, *Cirsium arvense* and *Papaver rhoeas*. Bio-belts perform many important functions in the landscape. In addition to soil protection, they increase the food offer and thereby have a significant benefits for animals living in agricultural landscape.

ACKNOWLEDGEMENTS

The research was financially supported by Mendel University in Brno as a part of the project IGA FA Mendel University in Brno no. IP 32/2017 „Botanical monitoring of selected agri-environment measures“.

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THE EFFECT OF DIFFERENT STRAW MANAGEMENT PRACTICES ON ORGANIC CARBON CONTENT AND HUMIC SUBSTANCES QUALITY

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Abstract: Influence of different straw management practices (SH – straw harvested; SI – straw incorporated; SB – straw burning) onto carbon stock and humic substances quality was studied. Stationary long-term field experiment was conducted at the Mendel University School Enterprise in Žabčice (under the guidance of Dept. of Agrosystems and Bioclimatology). Object of study was Fluvi-Eutric Gleysol under spring barley monoculture carried out in the long-term field stationary experiment since autumn 1969. Oxidimetric titration method was applied for organic carbon determination (soil samples was obtained almost after 50 years of trial). Fractionation of humic substances was determined by short fractionation method. Differences in total organic carbon content under different straw management were found out. Humic substances quality was higher under straw burning and incorporated to compare with straw harvesting. Statistically significant differences were achieved at $p \leq 0.05$.

Key Words: long-term field experiment, spring barley monoculture, straw management, organic carbon, humic substances

INTRODUCTION

Organic matter balance directly influence amount of organic carbon stock in soil. Crop management practices can enhance soil organic matter by optimal crop rotation, organic matter addition, optimal fertilization, and tillage systems. Sustainable agriculture and high productive soils are therefore strongly depended on soil organic matter input (Torresen et al. 2003, Banwart et al. 2015). Soil management becomes very important and should provide improving not only soil properties and production but also high carbon stock in soil. As quoted Hrubý et al. (1996) and Procházková et al. (2002) changes in economic conditions were accompanied by changes in the crop and livestock structure production. Concentration of cereals has been rising and a question how to use a straw for direct fertilization by appropriate techniques became discussed and important. Effect of various straw management practices was studied by Soon (1999) and under similar Czech experimental conditions Dryšlová (2008) and Procházková et al. (2011). They showed that straw fertilization often results in difficult establishment of stand and a higher amount of straw in the upper layer, or on the soil surface has usually a negative effect onto seeds germination. The inhibition is frequently combine with a negative biochemical effect, deficit of water, and water consumption for straw decomposition (Christian and Bacon 1991, Thomson 1992).

The objective of this study was to evaluate the long-term effect of different straw management practices (straw harvested, incorporated, and burning) on to organic carbon stock and humic substances content and quality.

MATERIALS AND METHODS

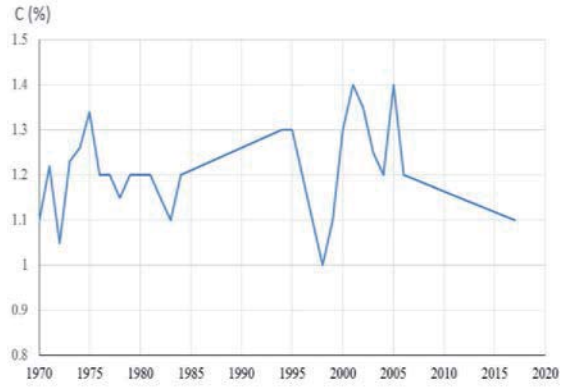
Fluvi-Eutric Gleysol (Žabčice, Czech Republic) belongs to the maize-growing area. Average annual temperature is 9.2 °C. Average annual precipitation 480 mm. Three different straw

managements were estimated: SH – straw harvested; SI – straw incorporated; SB – straw burning. Soil samples were taken by soil probe in 12 replications under each straw variant; upper layer of soil profile (0–0.1 m) were used for fractional composition. Basic soil properties were determined by standard methods. Soil reaction was determined by potentiometric method in distilled water and in 1M KCl solution (1:2.5). Total organic carbon content was determined by oxidimetric titration method (Nelson and Sommers 1996). Fractional composition of humic substances (HS), humic acids (HA) and fulvic acids (FA) ratio (HA/FA) were determined according to Kononova and Beltchikova method (1963). Detailed description of mentioned methods is given in Pospíšilová et al. (2016) and Pospíšilová and Vlček (2015). Data were statistically evaluated using one way ANOVA analysis, followed by Tukey HSD test ($p \leq 0.05$). Statistica CZ 12.0 software (StatSoft software Inc., Tulsa, Oklahoma, USA) was used.

RESULTS AND DISCUSSION

Fluvi-Eutric Gleysol was heavy textured (55–65% of clay particles less than 0.01 mm), weakly acid, with low humus content and middle substances quality. Studied soil is regarded as the medium quality arable soil. Dynamic of total organic carbon (TOC) content during experiment is demonstrated in Figure 1 (average value over all experiment treatments). TOC varied from 1 to 1.4% and obtained average values were typical for this soil type. The dynamic of TOC is not only influenced by organic matter input, fertilizing and tillage systems, but also by climatic conditions and sampling. Data represented in Figure 1 were measured twice a year since 1970 till 2006 (spring and autumn) then in cycle of 5 years. The decrease of TOC between 1995 and 1998 and its increase in 1998 and 2001 was affected by sampling and by the specific climatic conditions. The period from 1995 to 1996 was very dry, which caused higher rate of mineralization and TOC decreasing. The summer in 1997 and the next years were wet, which caused TOC increasing. Accuracy of sampling and soil heterogeneity also influenced the TOC dynamic. Statistically significant difference in TOC under different straw management in 2017 are given in Table 1 and 2 (Tukey test, $p \leq 0.05$). After straw burning (SB) and straw incorporating is evident a statistically significant increase of TOC.

Figure 1 Total organic carbon dynamic in Fluvi-Eutric Gleysol (long-term field experiment, Žabčice, Czech Republic)



Results of HS fractionation are given in Figure 2 and 3. Prevalence of FA was detected only under straw harvesting (SH) variant, which leads to lower values of HA/FA ratio. On the other hand, variants SI and SB showed increasing amount of TOC, HS, HA and FA. Consequently HA/FA ratio is higher. Results were statistically significant at $p \leq 0.05$ see Table 1– 4.

The similar positive effect of straw incorporation into the soil was published also by Hrubý et al. (1996) and Christian et al. (1991). They showed the straw incorporation to compare with straw harvesting caused increasing of humic substances content and humic substances quality. Soil monitoring data from long-term field experiments are important and required not only for soil quality evaluation but also for organic carbon protection. As it was stressed by Banwart et al. (2015) carbon dynamic directly influence also nitrogen and nutrients cycles in the soil. These data are also important for calculation of carbon stock and modelling. Suggested straw incorporation is one of the possibilities of agricultural practice, how to increase carbon stock in soil.

Figure 2 Average content of HA (long-term field experiment, Žabčice 2017, Czech Republic)

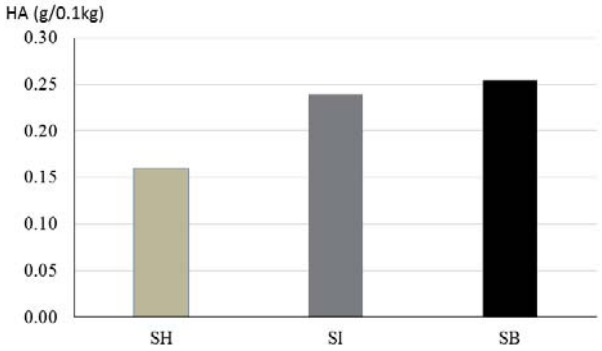


Figure 3 Average content of FA (long-term field experiment, Žabčice 2017, Czech Republic)

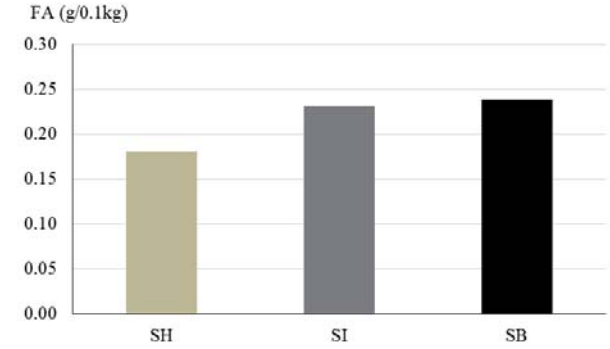


Table 1 Statistically significant differences in TOC content (long-term field experiment, Žabčice 2017, Czech Republic)

Straw variant	Value of TOC (%)
SI	1.11 a
SH	1.14 ab
SB	1.25 b
Average	**1.17**

Legend: Different letters (a, b) designate a significance level $p \leq 0.05$

Table 2 Statistically significant differences in HS content (long-term field experiment, Žabčice 2017, Czech Republic)

Straw variant	Value of HS (%)
SH	0.35 a
SI	0.47 b
SB	0.50 b
Average	**0.44**

Legend: Different letters (a, b) designate a significance level $p \leq 0.05$

Table 3 Statistically significant differences in HA content (long-term field experiment, Žabčice 2017, Czech Republic)

Straw variant	Value of HA (%)
SH	0.17 a
SI	0.24 b
SB	0.25 b
Average	**0.22**

Legend: Different letters (a, b) designate a significance level $p \leq 0.05$

Table 4 Statistically significant differences in FA content (long-term field experiment, Žabčice 2017, Czech Republic)

Straw variant	Value of HA (%)
SH	0.18 a
SI	0.23 b
SB	0.24 b
Average	**0.22**

CONCLUSION

Different straw management can directly influence soil organic carbon stock and content and quality of humic substances. Statistically significant increasing of TOC accumulation after straw incorporation (SI), straw burning (SB) was determined. Humic substances quality was higher under straw burning (SB) and incorporated (SI) variants to compare with straw harvesting (SH).

ACKNOWLEDGEMENTS

The research was financially supported by the National Agricultural Agency by the project QJ1210263 and QJ1610547.

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SELECTED SOIL PROPERTIES UNDER DIFFERENT TYPES OF MANAGEMENT

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Abstract: Basic soil properties such as porosity, soil reaction and humic substances content and quality were evaluated in Haplic Luvisol (Uhřice, Czech Republic) after conversion of ploughing soil into permanent grassland and forest. Soil porosity was calculated from physical cores. Soil reaction was measured by potentiometric method. Humic substances content was determined by short fractionation method. Generally, Haplic Luvisol (arable soil) was assessed as a high productive agriculture soil with good porosity, good humus content and quality, loamy textured, with weakly acid reaction, and high in nutrients content. Under different management systems all of studied properties have gradually changed. Three years of permanent grassland showed increasing of total organic carbon and humic substances content, which means potential for organic carbon accumulation increased. On the other hand there are some negative consequences of intensive soil exploitation such as pedocompation, decreasing of porosity and acidification after conversion. Statistically significant differences were found between arable soil and forest three years after conversion.

Key Words: organic matter, porosity, soil reaction, Haplic Luvisol, land-use change

INTRODUCTION

Content and quality of soil organic matter is a very important factor of sustainable agriculture. Loss of humus leads to decreasing of crop yields and consequently to the soil degradation. Land-use management can enhance soil organic matter by optimal crop rotation, organic matter addition, and optimal fertilization and tillage systems, or by conversion into permanent grassland (Banwart et al. 2015, Guimarães et al. 2013). According to Doran and Parkin (1994) and Sáňka and Materna (2004) healthy soil is defined as a soil, which is able to fulfil all of its functions (e.g. biomass production and its safety, filtration and accumulation functions, transformation and hygienic functions). Parameters of soil quality/health are usually divided into chemical physical and biological (Doran and Parkin 1994, Pospíšilová and Vlček 2015). The most important indicators of good soil chemical properties is appropriate soil reaction, buffering capacity, high cation exchange capacity and stable soil colloidal complex. Soil reaction directly influence soil sorption capacity, plant growth and nutrients regime, and others physical and biological soil properties (Thomas 1996). As quoted Swift (1996) the main components of humic substances (HS) are fulvic acids (FA) and humic acids (HA). Because of relatively long period of their decomposition their ratio (HA/FA) is regarded as an important factor of soil quality/health. Healthy soils have also high porosity, appropriate structure, water holding capacity, and are not compacted (Pospíšilová and Vlček 2015). Soil porosity is closely connected with water regime, soil texture, structure, and directly influence soil chemical and biological properties. Porosity limit for loamy soil is 45 % (Lhotský 2000). All of these properties are influenced by intensive agriculture and by land-use management. Biocorridors play an important role in the landscape, because of elimination of soil erosion and improving life condition for animals. Changes of soil properties after biocorridors construction are not well studied (Horáková 2017).

This study is focused on the effect of biocorridors onto selected soil chemical and physical properties. Main aim is to show how man activities and land-use changes in positive and negative ways affecting soil properties.

MATERIAL AND METHODS

Study was localized in biocorridor Uhřice (Kroměříž region, Czech Republic). Monitoring was carried out during 2014–2016. Soil properties were compared on arable soil, and after its conversion into forest soil and permanent grassland. Following soil properties were observed: texture, soil reaction, conductivity, humus content and quality, nutrient content, hydrophysical properties, and penetrometric soil resistance. In this paper are estimated only selected parameters – soil reaction, humic substances content and quality and porosity.

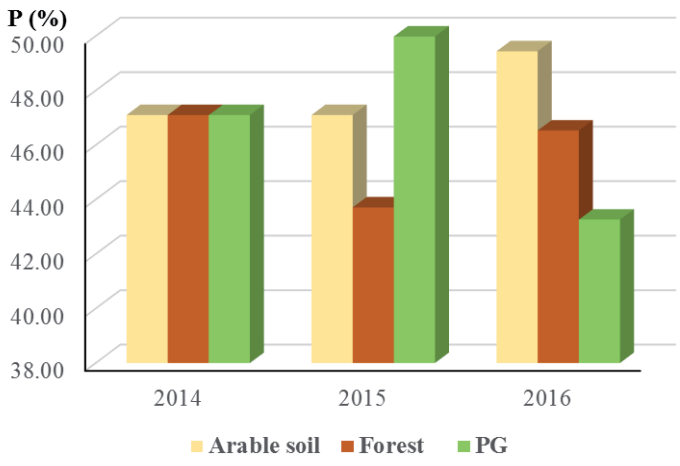
Figure 1 Locality Uhřice – young forest (photo: Eva Horáková)

Haplic Luvisol (locality Uhřice) belongs to the crop growing area. Average annual temperature is 8.5 °C. Average annual precipitation is 650 mm. Soil was sampled twice a year (spring and autumn) during 2014–2016 in the depth 0–30cm. In Figure 1 is illustrated view on the locality after conversion into forest soil. Basic soil properties were determine by standard method. Soil reaction was determined by potentiometric method in distilled water and in 1M KCl solution (1:2.5). Total porosity was calculated from physical cores (average from 3 replications, in the depth 0–30 cm). Total organic carbon content (TOC) was determined by oxidimetric titration method (Nelson and Sommers 1996). Fractional composition of humus was determined according to Kononova and Beltchikova method (1963). Detailed method´s descriptions is given in Pospíšilová et al. (2016). HA/FA ratio was calculated from data of humus fractionation. Humification degree was calculated as a ratio of HS/TOC content multiply by 100. One way ANOVA analysis and t-test were used for statistical data evaluation.

RESULTS AND DISCUSSION

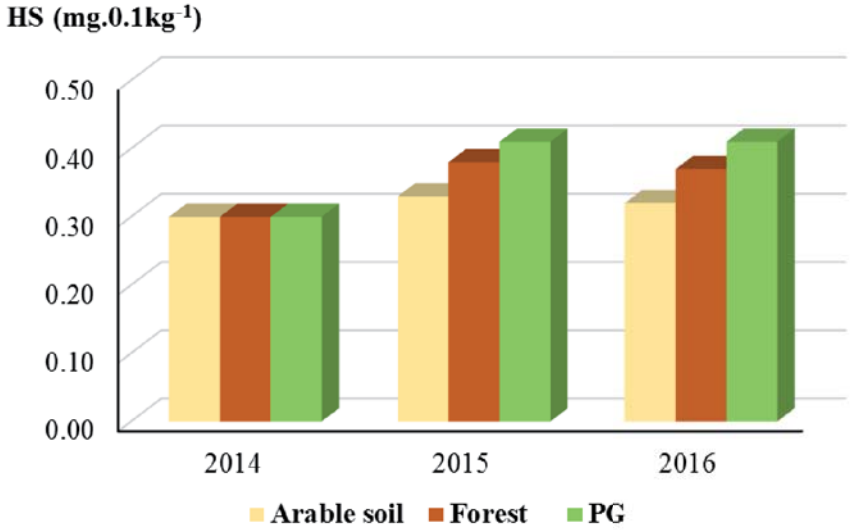
Studied soil was loamy textured, with good porosity, weakly acid, with middle content and low quality of HS. Changes of total porosity after land-use management changing is given in Figure 2.

Figure 2. Average values of total soil porosity (P, %)



Obtained porosity results showed that after conversion in some cases total porosity was lower than the limit (45 %) for agricultural soils. Low porosity and pedocompaction during first year of conversion into forest could be explain by biocorridor construction and using of machinery. We can also conclude that permanent grassland soil (2016) is much more compacted to compare with arable soil. On the other hand, total porosity in arable soil is less than 50 %, which is very close to limit values (45 %) for agricultural soils. It was recommended to improve soil structure and increase soil porosity by application of organic fertilizers.

Figure 3 Average content of humic substances (HS, mg.0.1/kg)



Statistically significant difference in porosity between variants were not found. Humic substances content during studied period is given in Figure 3. Prevalence of FA was determined, which indicate low quality of HS. HA/FA ratio was less than 1, which confirms low quality of HS. Humification degree was middle (30 %). Conversion directly influence HS content.

Statistically significant differences were found between arable soil and forest three years after conversion (LSD = 0.220), see Table 1. No differences were found between arable soil and permanent grassland three year after conversion, but we can say that there is a tendency of increasing of HS amount.

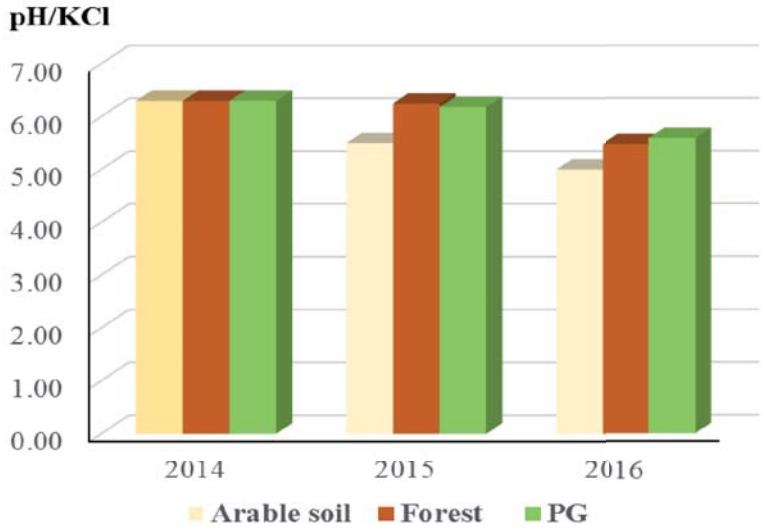
Table 1 Statistically significant differences in humic substances content

Source	*No*	*Sum*	*Average*	*Variance*		
HS (mg/0.1kg; arable soil)	4	1.42	0.355	0.0008333		
HS (mg/0.1kg; forest)	4	1.66	0.415	0.0005667		
Source of variability	*SS*	*Difference*	*MS*	*F*	*P*	F_{crit}
Between sources	0.0072	1	0.0072	**10.28571**	0.0184335	**5.987378**
All sources	0.0042	6	0.0007			
Total	0.0114	7				
LSD	0.220					

Legend: one way ANOVA analysis, $F_{crit} = 3.182$

Results of exchangeable soil reaction is given in Figure 4. Decreasing of soil reaction and increasing of soil acidification is a result of intensive agriculture. Haplic Luvisol after land-use conversion should be later carefully observed. Liming was advised for improving soil reaction in all types of land-use.

Figure 4 Average values of exchangable soil reaction (pH/KCl)



The results are taken from the diploma thesis of Eva Horáková. Other authors do not mention this locality.

CONCLUSION

Different type of land-use directly influence content and quality of humic substances, soil reaction and porosity. Statistically significant increasing of humic substances under forest soil, mainly fulvic acids, was documented. Higher organic carbon accumulation potential is expected in forest soil and under permanent grassland, but the results of three year´s experiment were not statistically significant. With respect to the soil type monitoring of soil physical and chemical properties is recommended. Further it is suggested to control porosity, soil reaction, humic substances content and quality and increasing of liming doses.

ACKNOWLEDGEMENTS

Financial support from the National Agricultural Agency project QJ1210263, and OP VaVpl CZ.1.05/4.1.00/04.0135 project Mendel University in Brno is highly acknowledged.

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CHANGES OF LAND USE IN THE HISTORICAL PERIOD 1845–2015 IN THE CADASTRAL AREA OF VĚTEŘOV

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Abstract: This paper deals with the evaluation of changes in land use in the cadastral area of Věteřov (Hodonín district). The aim was to find out how the use of land changed in the historical period 1845–1948–1990–2000–2015. The ecological stability of the cadastral area Věteřov was also evaluated in the article. An analysis of the data obtained from the Database of Long-term Changes in land Use of the Czech Republic was carried out. Changes were observed in the development of land use. A significant decrease of meadows occurred in the cadastral area of Věteřov. The area of the meadows was 17.60 ha in 1845, whereas no meadows were recorded in the area in 1990, 2000 and 2015. In the case of permanent culture, pastures, water areas, built-up areas and other areas, there was a slight increase in their area between 1845 and 2015. Ecological stability was determined using the Miklós (1986) and Míchal (1994) method. The results showed the differences between two methodologies. The calculation of the ecological stability coefficient according to Miklós showed almost no differences. The value of the ecological stability coefficient was same (0.46) for years 1845, 1990, 2000 and 2015 only in 1948 value of the ecological stability coefficient was 0.43. The results of the Míchal calculation of the ecological stability coefficient decreased from 1845 to 1948, whereas continual increasing was found for 1948 to 2015. Based on results of Míchal calculation is this cadastral area above average mainly used large-scale agriculture. Self-regulation processes of the ecosystem are considerably weakened. It causes ecological lability and requires high energy inputs.

Key Words: arable land, ecological stability, landscape fragmentation

INTRODUCTION

Today's Europe is a mosaic of landscapes, reflecting the evolving pattern of change that land use has undergone in the past. This change continues to alter our landscape and environment today, leaving large and often irreversible land-use footprints. Tensions are rising almost everywhere as society's need for both natural resources and space for settlement and infrastructure conflicts with the capacity of land to support and absorb these needs (EEA 2017).

Landscape fragmentation is important to all human activities that are related to the land (Forman 1995). In the last decades, the expansion of human needs has caused a dramatically higher consumption of the planet's resources with tremendous impacts on land use change and a considerable loss of habitats and biodiversity (Foley et al. 2005, Foley et al. 2011).

The European landscape is largely dominated by agricultural land uses; in fact, more than 35% of all land in the EU has an agricultural use. Thus agricultural land uses have a central role in terms of the potential impacts of land uses on the sustainability of the wider European environment. Land-use models can be used to capture the interactions between many factors that drive land-use changes, and can be used to predict future changes in the land-use patterns (Ustaoglu et al. 2016). Land use provides essential ecosystem services whose quantity and quality is changing according to the socio-economic, political and cultural conditions defined by humans (Verburg et al. 2015) and can therefore be considered a geo-manifestation of inherently spatial political, economic and cultural transitions (Aspinall 2004).

Land use is important for human life. Wu (2008) says, that land use is the backbone of agricultural economics and it provides substantial economic and social benefits. Land use change is necessary and essential for economic development and social progress.

Gutzler (2015) argues, that farmers' adaptation to growing demand may include changes in crops, crop rotation, utilization of crops, and intensification of production. A simple focus of agricultural management aimed solely at maximizing economic returns can lead to depletion of groundwater resources, erosion, loss of water quality, biodiversity loss and a reduction of socio-cultural services. Sustainable development therefore requires consideration of the balance between the economic production functions of agriculture and environmental and social services. Policies are implemented to incentivize farmers to respect this balance by remunerating for the provision of public goods.

In the Czech Republic, arable land account for approximately 60% of the state's area. The nature and character of the landscape are very significant in this way. That is why agriculture has a very important and irreplaceable landscape-building function. At present, in the period of high surplus agricultural products in Europe, the importance of the production function of agricultural production is diminishing, while the importance of other (non-productive) functions of agricultural farms is growing (Bičík and Jančák 2005).

In the second half of the 20th century, the destabilization and destruction trend prevailed in the Czech Republic of landscape systems. The landscape has adapted itself to unified technological processes of agricultural and forestry production and urbanization needs. This trend was reflected by creating large blocks of arable land, creating what longest straightened sections of waterways and creating forest monoculture (Buček 2009).

This has led to the disruption of the ecological stability of the landscape, the devastation of the agricultural land by water and wind erosion, the reduction of biodiversity and the disturbance of the landscape. This contribution is focused on the change of land use and change ecological stability between years 1848–2015 in the cadastral area of Věteřov. This cadastral area is covered mostly agricultural land and forests. Whole Hodonín district is also covered mostly agricultural land and forests. Cadastral area of Věteřov was chosen for evaluation because results from cadastral area of Věteřov can be generalized for Hodonín district.

MATERIAL AND METHODS

Characterization of selected locality

Cadastral territory of Věteřov lies in the district of Hodonín in the South Moravian Region in Czech Republic, about 6 km west of town Kyjov. The total area of the cadastral territory is 818 ha (data from 2015, Regional information service). The territory lies at an average altitude of 264 meters above sea level (Regional information service 2017). In the past (communist period) there was mining activity in this area. This activity strongly influenced the landscape.

Main type of soils are chernozems, rendzinas and pararendzinas and fluvisols. The territory belongs to the geomorphological unit Kyjovská pahorkatina and the geomorphological subcelle Věteřovská vrchovina (Inspire 2017). The cadastral area of Věteřov lies at the intersection of two climatic zones (T2 and T4 climatic zone). Climatic zone T2 is characterized by a long, warm and dry summer, a very short transition period with a warm to moderately warm spring and autumn and a short, slightly warm, dry to very dry winter with a very short duration of snow cover. The warmest month of the year is July with an average temperature of 18 °C to 19 °C; on the other hand, the coldest month is January with average temperatures from -2 °C to -3 °C. A very long summer is typical for T4, which is very warm and very dry. The transition period is very short with warm spring and autumn. Winter is short, warm and dry to very dry with a very short duration of snow cover (Quitt 1971).

Analysis of historical development in land use

Analysis of historical development of land use consisted in comparison of percentage representation of various parts of the land in a historic row from 1845 to 2015 and evaluation of the development of the landscape over time. The underlying data were obtained from the Database

of long-term changes in land use of Czech Republic (Bičík 2011). The underlying data for year 2015 were obtained from Czech Statistical Office. This year are also important in terms of landscape change. The period before 1948 was a period of collectivization. The Czech Republic originated after the end of socialism in 1990. After 2000, the Czech Republic joined the European Union.

Evaluation of ecological stability

The ecological stability coefficient (C_{es}) was calculated to assess ecological stability. For the calculation of the ecological stability coefficient, was used the method according Míchal (1994) and Miklós (1986). Method according Míchal (1994) expresses the ratio number and determines the ratio of the areas of the so-called stable and unstable landscaping elements in the studied area. The method is based on the unambiguous and final classification of a landscape element into a stable and unstable group and does not allow the assessment of a specific state of these elements (Sklenička 2003).

Calculation according to Míchal (1994):

$$C_{es} = \frac{\text{Stable ecosystem}}{\text{Unstabel ecosystem}} = \frac{FA + WA + PC + Pa + We + Or + Vi}{Al + HA + Hg}$$

An explanation of the abbreviations is as follows: FA forest areas, WA watter areas, PC permanent culture, Pa pastures, We wetlands, Or orchards, Vi vineyard, Al arable land, HA human areas, Hg hopgarden.

Method according Miklós was used for the next calculation. This methodology does not derive from the division of areas into stable and unstable, but differentiates their ecological significance by introducing numerical coefficients. The formulas for the calculation are as follows (Sklenička 2003):

Calculation according to Miklós (1986): $C_{es} = \frac{\sum p_{ni} \times \sum k_{pi}}{\sum p}$

An explanation of the abbreviations is as follows: p_{ni} – acreage of individual area; k_{pi} – the coefficient of ecologically significant areas; p – acreage of the area.

RESULTS AND DISCUSSION

Analysis of the historical development of land use

The following table (Table 1) shows data about land use in the cadastral area of Věteřov in 1845, 1948, 1990, 2000 and 2015. The table and figure are divided according to particular areas on permanent cultures (gardens, orchards, vineyards and hop gardens), different areas (built up, other, water areas) and agricultural land (arable land, permanent culture, meadows and pastures).

Table 1 Change in land use over the year 1845–2015

Year	1845	1948	1990	2000	2015
Specific type of land use	Size of area of specific land use (in ha)				
Agricultural land	**523.2**	**538.3**	**512.4**	**511.8**	**512.0**
Arable land	469,2	499,9	442,5	440,8	439.0
Permanent culture	32.1	22.1	46.1	47.2	45.0
Meadows	17.6	0.7	0.0	0.0	28.0
Pastures	4.3	15.6	23.8	23.8	
Forest areas	**274.8**	**257.9**	**258.2**	**258.9**	**260.0**
Different areas	**18.0**	**24.4**	**50.4**	**47.6**	**47.0**
Built up areas	8.9	7.2	10.4	10.6	10.0
Other areas	8.6	16.7	37.4	34.5	35.0
Water areas	0,50	0,50	2,60	2,50	2.0
Sum	816.0	820.6	821.0	818.3	819.0

Analysis of historical land use data pointed to some differences. A significant decrease in meadows areas occurred in the cadastral area of Věteřov. In 1845 the area of the meadows was 17.6 ha and in 1990 and 2000 no meadows were recorded in the area. A slight increase in the area can also be observed in arable land between 1848 and 1948. Then the area of arable land decreased between 1990 and 2015. In the case of permanent culture, pastures, water areas, built-up areas and other areas, there was a slight increase in their area between 1845 and 2015.

Evaluation of ecological stability

Calculations of ecological stability coefficients showed difference between the two methodologies (Table 2). The calculation of the ecological stability coefficient according to Miklós (1986) showed almost no differences. The value of the ecological stability coefficient was 0.46, except for the year 1948 (value of the ecological stability coefficient was 0.43). This methodology evaluates ecological stability on scale 0–1. The closer to value one, the territory is more stable. Based on the calculations made, it can be stated that the territory ranks among the less stable ones (Miklós 1986).

The results of the Míchal (1994) calculation of the ecological stability coefficient were already different. Based on results of Míchal calculation is this cadastral area above average mainly used large-scale farming. Self-regulation processes of the ecosystem are considerably weakened. It causes to ecological lability. Coefficient of ecological stability (according to both methodologies) of cadastral area of Věteřov points to ecological lability due to the high present arable land. In 2000 was average coefficient of ecological a stability according to Míchal in Czech Republic 1.03 (Czech Statistical Office, 2017). Cadastral area of Věteřov was stable less compare with Czech Republic average in 2000. In 2015 was average coefficient of ecological a stability according to Míchal in South Moravia region 0.73. Coefficient of ecological stability of cadastral area of Věteřov was comparable to this average (Czech Statistical Office, 2017).

Table 2 Results of ecological stability coefficient calculations

Method according to:	Miklós	Míchal
Year	Results	
1845	0.46	0.68
1948	0.43	0.57
1990	0.46	0.67
2000	0.46	0.68
2015	0.46	0.69

This paper deals with the evaluation of changes in land use in the cadastral area of Věteřov. The assessed area is made up of more than 50% of the arable land. Data analysis of land use pointed to certain changes. For example, a complete disappearance of grassland areas for the year 2000 and to rise in areas of pasture. In the case of permanent crops, pastures, water areas, built-up areas and other areas, there was a slight increase in their area between 1845 and 2015. The ecological stability calculations evaluated the area as stable less.

The ecological stability of the cadastral area Věteřov was also evaluated in the article. On the basis of calculations of the ecological stability coefficient (Míchal and Miklós), the cadastral territory of Věteřov can be considered as relatively stable. Lower ecological stability could have practical impacts on agricultural activity in the area. The manifestations of these impacts are mostly slow and unobtrusive, but they are manifested in increased soil erosion, which leads to a reduction in the fertility of arable land as well as its prices. Therefore, the importance of measures that can be implemented directly on arable land is growing. These include a set of agri-environmental measures.

CONCLUSION

Ecological stability can be defined according to Forman and Godron (1993) as landscape resilience to disruption and its recovery after disruption. Each landscape component has its degree of stability and the overall stability of the landscape reflects at the same time the ratio of all represented landscape types. The assessed area is made up of more than 50% of the arable land, which can be considered as ecologically unstable system. Arable land is dependent on the supply of external inputs from farmers. To maintain ecological stability, territorial systems of ecological stability are established. Agri-environmental measures can form part of the territorial system of ecological stability in the agricultural landscape and thus contribute to increasing its stability.

ACKNOWLEDGEMENTS

The research was financially supported by Mendel University in Brno as a part of the project IGA FA Mendel University in Brno no. IP 32/2017 „Botanical monitoring of selected agri-environment measures“.

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COMPARISON OF ACTUAL EVAPOTRANSPIRATION FROM ALEXI AND SOILCLIM MODELS

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Abstract: Actual evapotranspiration (ETa) determined by the Atmosphere-land Exchange Inverse (ALEXI) model and water balance model SoilClim was compared for selected districts of the Czech Republic. The ALEXI model uses the land surface temperature (LST) from remote sensing and provides information on ETa and subsequently the surface moisture status. The SoilClim is a dynamic model of water content in soil and represents a model based on water balance approach. The current version of the model is able to estimate the value of ETa, as well as soil moisture content in two layers of the soil profile. Investigated period of ETa comparison were years 2014 and 2015. Especially the year 2015 had a special relevance due to the significant summer drought that occurred in CR. Model performance was compared for the period when changes in vegetation are most significant – from April to August. Week sums of ETa from both models were compared at the district level for Vysočina, Jihomoravský and Olomoucký regions. The ETa values were generally higher from ALEXI as compared to SoilClim. ALEXI values were in some cases even two or three time higher. Moreover, the seasonal dynamics showed sometimes opposite trends. As this is a pilot testing of ALEXI based ETa in the conditions of Central Europe and show large differences as compared to well established methods, more detailed testing is required prior drawing any general conclusions.

Key Words: evapotranspiration, drought, remote sensing, land surface temperature, water balance

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). Water lost to the atmosphere through evapotranspiration (ET) has the effect of cooling the surface of the Earth. ET can be mapped while using thermal-infrared (TIR) remote sensing of land-surface temperature (LST). The LST is a valuable remote sensed indicator of both ET and the surface moisture status (Moran 2003). Soil moisture deficit in the root zone of vegetation (down to 1–2 m depth) lead to stomatal closure, reduced transpiration and higher canopy temperatures. These processes can be effectively detected from space in thermal wavebands (Anderson et al. 2007b). In comparison with standard water balance based approaches that model ET, TIR remote sensing provides diagnostic assessments of surface moisture conditions without the need to use precipitation data or information about soil texture and moisture holding capacity (Anderson et

al. 2011). As current available moisture to vegetation is derived directly from remotely sensed LST, this method can be used as a source that is not dependent on availability of information about soil and precipitation. This method can be also used for calibration of approaches based on water balance. One of such models is the Atmosphere-land Exchange Inverse (ALEXI) model developed by Agricultural Research Service (ARS) in the United States Department of Agriculture (USDA) (Anderson et al. 1997).

The goal of the paper is to investigate use of ALEXI in conditions of the Central Europe as a global ALEXI ET model is relatively new and not yet well tested out of the United States. Results of the study can be therefore used for ALEXI implementation in the conditions of the Central Europe where landscape structure is more heterogeneous than in the United States. Important part of the study is to compare different behavior of models, especially during sensitive parts of the growing season or when a significant drought event was recorded.

MATERIAL AND METHODS

ETa estimation from two models based on different approaches was compared. ALEXI is a model based on remotely sensed LST while SoilClim represents water balance based approach.

As it was previously mentioned, ET can be mapped while using TIR remote sensing of land-surface temperature (LST). TIR remotely sensed data can provide useful information about the surface moisture conditions due to the fact that evaporation has influence on land surface temperature. This approach is represented in this study by the ALEXI model that can provide data related to the surface moisture status (Anderson et al. 2007a). The ALEXI model was specifically designed to minimize the need for additional meteorogical data while still maintaining a physically realistic representation of land-atmosphere exchange over various vegetation cover conditions (Anderson et al. 2011). The ALEXI model uses measurements of the morning LST rise, typically provided by geostationary satellites in order to map daily ET and other surface fluxes (Anderson et al. 2015). In this study, global ET model at 5 km resolution was used. ALEXI is a two-source model of surface energy balance. These two sources represent soil and vegetation. The model is based on principle that wetter surfaces warm less rapidly during the morning hours (Anderson et al. 2015). The ALEXI model is able to estimate evapotranspiration (ET) using LST maps retrieved from TIR imagery generated from satellites (Anderson et al. 2015). Negative anomalies in ET can be a signal of drought or canopy stress. The important product of the ALEXI model is the Evaporative Stress Index (ESI) that is an indicator of agricultural drought. The ESI is expressed as standardized anomalies in the ratio of actual-to-potential ET ($f_{RET} = ET_a/ET_{ref}$). It is retrieved by using LST based energy balance algorithm (Anderson et al. 2011).

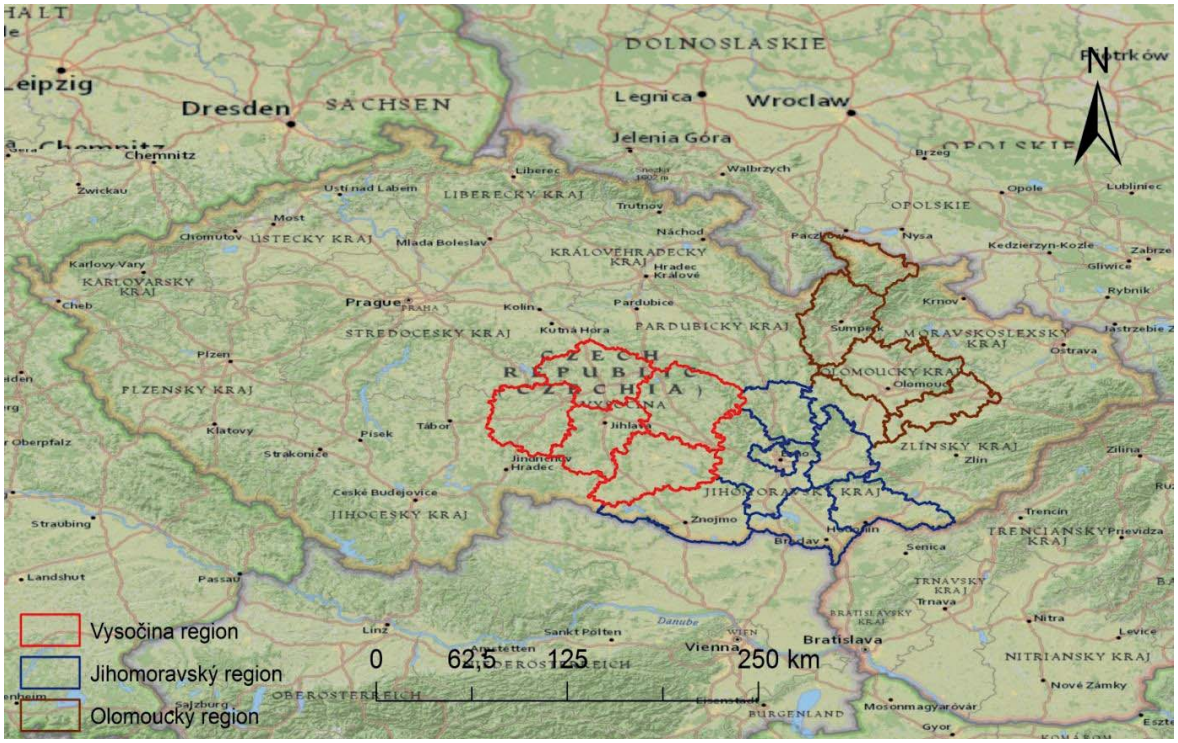
The SoilClim is a dynamic model of water content in soil and represents a model based on water balance approach (Hlavinka et al. 2011, Trnka et al. 2013). It was developed in coordination of Global Change Research Institute (CzechGlobe) and Mendel University. The SoilClim model is based on the work of Allen et al. (1998, 2005) but it also includes many modifications and adaptations to correspond with the conditions of the Czech Republic. The current version of the model is able to estimate the value of ETa and reference evapotranspiration (ETr), as well as soil moisture content in two layers of soil profile for 11 types of vegetation. The top layer includes the first 40 cm of the soil profile (topsoil and subsoil adjacent) and the second layer includes soil from the depth of 40 cm to 100 cm. The parts of the model are a dynamic crop model and a phenological model. Model considers interception, as well as changes of land surface albedo during the growing season. In the grid features, it also takes into consideration influence of slope orientation, inclination or different snow distribution during winter. SoilClim model is at spatial resolution of 500 m (www.intersucho.cz). Outputs of the model can be regularly followed on the website www.intersucho.cz and are weekly updated. Various indicators dependent on soil moisture are available on the website even for past months or years.

Comparison of ETa estimation from these two models was done at the district level. Model behavior was compared for districts of three regions of the Czech Republic located in the eastern part of the country – Vysočina, Jihomoravský and Olomoucký regions (Figure 1). Jihomoravský and Olomoucký regions are located at low elevations of the country when intensive crop production

prevails. Especially southern part of the Jihomoravský region (the Znojmo district) is known for its sensitivity for severe drought events (Zahradníček et al. 2014). The Vysočina region is located at higher elevations and amount of precipitation is here higher than in other two regions.

ETa comparison was done for the years 2014 and 2015. In 2015, the significant summer drought appeared in the Czech Republic. Week sums of ETa generated by models in mm were compared. SoilClim ETa values were in mm while ALEXI values needed to be converted. Original units of AXEXI model were MJ per m^2. The analyzed period was from April to August, it covered ETa values from 92th to 232nd day of the year (DOY). This part of the growing season represents the most sensitive period when changes in vegetation development are most significant. Week ETa sums were calculated by ArcGIS (ESRI, USA) and the area of the whole districts was used for calculation. There wasn't done any land use distinction during calculation.

Figure 1 A map showing districts of 3 studied regions of the Czech Republic



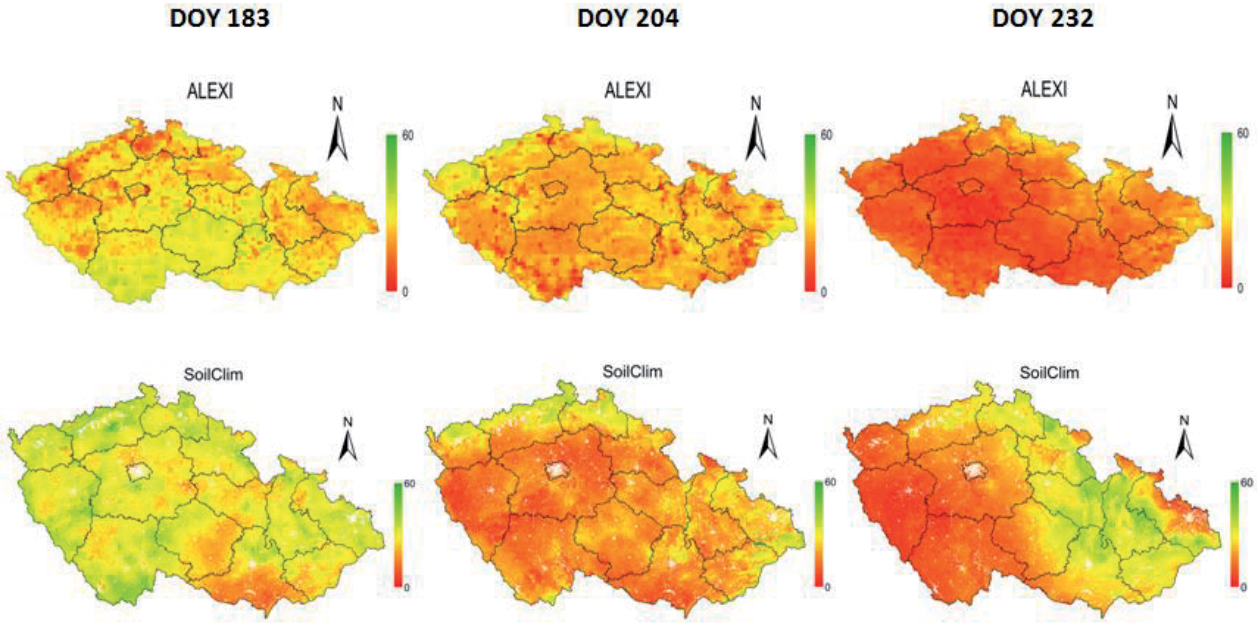
RESULTS AND DISCUSSION

ALEXI and SoilClim ETa maps for particular days of the year 2015 can be seen in Figure 2 (for DOY 183, 204, 232). Upper pictures come from ALEXI and lower ones are from SoilClim. DOY 183 is the beginning of July, DOY 204 is the end of July and DOY 232 is the end of August. Both models captured the drought event of 2015 in a different way. Differences are visible for all three dates, but mostly for 183 day and 232 day. For 183 day, ALEXI didn't capture lower ETa in the southern part of Moravia. For 232 day, ALEXI showed lower ETa in the whole country but SoilClim captured lower ETa mostly in western part of the Czech Republic. ETa maps shown in Figure 3 represent the week sums in the range of 0 to 60 mm per week.

Extracted ETa values from both models were compared for individual districts. Figure 3 shows graphs for selected districts of three regions of the Czech Republic. In certain parts of the year, ALEXI ETa values differed significantly from values obtained from SoilClim. In some cases, ALEXI values were even tree times higher than values from SoilClim. Also, the course of curve during the compared period (April to August) seemed to be quite different. In particular dates, ALEXI and SoilClim ETa curves showed an opposite direction – i.e. one was decreasing while another was increasing. This phenomenon appeared for both compared years during different parts of the growing season. However, both models captured certain parts of the growing season similarly, although the magnitude of ETa differed. ALEXI and SoilClim ETa dynamcs agreed better for the dry year 2015

than for the year 2014.

Figure 2 Comparison of ALEXI and SoilClim ETa during the part of the growing season of 2015 (for 183rd, 204th and 232nd day of the year). Upper maps come from ALEXI and lower ones from SoilClim



Generally, there are various reasons for different model behavior. First, ALEXI values were extracted from an ALEXI ET global product and this study represented its regional check. Each new checked region presents unique challenges and requires fresh evaluation as showed previous studies (e.g., in Brazil) (Anderson et al. 2015). This testing is valuable for further research and development of the product. Secondly, higher ALEXI values might be partially caused by different model resolution. ALEXI is at a spatial resolution of 5 km while SoilClim works at a resolution of 500 m, therefore ALEXI can have difficulties to distinguish different landscape features that respond differently to soil moisture while SoilClim can fail to have exact actual information about the surface parameters that is modeling. Thirdly, the excision of land use (in the case of ALEXI) can also play role because SoilClim considers runoff, deep percolation, simplified macropore water flow, dynamically simulated vegetation cover and multiple vegetation cover types (e.g., spring and winter field crops, permanent grasslands, evergreen and deciduous temperate forest) (Trnka et al. 2013). SoilClim can be considered as a reliable tool for soil moisture simulations as it was developed, calibrated and extensively validated in the conditions of Central Europe (Hlavinka et al. 2011, Trnka et al. 2013). However, no detailed validation of ETa itself has been done and should be considered in the next research activities.

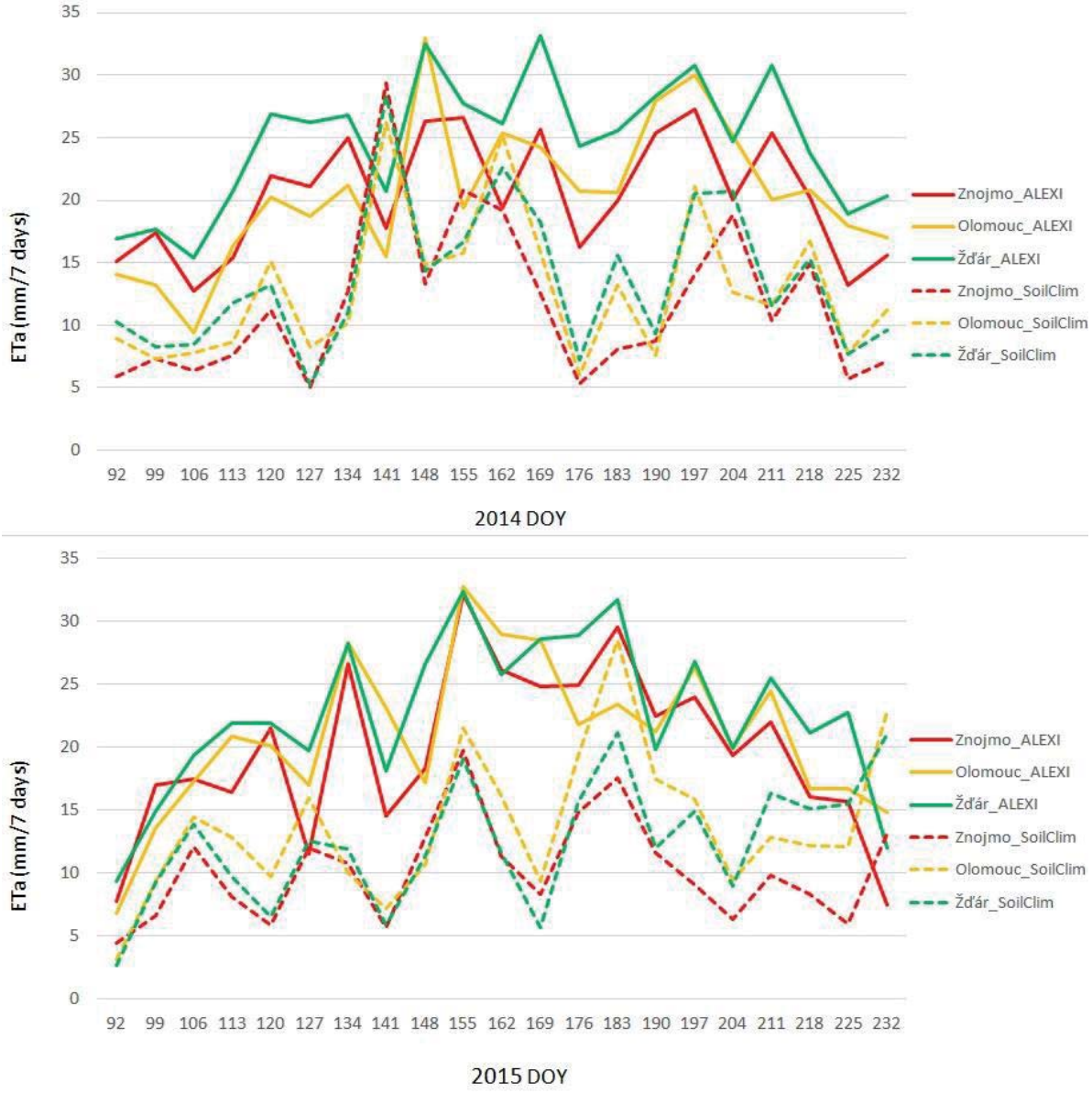
CONCLUSION

Results of the study showed that further checking and calibration of ALEXI is needed because it is a new model that hasn't been yet tested in conditions of Central Europe. The next step should include longer time period for comparison, as well as the year 2016. Employing a spatial flux disaggregation technique derived from ALEXI (DisALEXI; Norman et al. 2003), which uses air temperature diagnoses from ALEXI along with higher resolution TIR imagery, can be very useful for further investigation.

Due to different behavior of models related to ETa, it seems that further investigation and rigorous testing of both models is needed. ALEXI is a new model recently tested in conditions of Central Europe while SoilClim is a well-tested and validated tool for predictions of the soil moisture. Despite that, careful consideration is needed prior drawing general conclusions resulting from this first comparison of ETa. The next step should include step by step testing of the particular

assumptions present within both models. Employing a spatial flux disaggregation technique derived from ALEXI (DisALEXI; Norman et al., 2003), which uses air temperature diagnoses from ALEXI along with higher resolution TIR imagery, can be very useful for further investigation testing because in this way the ALEXI approach can be tested by independent *in-situ* observation using e.g. eddy covariance or scintillometer techniques.

Figure 3 Week ETa sums for selected districts of studied regions of the Czech Republic. Compared period is from 92nd to 232nd day of years 2014 and 2015



ACKNOWLEDGEMENTS

This research was conducted at Mendel University in Brno as a part of the project IGA AF MENDELU no. IP 9/2017 with the support of the Specific University Research Grant, provided by the Ministry of Education, Youth and Sports of the Czech Republic in 2017.

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SIGNIFICANT DECREASING TREND OF MOISTURE CONDITIONS DURING THE GROWING SEASON IN THE CENTRAL EUROPE

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Abstract: The aim of this work was to evaluate the moisture conditions in growing season in the Czech Republic for the period 1971–2010 by means of the P–E indicator. In all seasons during the monitored period 1971–2010, the growing period was evaluated across 14 localities from the 61st to the 180th day of the year (12 decades) using the percentile method. The ten–day indicator values were compared with acquired 2nd, 15th, 45th, 55th, 85th, and 98th percentiles. Both the vegetation seasons with unfavourable moisture conditions: 1976, 1993 and 2003, and the seasons with favourable moisture conditions: 1987, 1995 and 2010 were determined. Trend analysis of ten–day indicator values across all monitored localities was performed through the Mann-Kendall test. Statistically significant decreasing trend ($P < 0.05$) was found for the VI decade (days 111 to 120), in which important vegetative growth phases of agricultural crops take place in Central Europe. Unfavourable moisture conditions in this decade could reduce crop yields. Information about the frequency and intensity of drought contribute to the development and localization of appropriate adaptation measures.

Key Words: drought, growing season, P–E indicator, prediction

INTRODUCTION

According to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change (IPCC), average annual precipitation totals at higher altitudes should be higher, while in subtropical areas, particularly on the land, they should be lower. Furthermore, floods are supposed to be more frequent as well as longer and more intensive drought periods (IPCC 2013).

Drought is a result of a number of factors and meteorological elements (precipitation, vaporization, length and intensity of solar radiation, temperature, humidity, and air flow). As a temporary climatic anomaly, drought can occur in all climatic zones (precipitation regimes). According to Wilhite and Glantz (1985), the intensity and duration of drought periods can vary from short term droughts to periods with low precipitation totals lasting several months or even years.

Drought can have different impact on different agricultural crops, based on time of occurrence, the monitored crop and stage of its growth etc. Due to this fact, the methods of drought evaluation can differ greatly and their outcomes can thus differ as well. Simple evaluation of precipitation totals is not a sufficient indicator of moisture conditions. For the exact evaluation of moisture conditions, it is advisable to use a costly direct herbage monitoring. Thus, it is necessary to use sophisticated models concerning a wide range of meteorological elements and biological characteristics of the monitored crop, in order to objectively evaluate the moisture conditions in the soil-plant-atmosphere system. With the results applied, it is possible to directly design and localize adaptation agro-technical (Bodner et al. 2015) or cultivation measures (Lazarova et al. 2016, Klimesova et al. 2015).

MATERIAL AND METHODS

Climate data and the study area

The data from the technical series of climatic elements were used to analyze moisture conditions during the vegetation period of the localities concerned between days 61 and 180 of the year.

The series is based on the data measured across the station network of Czech Hydrometeorological Institute (CHMI). The outcome is a set of complete homogeneous station series, which were used to calculate the series of climatic elements on a daily basis for grid points 10 km far from one another (Štěpánek et al. 2011). From the technical series database 14 representative grid points were selected in order to evaluate moisture conditions in the vegetation period in a significant part of the area of the Czech Republic for the period 1971–2010. Technical data series were created at the grid points of the outcomes of the regional climatic model ALADIN-Climate/CZ. The characteristics of the individual CHMI grid points are demonstrated in Table 1.

The P–E indicator

In order to evaluate moisture conditions, out of a number of characteristics the P–E indicator was selected, which is expressed in mm. This drought indicator is calculated as a difference between precipitation totals (P) and potential evapo-transpiration (PET). To assess the P–E indicator for individual seasons at each grid point, it was crucial to calculate the potential evapo-transpiration, which is calculated with the agro-meteorological model AVISO for each decade of the vegetation period (days 61–180). The main phenological phases of the crops in the Central Europe take place during this period). The AVISO model is based on the MORECS model (Hough et al. 1997), but differs in the way meteorological data is collected, the outcoming sets, and in a number of program adjustments, which were made on the basis of experimental measurements. Both models are based on a combined Penman-Montei formula for calculating evapo-transpiration in a modified way. The input meteorological data are variables (temperature, moisture in form of water vapour, the time of solarification, speed of wind, and precipitation). AVISO was modified and adjusted to the conditions in the Czech Republic and has regularly been perfected and optimized (Kohut 2007).

Table 1 Characteristics of all grid points from the network of CHMI

Locality	District	Altitude (m)	Long-term average temperature t_{30} (°C)	Long-term average precipitation p_{30} (mm)
Brno-Chrlice	Brno–město	211	9.0	451
Čáslav-Filipov	Kutná Hora	247	8.9	555
Hradec nad Svitavou	Svitavy	489	7.4	616
Chrastava	Liberec	434	8.0	738
Jaroměřice n. Rokytnou	Třebíč	462	8.0	471
Lednice	Břeclav	167	9.6	461
Lípa	Havlíčkův Brod	515	7.5	594
Pusté Jakartice	Opava	322	8.3	584
Staňkov	Domažlice	417	8.1	537
Uherský Ostroh	Uherské Hradiště	208	9.1	521
Věrovany	Olomouc	219	8.7	502
Vysoká	Příbram	561	7.1	611
Znojmo-Oblekovice	Znojmo	233	9.3	435
Žatec	Louny	250	9.0	439

Legend: t_{30} and p_{30} – the long-term average temperature t_{30} and the long-term average precipitation p_{30} (1971–2000)

Evaluation of moisture conditions

The moisture conditions of the vegetation period between days 61 and 180 of the year (12 decades) were evaluated for the period 1971–2010 across all the localities with the help of the percentile method. This method supposes a gamma distribution of precipitation totals and evapo-transpiration data. The moisture indicators can be well characterized by this asymmetrical distribution. The moisture conditions were evaluated on the basis of comparison of the P–E indicator values of each decade with the calculated 2nd, 15th, 45th, 55th, 85th, and 98th percentile. Decade P–E indicators lower than 2nd percentile represent very dry conditions (VDC), lower than 15th percentile represent moderately dry conditions (MDC) and lower than 45th percentile represent slightly dry

conditions (SDC). Similarly, categories above 55th, 85th, and 98th percentile are characterized as slightly humid conditions (SHC), moderately humid conditions (MHC) and very humid conditions (VHC).

The trend analysis of the time lines was performed via a non parametric Mann-Kendall test of statistical significance of the trend (MK test) using XLSTAT software (Mann 1945, Kendall 1976). The trend was evaluated in all monitored decades of the vegetation period.

RESULTS AND DISCUSSION

Figure 1 shows in detail the evaluation of moisture conditions across the localities during the vegetation period between days 61 and 180 of the year in the period 1971–2010. These decade P–E indicator values were categorized the percentile values. They were calculated as average values of all the localities situated at different altitudes. They represent the moisture conditions of the vegetation periods, during which crucial growth and development phases take place, of the agricultural crops grown in the Czech Republic. Nevertheless, it is possible to identify a significant variability in moisture conditions in the individual decades of the vegetation period.

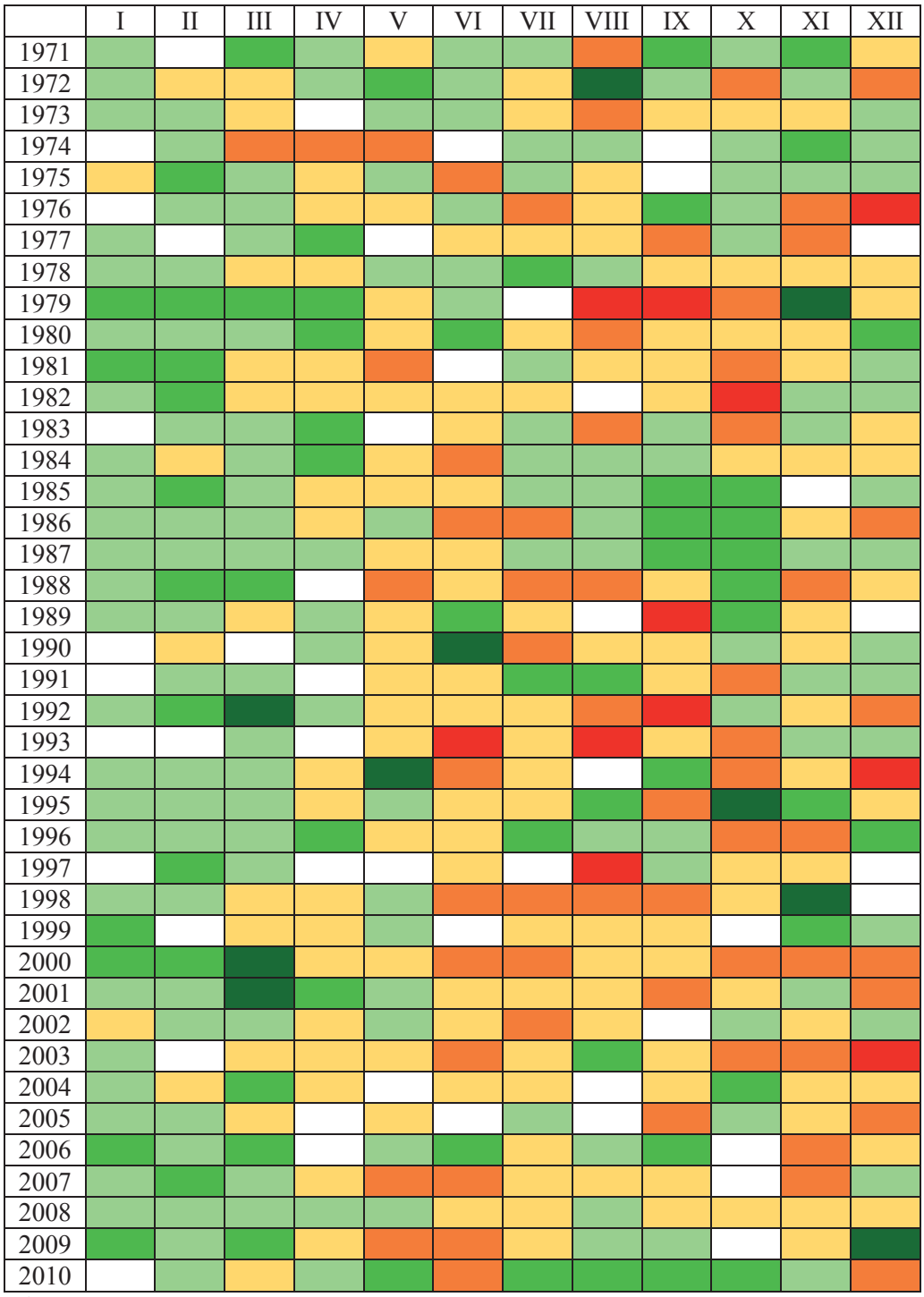
The maximum average long-term value of the P–E indicator was recorded in the decade between days 171 and 180 in 2009 (47.20 mm), while the minimum average value was recorded in the decade between days 171 and 180 in 1994 (-47.98 mm). The driest vegetation decade of all the monitored period 1971–2010 was the VI decade (days 111–120), when the average value was -11.82 mm. On the contrary, the most humid vegetation decade was the I decade (days 61–70), when the average value was 1.45 mm. Dry vegetation periods were recorded in the seasons 1976, 1993, and 2003, when the average value of the P–E indicator of the whole vegetation period was below -10 mm. Humid vegetation periods were recorded in the seasons 1987, 1995, and 2010, when the average value was above 1.5 mm.

Table 2 Trend analysis of P–E values for decades of growing season (P < 0.05)*

Decades (ten days)	P-value	Trend – MK test
I	0.258	–
II	0.239	–
III	0.463	–
IV	0.213	–
V	0.522	–
VI	*0.015	negative
VII	0.152	–
VIII	0.121	–
IX	0.568	–
X	0.600	–
XI	0.159	–
XII	0.435	–

An analysis of the development trend of the decade values of the P–E indicator was carried out using the Mann-Kendall test for the period 1971–2010 across all the experimental localities (Table 2). A statistically significant decreasing linear trend at the level of reliability P < 0.05 was recorded in the VI decade of the vegetation period, 111th–120th day of year (the second half of April). In this time, the phenological phases emergence and tillering of the cereals take place in Central Europe. According to Haberle et al. (2008), the occurrence of drought during sowing and the vegetative phases of the growth of the cereals influences the emergence of the herbage and the following reduction of the tillers. The phenophase of the tillering determines the number of spikes and the of secondary roots. Spinoni et al. (2014) investigated the causes and mechanisms of the drought occurrence in different parts of Europe, and discovered that the distribution of precipitation in Central Europe is increasingly irregular while the temperature of air is increasing as well. Podhrázská et al. (2013) and Středová et al. (2013) discovered an increase in potential evapo-transpiration and thus a higher risk of drought in areas of intensive farming in Central and South Moravia and Central Bohemiabe in the period 1961–2010, compared to the average values between the years 1901 and 1950.

Figure 1 Ten-day values of P–E indicator within the growing season during 1971–2010



Legend to Figure 1:

Colour	*Range of percentiles*	*Abbreviation*	*Categories*
	≤ 2	*VDC*	*very dry conditions*
	≤ 15	*MDC*	*moderately dry conditions*
	≤ 45	*SDC*	*slightly dry conditions*
	45 to 55	*NC*	*normal conditions*
	≥ 55	*SWC*	*slightly wet conditions*
	≥ 85	*MWC*	*moderately wet conditions*
	≥ 98	VWC	very wet conditions

CONCLUSION

On the basis of the P–E indicator a significant variability of the moisture conditions in the individual decades of vegetation was recorded in the period 1971–2010. Dry vegetation periods were recorded in the seasons 1976, 1993, and 2003, while humid vegetation periods were recorded in the seasons 1987, 1995, and 2010. In the decade between days 111 and 120 of the year, a statistically decreasing linear trend was discovered ($P < 0.05$), thus drier moisture conditions are likely in a significant area of the Czech Republic. In this decade, important vegetative phases of the growth of the agricultural crops take place in Central Europe, which can have a negative impact on the crop yield.

ACKNOWLEDGEMENTS

This article was written at Mendel University in Brno as a part of the project IGA FA MENDELU no. IP_1/2017 with the support of the Specific University Research Grant, provided by the Ministry of Education, Youth and Sports of the Czech Republic in the year of 2017.

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DOES THE ROOT SYSTEM SIZE AND SEED VIGOUR AFFECT THE DROUGHT TOLERANCE OF WHEAT?

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Abstract: Drought is one of the most serious abiotic stressors reducing the yield of cereal crops worldwide. Plants can defend water scarcity by „drought escape" thanks to more vigorous seed or by „dehydration avoidance" via development of larger and deeper root system in crucial vegetation stages. The root system size (RSS) and seed vigour of 14 winter wheat genotypes were tested in a two-year field experiment. RSS was evaluated by measuring its electrical capacitance in three vegetation stages. The seed vigour was tested in conditions of cold (10 °C) and drought stress (at -0.5 MPa). RSS was significantly affected by genotype in stem elongation phase (11.4%) and in the average of vegetation period (8.2%). The seed vigour was significantly affected by the interaction of the genotype with the year (40.4%) and genotype (37.3%). Correlation analysis of seed parameters and RSS and grain yield respectively did not show a clear relationship of the monitored traits. However, the grain yield was significantly affected by the root system size, especially in the heading phase in dry conditions. Hence, larger root system could be responsible for improved drought tolerance of wheat.

Key Words: climate change, phenotyping, seed germination, wheat, grain yield

INTRODUCTION

The occurrence of drought and its effects have currently been widely discussed among researchers, farmers and professionals. As the Assessment Report IPCC (2007) shows, the air temperature has increase by 0.74 °C in the 20th century, and according to the scenarios, it is possible to expect its further rise. Similarly, it is possible to expect a change of the amount and distribution of precipitation. In the Czech Republic, the amount of days without precipitation can rise from today's 79.9 up to 141.6 in the period 2071–2100, however the annual precipitation total should not be significantly affected (Spáčilová et al. 2014). The global warming will presumably cause a 20% increase in the lack of water, not only in the areas with regular drought occurrence (de Almeida Silva et al. 2012).

Breeding for the drought tolerance is complicated by a genetic disposition of this polygenic trait, typical for its low heritability. Along with this, it is the time of the drought occurrence, its duration and intensity, which prevent scientists from finding the traits characteristic for the tolerant plant genotypes. The selection based on the traits determining high yield in stress conditions can only be applied in those production areas, where a particular kind of stress occurs regularly every year in the same phase of the vegetation period (Cattivelli et al. 2008). One of the aims is to create such genotypes, which would be able to withstand drought during the vegetation period without lowering of the yield potential in stress-free conditions. These genotypes should be characterized by a phenotypic plasticity mirroring the interaction of the genotype with the environment. However, from the aspect of plant physiology, the genotypes with high yield and effective nutrient uptake are often less tolerant to the lack of resources (de Almeida Silva et al. 2012).

Out of many plant characteristics related to drought tolerance, such traits are selected, which present clear information of the phenotype of an individual plant exposed to the lack of water. Development of a large root system and seed vigour can represent tools of the stress avoidance strategy.

The genotypes with more vigorous seeds will supposedly increase their tolerance of drought. More vigorous seedlings are more likely to resist potential drought in the initial vegetation phases

(drought escape), will create a larger root system and will survive drought in the following vegetation phases as well. Availability of soil moisture is essential for seed germination (Benett 2004). Water potential of the soil lower than -2 MPa is a critical boundary for germination of the wheat seeds (Lindstrom et al. 1976), however some genotypes experience stress even at -0.5 MPa. Bertholdsson and Brantestam-Kolodinska (2009) discovered, that early vigour of the root system and development of more fine roots is important for barley drought tolerance. The length of the germinal roots is used for evaluation of the growth of the germinating seeds (Kakhki et al. 2008), hence the seed vigour (Klimešová et al. 2015, Vintrlíková et al. 2015).

Early development of the roots and their presence in deeper soil profile layers can be an advantage in case of the drought stress. Wheat and barley varieties with a larger root system use the water and nutrient resources more effectively than the varieties with a smaller root system, which results in higher grain yield (Chloupek et al. 2010, Středa et al. 2012, Svačina et al. 2014, Heřmanská et al. 2015). For instance, when 12 barley populations crossbred with regard to the root system size were selected, in the F3 generation, the root system grew by 3.9%, which was related to the increase in yield by 8.1% (Svačina et al. 2014). Higher root length density in the soil profile layer of 30–50 cm was statistically significantly related to higher barley grain yield in drier conditions (Klimešová and Středa 2013).

The aim of this work was to (i) analyze the differences in the root system size in 3 phenological phases, and in seed vigour or germination of selected wheat varieties, quantify the relationship between (ii) the root system size and seed vigour, and (iii) RSS and seed vigour, respectively and grain yield in a two-year field experiment.

MATERIAL AND METHODS

Root system size (RSS), seed vigour and germination were evaluated in 14 or 13 winter genotypes of wheat (Table 1) in a field experiment at Branisovice locality (South Moravia, Czech Republic) in 2015 and 2016. Root system size (nF) of 672 plants in 4 replications was measured using the electrical capacitance method according to Chloupek (1972) in the stem elongation (BBCH 30), heading (BBCH 50) and grain filling phase using the LCR device (Extech instruments, NH, USA) at the frequency of 1 kHz. At the same time, germination and vigour of the seeds of the same genotypes in laboratory conditions were tested. Vigour was established as a percentage of germinated seeds in stress conditions: temperature of 10 °C and physiological drought -0.5 MPa in the water solution of polyethylene glycol (PEG 6000). A control variant was established at the same time, with optimum water regime at the temperature of 10 °C (germination). Both variants were established in germination chamber on the filter paper with 50 grains and replicated 3 times. Only the seeds with a sprout at least as long as half of the grain and with at least 3 roots were considered properly germinated. The number of germinated seeds were evaluated after 2 weeks. Grains were harvested in fully ripe phase (BBCH 90). The analysis of variance ($p \leq 0.05$) and the correlation analysis were processed with the STATISTICA 12 software (Statsoft Inc., Tulsa, OK, USA). The effect of experimental factors (%) on the variability of data was quantified.

Table 1 Earliness of wheat genotypes tested for RSS, seed vigour and germination

Wheat genotype	501	502	504	507	509	510	517	523	527	528	530	531	533	538
Earliness	L	E	E	E	E	E	L	E	L	L	L	E	E	E

Legend: Earliness: L (Late), E (Early)

RESULTS AND DISCUSSION

Root system size

Root system size of winter wheat was statistically significantly affected by the genotype in the stem elongation phase (11.4%) and during the vegetation period on average (8.2%). In the heading and grain filling phase, the differences between genotypes were not statistically significant. A statistically significant effect of a year on the RSS values reached 76.2–84.5% in all

the phenophases. The considerable effect of a year on the RSS is based on the measurement method, hence it will not be considered further. Relative values of the root system size of the monitored genotypes in 2015 statistically significantly correlated with the values in 2016 in the stem elongation phase (r = 0.548) and on average during vegetation (r = 0.604). Thus, genotypes maintain a stable root system size during the vegetative growth without a significant interaction with the environment (6.5%). Higher RSS values of both years in the stem elongation phase were recorded mainly in late genotypes, however in the grain filling phase this tendency did not occur. Statistically significantly larger root system was developed by the genotypes 517 (2.45 nF), 527 (2.34 nF) and 501 (2.53 nF), in comparison to 533 (1.55 nF) in the stem elongation phase. The genotype 538 (0.44–2.24 nF) showed constantly higher values of RSS during all the vegetation, on the other hand, low values of RSS were recorded in 523 (0.28–1.7 nF). Root system size was variable in some genotypes during the vegetation.

Seed vigour and germination

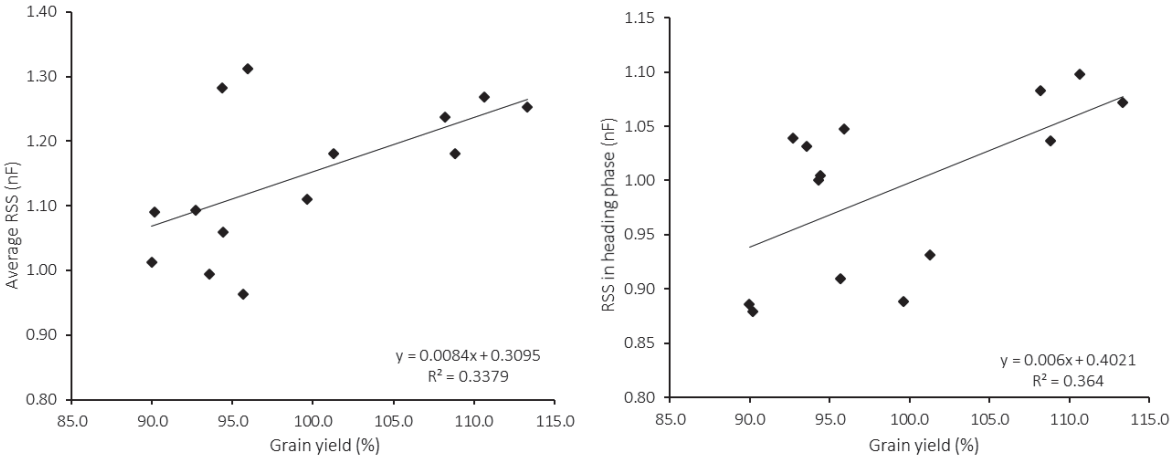
Seed vigour and germination of 13 genotypes of wheat in the control variant was monitored for two years. The genotype 538 was not included in the experiment in 2015. Natural conditions (drought stress and cold stress) were simulated in order to evaluate the seed vigour during the germination. A significant effect of a genotype (37.3%) and the interaction of a genotype and year (40.4%) on the values of seed vigour was found, however the weather during the vegetation period influenced neither germination, nor vigour (2.4%). The genotypes reached the germination/vigour of 99/87% (in average). Seed germination was not affected by any experimental factor. The lowest vigour values were during the two-year monitoring observed in the genotypes 527 (73%) and 510 (78%), statistically significantly better seed vigour was typical for the genotype 530 (95%).

The relationship of the root system size and seed parameters

The relationship of a genotype earliness and vigour or germination values was not confirmed. The relationship of seed vigour, germination and root system size is vague as well. In 2015, the lowest seed vigour value was recorded in the genotypes with a small root system size (533 and 509), while in 2016 these genotypes developed the most vigorous seeds. The genotype 527, typical for its large root system size in the stem elongation and the grain filling phase developed the least vigorous seeds in both years. The relationship of the RSS and seed vigour/germination has not been confirmed.

The relationship of the RSS and seed vigour of similar plants (the relationship of the parental RSS and progeny germination) was not significant. What is remarkable, is the mostly negative relationship of the monitored traits. Higher values of negative correlation coefficients were found for the relationship of germination and RSS in both years (r = -0.249 to r = -0.436). A faster growth of a shoot and germinal roots of the vigorous seedlings presumably does not relate to the root system size in the latter phases of the wheat development. Contrary to these results, a positive relationship was observed between RSS and seed vigour of barley, expressed by the length and surface area of the roots and shoot (Klimešová et al. 2015).

Figure 1 The root system size (nF) of 14 wheat genotypes in heading phase and during the vegetation period (average from the three phenophases) in relation to grain yield (%) in 2015–2016



Relationship of the grain yield, RSS and seed parameters

Wheat grain yield at the monitored locality with frequent drought occurrence during the two-year observation was affected by the root system size. Especially in 2015, when wheat canopies was affected by a short-term drought in May and June, more yield was provided by the varieties with larger root systems in the heading phase (r = 0.533) (Table 2). RSS values in the heading phase affected mainly the yield of the late varieties (r = 0.965), for the yield of the early varieties, RSS in the stem elongation phase was important (r = 0.771). In 2015–2016, the higher grain yield was statistically significantly related to higher RSS in the heading phase (r = 0.603), and average RSS during the vegetation period (r = 0.581) (see Figure 1), mainly in the early genotypes (r = 0.784). Seed vigour and germination was not related to the grain yield (Table 2). This may be caused by the long vegetation period of the winter wheat, which is strongly affected by the course of weather in the winter months.

Table 2 Correlation coefficients of the relationship of the root system size of wheat genotypes (total: n = 14; early: n = 9; late: n = 5) in two years in three vegetation phases (stem elongation, heading, grain filling, average) and seed characteristics (vigour, germination) to grain yield

		Root system size				Seed vigour	Seed germination
		Stem elongation	Heading	Grain filling	Average		
2015	Total	0.499	0.533*	0.406	0.545*	0.045	-0.062
	Late	-0.418	0.965**	0.866	0.285	0.393	-0.185
	Early	0.771*	0.428	0.276	0.731*	0.084	-0.297
2016	Total	0.378	0.513	-0.052	0.487	-0.200	-0.214
	Late	-0.622	0.181	-0.581	-0.555	-0.367	0.001
	Early	0.452	0.579	0.066	0.661	0.316	-0.278
Both years	Total	0.472	0.603*	0.362	0.581*	-0.297	-0.244
	Late	-0.801	0.747	0.047	-0.685	-0.385	-0.163
	Early	0.663	0.439	0.413	0.784*	-0.424	0.321

*Legend: Statistically significant values of correlation coefficients are marked by * and ** resp. at p≤0.05 and p≤0.01 resp. Correlation coefficients were determined for seed vigour and seed germination in 13 genotypes in total, and in 8 early genotypes in 2015 and in both years.*

CONCLUSION

The root system size and seed vigour of wheat vary across the genotypes. RSS in the stem elongation phase was during the two-year observation significantly affected by the genotype. Hence it is possible to select wheat genotypes by the root system size as soon as in the vegetative development phases. In comparison to the germination values, seed vigour is significantly affected by the interaction of genotype and year, and a genotype, which implies the possibility of selection of suitable genotypes with a higher seed vigour on the basis of long-term experiments. Correlation analysis of the relationship of the parameters of the seeds and RSS or grain yield did not confirm a clear connection of the monitored traits. However, the grain yield was statistically significantly affected by the root system size mainly in the heading phase. Hence, larger root system can be responsible for improved drought tolerance of wheat.

ACKNOWLEDGEMENTS

The research was financially supported by the project QJ1510098 of the National Agency for Agricultural Research.

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EVALUATION OF CROP YIELD SPATIAL VARIABILITY IN RELATION TO VARIABLE RATE APPLICATION OF FERTILIZERS

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Abstract: The application of nitrogen fertilizers is a challenge both in environmental and economic terms. The solution to the issue of rational nitrogen management is the variable rate application (VRA) of nitrogen fertilizers, which respects site specific soil conditions and the nutrient status of plants. The methodology of the work was based on the collection of data from the Isaria crop sensor system (IRMI, IBI values and N rate), yield maps of winter wheat from the harvesters, Sentinel–2 NDVI image and digital elevation model of the fields. All experimental work was carried out in Zdounky (Kromeriz) during the year 2016. These data were processed and analyzed by using geographic information systems and then statistically evaluated the relationships between variables. There was found a moderate positive relationship between the Sentinel NDVI imagery and the IBI and IRMI indices in both application. Also, high correlation between crop yield and N rate in second application confirmed the influence of N doses for yield production. Furthermore, the negative effect of elevation on the crop yield was observed.

Key Words: crop sensing, variable rate application, precision agriculture, yield mapping

INTRODUCTION

The most important yield formation factor of cereals is nitrogen fertilizing. The application of nitrogen fertilizers is a challenge both in environmental and economic terms, as fertilizer prices have increased many times over the last twenty years. The solution to the issue of rational nitrogen management is the variable rate application (VRA) of nitrogen fertilizers, which respects site specific soil conditions and the nutrient status of plants. VRA of fertilizers should result in a higher efficiency in the use of nitrogen, which is important for the environment (Klír 2002). According to Heege (2013), higher accuracy in application and increased efficiency of nitrogen utilization is required to achieve a higher yield while decrease of nitrogen costs and water contamination by nitrates.

Nowadays, the VRA of nitrogen topdressing is based on the crop sensing by remote sensing, such as aerial, unmanned or satellite survey, or by on-the-go proximal sensing. Crop sensors are mounted on the machinery and use information about the reflectance of visible and near-infrared spectrum, which is related to the crop parameters, such as LAI, chlorophyll content, aboveground crop biomass and other.

Some of crop sensor systems can react not only to the actual crop status, but also to combine that with the information about site specific productivity of soil – in form of yield potential maps. Yield potential maps are produced from time-series of yield maps and describes the spatial heterogeneity of yield trends. Blackmore and Larscheid (1997) proposed a method for classification of yield zones based on the normalized yield maps - the yield level and its stability. This method divides the field area into three categories based on the variation of each site in recent years: (a) high and stable yields, where the crop inputs shouldn't limit the crop yields; (b) low and stable yields where the crop inputs should be limited until the cause of low yield will be investigated and managed; and (c) areas

with unstable yields, where the high intensity of crop management should be considered according to the weather condition.

The aim of this study is to investigate the spatial variability of crop parameters measured by crop sensing system Fritzmeier Isaria, the recommendation of N application and to evaluate its relation to the crop yield heterogeneity.

MATERIAL AND METHODS

Study area

Input data of winter wheat were acquired during the year 2016 by mapping of fields with total area 248 ha at farm company SALIX MORAVA a.s. (part of Spearhead Czech s.r.o. holding). Zdounky area is located in the sugar beet production area in district Kromeriz. The climatic condition of the region is slightly warm to warm and slightly damp. The long-term average annual temperature is 8.1 °C and the average precipitation is 550–700 mm. The fields are located at an altitude of 205–320 m a.s.l. The soil types are Chernozem, Haplic Luvisol, Cambisol and Fluvisol with medium to deep soil depth. The humus content is moderately high, equal to 2–3%. Soil pH value ranges between 6.6–7.2. Fields are flat to moderately sloped.

Variable application of fertilizers by Fritzmeier Isaria

Application of nitrogen fertilizers to winter wheat crop during vegetation was carried out as variable rate application (VRA) of Urea Stabil (N2–2nd application, BBCH 29–30) and ammonium nitrate with dolomite (N3–3rd application, BBCH 44) by John Deere 6195R with the spreader Amazone ZA-TS 4200 and crop sensing system Fritzmeier Isaria (Figure 1). Nutrient status of plants is evaluated based on the spectral measurement of crops by active LED lighting in four spectral wavelengths (660–780 nm). Two vegetation indices are calculated - Isaria Reflectance Measurement Index (IRMI), which is related to chlorophyll content, and Isaria Biomass Index (IBI) related to crop biomass. Spreader was under full control of CCI terminal with automatic detection of swath overlaps. Isaria system works in various modes–for this study application of fertilizers under absolute mode with/without yield potential maps was used.

Figure 1 Nitrogen application by Isaria system on the field (left) and board terminals in tractor cabin (right). Photo by V. Lukas

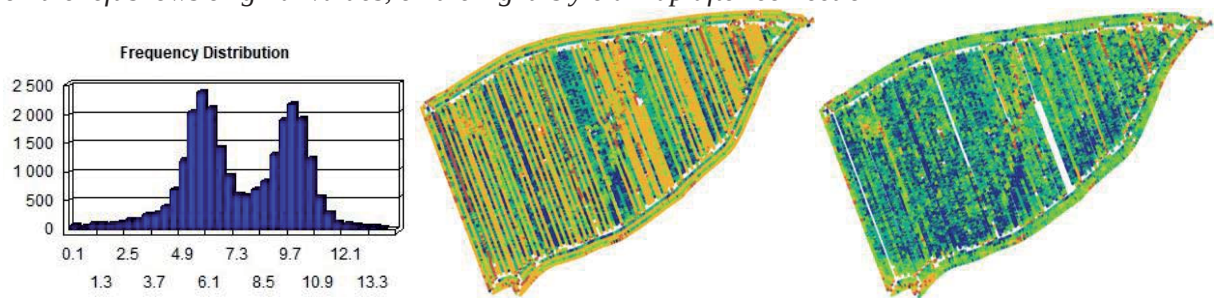
The Isaria system uses the yield potential maps that are determined by spatial analysis of the multi–temporal series of multispectral satellite data. It is the identification of yield above–average and below–average area within the field and their percentage expression with respect to the average value of the vegetation index sensitive to changes in biomass (Řezník et al. 2016). The maps were in two variants: (1) provided by Fritzmeier company and (2) calculated by Department of AgroSystems and Bioclimatology of Mendel University in Brno according to their own algorithm.

Records of vegetation indices and applied N rates were downloaded from board computer and processed in ArcGIS 10.3 application (ESRI. Redlands. USA). Files were merged based on the field identification and interpolated by spatial interpolation techniques to continuous raster maps. In this Empirical Bayesian Kriging (EBK) method was used to reach the most optimal results of interpolation.

Crop yield mapping by harvesters

Yield data of winter wheat were recorded during the harvest 2016 carried out by Claas Lexion 670 harvesters. Each harvester was equipped by DGPS, grain flow and moisture sensors. The results are point data with location, grain moisture, wet and dry yield, width and other parameters. The data were processed in ArcGIS. In some cases where two harvesters were on the field, the records needed to be additionally calibrated to obtain comparable results from both harvesters. Second step was to filter outliers and error values and spatial interpolation. In this case ordinary kriging technique was applied to smooth the differences at small scale level.

Figure 2 Example of histogram offset caused by combination of records from two harvesters. Map on the left shows original values, on the right is yield map after correction



Other data used in the study

Additionally, NDVI image from satellite Sentinel–2 with spatial resolution of 10 m per pixel, acquired in 23 May 2016 (BBCH 44–47), was used for estimation of crop variability. To obtain information of field topography, the online available DMR4G provided by Czech Office for Surveying, Mapping and Cadastre (CUZK) was analyzed in Geographic Information System (GIS).

RESULTS AND DISCUSSION

Mapping of crop and yield variability

The results of crop mapping by Isaria system in form of IRMI, IBI values and N rate for both terms of application were analyzed by GIS. The results of winter wheat grain yield recorded by harvesters and their basic statistics for observed fields are listed in the Table 1. Average yield varied among the fields from 7.22 t/ha (Field 8102/1) to 10.47 t/ha (Field 4002/1). Although coefficient of variability, considered as the indicator of within field yield heterogeneity, gains range which could be considered according to Blackmore et al. (2003) as the lower spatial variability (8.32 to 13.98%, both bellow threshold 30%), the whole dataset of yield starts at 3.65 t/ha and finishes at 12.39 t/ha.

Figure 3 Yield maps and box plot graph of grain yield ranges for observed fields

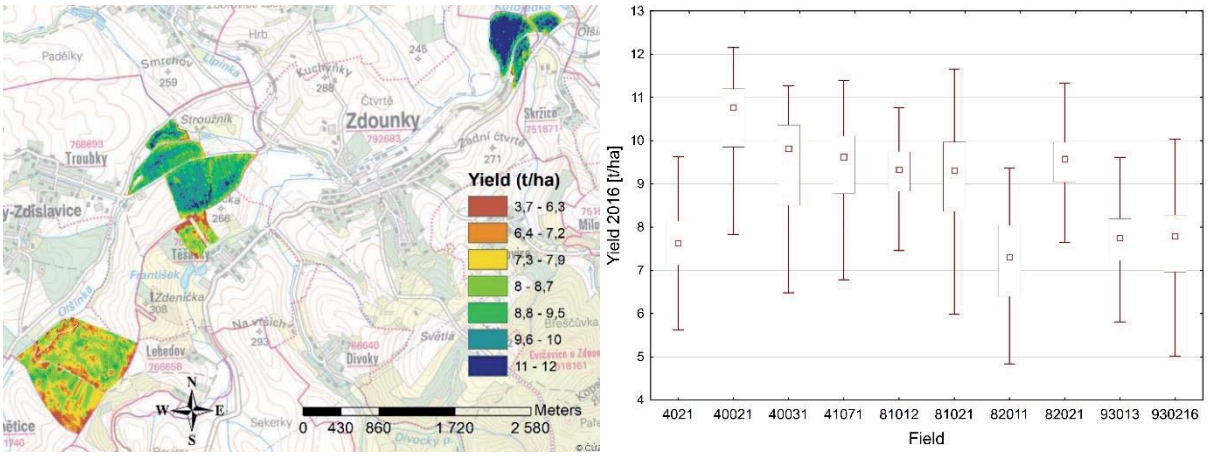


Table 1 List of the observed fields and basic statistics of winter wheat grain yield recorded by harvester

Field ID	Area [ha]	Grain yield [t/ha] Min	Max	Range	Average	St. dev.	Sum [t]	CV [%]	Variety
0402/1	41.23	3.94	10.50	6.56	7.58	0.82	312.59	10.80	Viriato
4002/1	20.01	6.65	12.20	5.55	10.47	0.96	209.47	9.15	Tobak VO
4003/1	6.47	4.84	12.39	7.55	9.44	1.15	61.11	12.15	Tobak VO
4107/1	7.12	4.35	11.41	7.05	9.33	1.13	66.45	12.12	Tobak VO
8101/2	21.61	5.86	10.92	5.06	9.21	0.78	199.13	8.49	Matchball E
8102/1	8.07	4.63	11.85	7.22	9.06	1.22	73.20	13.41	Matchball VO
8201/1	4.8	4.57	9.56	4.98	7.22	0.98	34.68	13.59	Rebel
8202/1	44.02	5.55	11.53	5.98	9.43	0.78	415.20	8.32	Rebel
9301/3	57.44	3.65	10.68	7.03	7.67	0.80	58.30	10.43	Matchball
9302/16	7.73	3.81	10.14	6.32	7.54	1.05	440.60	13.98	Rebel

Legend: St. dev. – standard deviation; CV – coefficient of variation

The map of crop yields and box plot graph in Figure 3 illustrates the yield heterogeneity and ranges over each observed field. Coincidence of within field yield distribution to other measured crop and site characteristics is presented and discussed in next section.

Evaluation of relationship among crop, yield and site parameters

The relationship among observed vegetation parameters, crop yields and topography characteristics were evaluated based on the results of correlation and regression analysis. The variables without normal distribution were calculated by Spearman correlation. The matrix of Spearman correlation coefficients is shown in Table 2. All results were significant at 95% level due to the huge number of records.

Table 2 Correlation matrix of Spearman correlation coefficient (r)

Variable	NDVI (23 May)	Grain yield	N2 IRMI	N2 IBI	N2 apl.N	N3 IRMI	N3 IBI	N3 apl.N	DEM	YP
NDVI (23 May)	1.000	0.212	0.488	0.435	0.123	0.627	0.620	-0.436	-0.013	0.285
Grain yield	0.212	1.000	0.136	0.292	0.638	0.528	0.485	-0.309	-0.624	0.059
N2 IRMI	0.488	0.136	1.000	0.878	0.070	0.704	0.768	-0.383	-0.176	0.254
N2 IBI	0.435	0.292	0.878	1.000	0.244	0.688	0.752	-0.291	-0.324	0.229
N2 apl.N	0.123	0.638	0.070	0.244	1.000	0.368	0.334	-0.142	-0.541	0.168
N3 IRMI	0.627	0.528	0.704	0.688	0.368	1.000	0.952	-0.619	-0.409	0.226
N3 IBI	0.620	0.485	0.768	0.752	0.334	0.952	1.000	-0.544	-0.350	0.227
N3 apl.N	-0.436	-0.309	-0.383	-0.291	-0.142	-0.619	-0.544	1.000	0.329	0.127
DEM	-0.013	-0.624	-0.176	-0.324	-0.541	-0.409	-0.350	0.329	1.000	0.027
YP	0.285	0.059	0.254	0.229	0.168	0.226	0.227	0.127	0.027	1.000

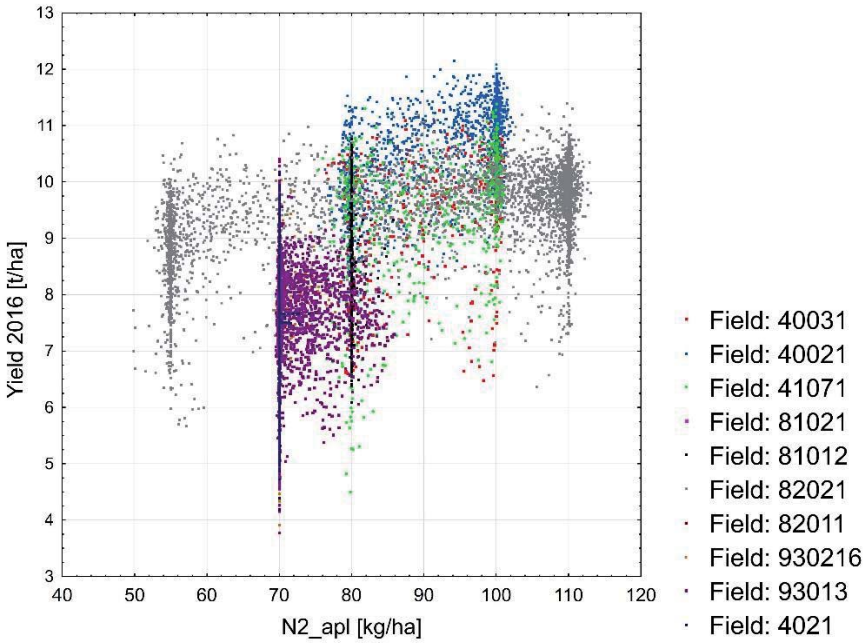
Legend: N2, N3 – second and third nitrogen application; YP – yield potential map (MENDELU); DEM – digital elevation model

Highest correlation to grain yield was reached by N rate values in second application (N2), while the N3 application rates had negative relationship to crop yield. This confirms the effect of second nitrogen application on the yield formation, while the third application aims on the grain quality parameters.

The Sentinel–2 NDVI reached highest level of correlation with the Isaria vegetation indices (IRMI, IBI) at N3 application, which is in the same vegetation period (23 May 2016) and coincide by spectral measurement. Lower correlation was found to the previous measurement of Isaria (N2). Although relation to crop yield and yield potential maps (YP) was significant, it is very low to explain the measured variability. Comparing of N2 and N3 Isaria indices shows good repeatability of measurement and high sensitivity to the crop spatial variability.

The higher negative correlation of crop yield and field topography (r = -0.624), represented by digital elevation model, claims the increased yields in the lower terrain elevation. This is confirmed also by study of Kumhalová (2010), where crop yields and topography derivate were analyzed. Topographic parameters influence the soil parameters, such as water availability, soil texture and organic matter content.

Figure 4 Scatter plot of crop yield and N rate by second nitrogen application



The scatterplot in Figure 4 shows the relationship between the yield and the applied amount of nitrogen in the second application. The value of the correlation coefficient r = 0.62 indicates a relatively high degree of direct dependence. Based on the linear regression equation (yield = 4.0388 + 0.0574 × N2_amount), increase of 10 kg N rate led to increase of yield 0.574 t/ha. Depending on the index of determination, the model R2 explained 38.8% of the data variability. The vertical points for some fields (402/1, 8101/2, 9302/16) indicate a constant N rate by non-optimal settings of Isaria. Thriwakala et al. (1997) noted that on the high and moderate productive soil should be variable application set to higher doses. The study of Hrůza (2008) showed an economic benefit of fertilizers at less productive areas. As shown by Galambošová et al. (2015), variable nitrogen applications based on the Isaria system together with yield potential maps brought a significant economic benefit in the form of an increase in yield of 1.25 t/ha. The increase was due to higher doses of fertilizer by an average of 49 kg N per ha, which was utilized by plants.

CONCLUSIONS

The evaluation of crop and yield mapping reveals significant spatial differences both in the assessment of vegetation status and crop yields recorded by harvester. The crucial step is the high quality of data pre-processing, which in the case of yield data can omit many errors. The analysis of interdependencies has shown the connection between the observed characteristics of the crop stand, yield and topographical properties of fields. A moderate positive relationship was found between the Sentinel NDVI imagery and the IBI and IRMI indices in both application. Also, high correlation between crop yield and N rate in second application confirmed the influence of N doses for yield production. Furthermore, the negative effect of elevation on the crop yield was observed.

Based on the results obtained, it can be stated that the precision farming system is able to provide sufficient information on the field heterogeneity with the potential to optimize input control in crop production. However, the economic effect is long-term and depends on the skills of the farm workers to utilize the precision farming technologies.

ACKNOWLEDGEMENTS

This study was supported by research projects NAZV QJ1610289 "Efficient use of soil productivity by site specific crop management" and TACR ALFA TA04021389 "Development of the system for variable rate application of pesticides and fertilizers using crop monitoring". Data from field experiments were provided by SALIX MORAVA a.s. and Spearhead Czech s.r.o. We thank also Vladyslav Shpakovskyi from Fritzmeier Umwelttechnik company for his support in evaluation of Isaria results.

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INFLUENCE ON ONION (*ALLIUM CEPA*) YIELD AND INTERNAL QUALITY OF BIOADDITIVE TREATMENT

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Abstract: For organic growers is very important to increase yield and quality of production by using suitable bioadditives. Onion is the most economically significant member of the *Asparagales*. Also, it is a good source of vitamins, minerals, polyphenols as well as carotenoids and antioxidants. The field experiment with total size of 128 m^2 was conducted to study the effect of three bioadditives on yield and quality of two cultivars of onion *Allium cepa* L., Stuttgarter riesen, and Rote Laaer during 2016 in Lednice, Czech Republic. The experiment was set according to the Latin square system in four repetitions including control. Bioaditives, B-Stimul, EkoBooster 2 and Vermifit A were used during vegetation period. Bioaditives, B-Stimul contains *Azotobacter*, *Azospirillum*, *Herbaspirillum*, *Bacillus, Chlorella*. EkoBooster 2 which contains organic matter, N, P, K stimulates, and Vermifit A composition nutrients in immediately acceptable forms, plant hormones, enzymes, coenzymes, sugars, extract of compost of Californian earthworm. The treatment Vermifit A was applied four times during vegetation period, while B-stimul and Ekobooster 2 three times. The total and market yield and the content of vitamin C and carotenoids were determined. The influence of the treatments on those parameters was evaluated. The bioadditives treatments affected content of vitamin C, carotenoids, as well as marketable yield. The results indicated in the cultivar Stuttgarter riesen the highest content of vitamin C (46.05 mg/kg), and carotenoids (14.60 mg/kg) found in B-stimul. In the cultivar Rote Laaer the highest content of vitamin C was found in control (17.47 mg/kg), and the highest content of carotenoids in EkoBooster 2 (7.54 mg/kg). Also, this cultivar showed the highest market yield in control (3.12 kg/m^2), while cultivar Stuttgarter riesen the highest market yield in EkoBooster 2 (3.48 kg/m^2). The study showed that between cultivars and treatments there were no statistically significant differences in total yield.

Key Words: bacteria, organic agriculture, vitamin C, carotenoids, yields

INTRODUCTION

The common onion (*Allium cepa* L.) is one of the most popular and important vegetable crops worldwide. Onion production and consumption are steadily increasing globally (Hassan 2008), and it is widely used as a vegetable by almost all classes of the society (FAOSTAT 2005).

Organic farming reduces the cost of production by utilization of organic wastes as fertilizers which are said to be the potential source of pollution unless they are used in a productive and efficient way (Banjare et al. 2015). For sustainable production and productivity as well as quality, organic farming may be the alternative means. Only a few researchers like Yadav et al. (2004), Jha et al. (2006), Balemi et al. (2007), studied the effect of bio-fertilizers on an onion. Yassen and Khalid (2009) showed that all organic fertilizer treatments improved vegetative growth characters, some of the main constituents of essential oil and N, P, K contents.

This experiment was based on an evaluation of organic onion performance with using different additions containing bacteria (*Azotobacter* sp., *Azospirillum*, *Herbaspirillum* sp., *Bacillus* sp., *Chlorella* sp., *Bacillus coagulans*), compost (which contain earthworms) and organic matter with content of N, P, K. For the production of biofertilizers and active cultures of bacteria were used, *Bacillus subtilis* and *Bacillus megatherium* var. *phosphaticus*. These bacteria, produce vitamins and other biologically active compounds which are stimulative for plant growth (Compant et al. 2010). The compatible combination of selected bio-additives for a particular plant species or genotype can

lead to numerous improvements of crop production including plantlet survival, increased plant health and resistance to environmental factors, and yield increase (Vosátka et al. 2014). Compost addition is also known to enhance microbial biomass and soil respiration (Bhattacharyya et al. 2003). Soil microbial health can be related to soil enzyme activity which is enhanced by fermentation of compost (Crecchio et al. 2001). The earthworms fragment the organic waste substrates, stimulate microbial activity greatly and increase rates of mineralization, rapidly converting the wastes into humus-like substances with a finer structure than composts but possessing a greater and more diverse microbial activity, commonly referred to as vermicomposts (Atiyeh et al. 2000). There is a good scope of increasing onion yield and quality for which nutrient management is one of the most important considerations under organic production system (Patel et al. 2005).

The aim of this study was to evaluate the effect of selected bioadditives on content of vitamin C, carotenoids and yield on different cultivars of onion.

MATERIALS AND METHODS

Experiment took place in 2016 on the experimental field of the Faculty of Horticulture in Lednice, Mendel University in Brno, Czech Republic. The trials were conducted according to the Latin square system in four repetitions including control. Sowing was done on 15 March on the depth: 1.5–2 cm in a greenhouse in containers while transplanting was done on 4 May, respectively. Each cultivar contained 16 plots with a size of each 4 m² (2 x 2 m) per plot. The spacing was 0.3 × 0.035 m (270 plants). Harvesting was done on 9 August.

Materials

For the experiment were used two onion cultivars: Stuttgarter riesen, yellow onion and Rote Laaer, violet-red onion (Permaland, Czech Republic). Treatments B-Stimul, Ekobooster 2 and Vermifit A were used. B-Stimul (Rawat, Czech Republic) contains a mixture of the following bacterial and algal cultures: *Bacillus licheniformis*, *Bacillus megatherium*, *Azotobacter* sp., *Azospirillum*, *Azotobacter*, *Herbaspirillum* sp. and *Chlorella vulgaris* at a concentration of 10^7 cfu/g. EkoBooster 2 (Ekopatent, Serbia) with content of organic matter 7.8%, N-9%, P-1%, K-4%. Vermifit A (Primrose, Czech Republic) is extract of compost from California earthworms and peat, contains nutrients in immediately acceptable forms, plant hormones, enzymes, coenzymes, amino acids and sugars. Composition is as follows: pH/H_2O 8.2, total nitrogen N 1.9, total potassium as K_2O 35.6, total phosphorus as P_2O_5 2.8, dry matter 0.95%. The treatment Vermifit A was applied four times during vegetation period while B-stimul and Ekobooster 2 three times. The application was foliar for each treatment with dose for 1 m^2: Vermifit A 0.4 ml dissolved in 39.6 ml of water, B-stimul 3.38 g dissolved in 3.38 l of water, Ekobooster 2 0.125 ml dissolved in 25 ml of water. Analyses for internal quality and economic parametric were done at the laboratory of Faculty of Horticulture. All bulbs for analyses were medium size, peeled and not damaged.

Vitamin C (ascorbic acid)

The concentration of vitamin C was determined by HPLC according to Arya and Mahajan (2000) with slight modification. A fresh sample of onion bulbs (20 g) was homogenized in a blender with 20 ml oxalic acid. Samples medium size, were taken from each repetition. The homogenate was topped up with oxalic acid to the volume of 100 ml, filtered, centrifuged (3800 rt/min for 10 minutes at room temperature) and the supernatant was used for measurement. The amount of ascorbic acid was expressed as mg/kg fresh mass.

Chromatographic analysis: The analyses were performed by HPLC (ECOM, ECB 2000 Praha, Czech Republic) at 254 nm using Knauer detector. Analytical column YMC - Triart C18 150 x 4.6 mm. D. S – 5 µl 12 nm. TA12SO5 1546WT, pre-colonies CGC 3x30 Separon SGX 18.7 nm, isocratic mode of mobile phase (tetrabutylammonium hydroxide), oxalic acid, distilled water, 10:20:70. Column flow rate 0.5 ml/min, injection volume 20 µl, wavelength 254 nm.

Carotenoids

0.2 g dry weight of the aerial parts of onion was homogenized and extracted with acetone using microwave extraction system (Start E, Milestone, Germany). 20 minutes was carried out the extraction process. After cooling, each sample was kept in dark room for 24 hours. Spectrophotometric

measurements were performed in cuvettes using a spectrophotometer (Specord 50 PLUS, Analytik Jena, Germany). Absorbance was measured at the wavelength of 440 nm for carotenoids.

Determination of yields (total and marketable)

After harvest, the yield of each plot was kept under ambient conditions. Determination of economic parameters by evaluating the total and marketable yields per plot was expressed as kg/m^2. Before determination onion was cleaned from roots and leaves. The quality of production was evaluated according to Czech quality standards (ČSN 46 3161). The onion bulbs were graded in different categories according to their bulb size: 0–20 mm for small bulbs, 20–40 mm for medium bulbs, and 40–70 mm for big bulbs.

Statistical analysis

Data were evaluated by two-way analysis of variance (ANOVA) using PC software Statistica Cz v. 12 (StatSoft). Differences in content levels among the varieties were estimated through Fisher's LSD test at P=0.05.

RESULTS AND DISCUSSION.

Determination of internal quality

The results of the analysis of vitamin C and carotenoids content in the selected onion cultivars are shown in Table 1 and Figure 1 and 2.

The vitamin C contents in Stuttgarter riesen ranged from 14.93 to 46.05 mg/kg. The lowest content of vitamin C was found in control (14.93 mg/kg), while the highest content in B-stimul (46.05 mg/kg). The content of carotenoids in Stuttgarter riesen ranged from 2.80 to 14.60 mg/kg. The lowest content of carotenoids was found in EkoBooster 2 (2.80 mg/kg), while the highest content in B-stimul (14.60 mg/kg).

Table 1 Determination of Vitamin C and Carotenoids in onion (mg/kg)

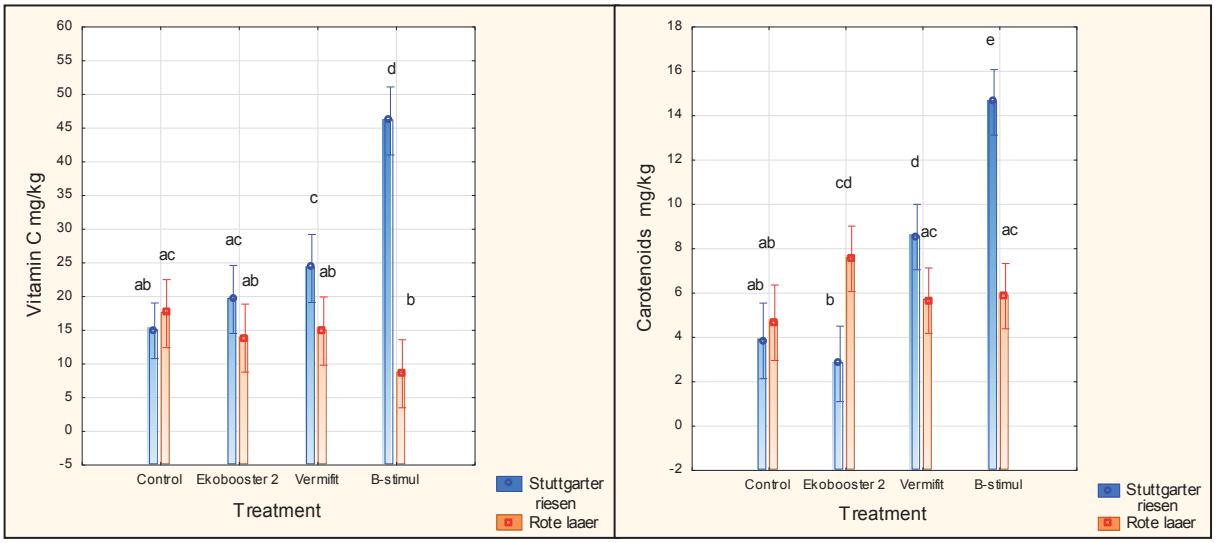
Cultivar	Treartment	Vitamin C mg/kg	Carotenoids mg/kg
Stuttgarter risen	Control	14.93 ab	3.84 ab
Stuttgarter risen	EkoBooster 2	19.57 ac	2.80 b
Stuttgarter risen	Vermifit A	24.18 c	8.52 d
Stuttgarter risen	B-stimul	46.05 d	14.60 e
Rote Laaer	Control	17.47 ac	4.66 ab
Rote Laaer	EkoBooster 2	13.84 ab	7.54 cd
Rote Laaer	Vermifit A	14.88 ab	5.65 ac
Rote Laaer	B-stimul	8.55 b	5.86 ac

Legend: The letter indicates the significant difference according to the LSD test at P=0.05 between treatments and cultivars.

The lowest content of vitamin C in cultivar Rote Laaer contained in treatment B-stimul (8.55 mg/kg), while the highest in control (17.47 mg/kg). The content of carotenoids in Rote Laaer ranged from 4.66 to 7.54 mg/kg. The lowest content of carotenoids was found in Control (4.66 mg/kg), while the highest in EkoBooster 2 (7.54 mg/kg). The content of vitamin C in both cultivars was not higher compared to USDA (2017), where the content of vitamin C in raw onion is 7.40 mg/100g. At present, using organic manures and biofertilizers such as vermicompost and nitrogen fixing bacteria contain *Azotobacter* and *Azospirillum* have led to a decrease in the application of chemical fertilizers and has provided high-quality agricultural products (Migahed et al. 2004, Mahfouz and Sharaf Eldin, 2007). According to Gupta et al. (as cited in Yohannes G. et al. 2017), there is a great scope for improving the yield, quality and shelf life of onion with integrated nutrient management using organic fertilizer. Results showed that content of carotenoids was not significantly different between both cultivars in control. The bioadditive comprising of *Azotobacter*,

Azospirillum, *Herbaspirillum*, *Bacillus*, *Chlorella* showed significant differences on the quality parameter of cultivar Stuttgarter riesen within the content of vitamin C and carotenoids.

Figure 1 Content of vitamin C in onion. *Figure 2 (right) Content of carotenoids in onion.*



Determination of yield (market and total)

The results of the analysis of marketable and total yield in the selected onion cultivars are shown in Table 2. The study showed that between cultivars and treatments do not exist statistically significant differences in total yield. In the experiment, results showed the highest market yield in cultivar Stuttgarter riesen in EkoBooster 2 (3.48 kg/m^2).The lowest market yield results showed in Vermifit A (2.99 kg/m^2) but it was not significantly different comparing to control and B-stimul. The treatment EkoBooster 2 comprising of organic matter and N P K showed the best effect on cultivar Stuttgarter riesen with 15% higher market yield comparing to Vermifit A, 13%, comparing to control and 10%, comparing to B-stimul. The results indicated market yield of 38 tons per hectare, which is more than 50% higher comparing to FAOSTAT 2014 results, which showed market yield of onion per hectare averages 18.45 tons.

The cultivar Rote Laaer contained the highest market yield with control (3.12 kg/m^2) but it was not significantly different compared to Vermifit A. These results showed market yield in control 15% higher comparing to EkoBooster 2.5% comparing to Vermifit A, and 20% comparing to B-stimul.

*Table 2 Determination of market and total yield in onion (kg/*m^2)

Cultivar	Treatment	Market yield kg/m^2 $\overline{X}$	Total yield kg/ m^2 $\overline{X}$
Stuttgarter risen	Control	3.04 ab	3.14 a
Stuttgarter risen	EkoBooster 2	3.48 b	3.48 a
Stuttgarter risen	Vermifit A	2.99 ab	3.31 a
Stuttgarter risen	B-stimul	3.14 ab	3.16 a
Rote Laaer	Control	3.12 ab	3.37 a
Rote Laaer	EkoBooster 2	2.65 a	2.92 a
Rote Laaer	Vermifit A	2.98 ab	3.39 a
Rote Laaer	B-stimul	2.50 a	2.88 a

Legend: The letter indicates the significant difference according to the LSD test at P=0.05 between treatments and cultivars.

In both cultivars results showed that it was no significant difference between control and Vermifit A in market yield. Magdi et al. (2009) reported that the yield and quality of onion were significantly influenced by fertilizer types. In general, the significant improvement in yield attributes of onion with organic nitrogen fertilization could be ascribed to an overall improvement in crop growth. Increasing levels of organic nitrogen also increase onion bulb. However, the use of organic fertilizer increases the height of the bulb, as reported by Jayathilake et al. (2003) and Akoun (2005).

Similar result was also reported by Sharma et al. (2003). They found that animal manure applications increased onion yield. In some studies, P fertilizer applied to onions provided a slightly positive effect, resulting in an increase in bulb yield as compared with no P fertilization (Amin et al. 2007), whereas, in others, there were no differences (Boyhan et al. 2007). In the cases of K fertilization levels, some researchers (Aisha and Taalab 2008, El-Bassiony 2006), reported that the highest bulb yield and quality were observed with increased potassium sulfate level in the clay or clay loamy soil of Egypt.

CONCLUSIONS

For consumers, the most important thing for vegetables is how healthy they are and which nutrients and vitamins they provide. The vitamin C content and carotenoids were studied in two onion cultivars, using different treatments of bioadditives. According to year 2016, the cultivar Stuttgarter riesen contained the highest content of vitamin C and the highest content of carotenoids in treatment B-stimul. The cultivar Rote Laaer contained the highest content of vitamin C in control and the highest content of carotenoids in treatment EkoBooster 2. Results showed that use of bioadditives can be useful in the organic production of onion for the farmers in case of yields. The study concluded that market yield in the cultivar Stuttgarter riesen was significantly increased due to the application of EkoBooster 2, while in cultivar Rote Laaer the highest market yield was found in Control. Also, the study showed that between cultivars and treatments there are no statistically significant differences in total yield.

ACKNOWLEDGEMENTS

This work was supported by IGA Project No. 7/2016/591 of the Mendel University in Brno.

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REACTION OF ZYMOSEPTORIA TRITICI ISOLATES COLLECTED IN THE CZECH REPUBLIC DURING THE YEAR 2017 TO AZOXYSTROBIN

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Abstract: The most frequent technique used among the wheat (*Triticum aestivum*) growers, in order to fight with fungal pathogens, is treating plants with fungicides. Unfortunately, due to the developed resistance of those pathogens to some active ingredients of fungicides, this method is not always effective. The aim of this study was to detect the resistance of *Zymoseptoria tritici* isolates collected in the Czech Republic during the year 2017 to strobilurin, by laboratory agar dilution biotest and molecular methods as a CAPS marker and qPCR. Resistance to strobilurin fungicides was found in 54% of total 66 analysed isolates. The presence of G143A mutation in the resistant isolates was confirmed.

Key Words: Septoria tritici blotch, *Mycosphaerella graminicola,* agar dilution method, quinone outside inhibitors, cytochrome *b*

INTRODUCION

In agriculture, the main focus is on producing more food with better quality. Due to the climate change and many other environmental factors, such are diseases and pests, this task became more challenging. Wheat (*Triticum aestivum*) is produced for its grain so it is of great importance to increase the yield. One of the main factors causing yield loss are plant diseases and it is essential to find an effective method for disease control. Winter wheat is during different stages of its development attacked by many infectious agents causing a disease. One of the most common is *Zymoseptoria tritici,* the causal agent of Septoria tritici blotch (STB). *Z. tritici*, anamorph of filamentous ascomycete (Wittenberg et al. 2009) with teleomorph stage *Mycosphaerella graminicola* ((Fuckel) Schröter in Cohn) belongs to the genus *Mycosphaerella* (Quaedvlieg et al. 2011) and it is one of the best studied *Mycosphaerella spp.* fungi (Rudd et al. 2015). *Z. tritici* causes significant yield losses worldwide (Eyal 1999), including the Czech Republic (Drabešová et al. 2013, Matušinsky et al. 2011, Tvarůžek et al. 2015, Tvarůžek et al. 2016). This fungal pathogen evolved resistance to some chemical compounds of fungicides.

The most frequently used fungicides in wheat disease management are quinone outside inhibitors (QoIs or strobilurins) that are affecting the respiratory chain of phytopathogenic fungi. The evolved resistance of *Z. tritici* isolates to strobilurins is linked to the point mutation in the mitochondrial cytochrome *b* gene, resulting in the amino acid substitution from glycine to alanine at codon 143 (G143A) of the cytochrome *b* (Fraaije et al. 2003). The G143A mutation is preventing quinone outside inhibiting (QoI) fungicides to bind to the possible fungicide binding site (ubiquinol oxidation (Qo) site), letting on fungi to continue mitochondrial respiration. In a population of *Z. tritici* are thus two alleles, wild type (standard) allele with glycine (G143) and the resistant (mutant) allele with alanine (A143). The strobilurins fungicides were introduced for the first time in 1996 (Morton

and Staub 2008) and the first occurrence of this mutation in *Z. tritici* genes associated with above-mentioned resistance was reported in Europe in 2001 (Fraaije et al. 2005).

Due to a frequent application of this group of fungicides on the territory of the Czech Republic, the amount of QoI fungicide resistant populations highly increased during the years from 2005 to 2011 (Drabešová et al. 2013). The most recent significant increase of resistant *Z. tritici* isolates in the Czech Republic was also detected in 2015, when it was confirmed in 47.3% of tested isolates (Tvarůžek et al. 2016).

MATERIAL AND METHODS

Sampling

Plant material was obtained from different locations, across the Czech Republic. Around 250 samples were collected during the April 2017, when a winter wheat is in the phase of its regeneration, producing tillers (BBCH 21–29). One leaf sample for qPCR analysis was collected during stage BBCH 85.

Obtaining isolates

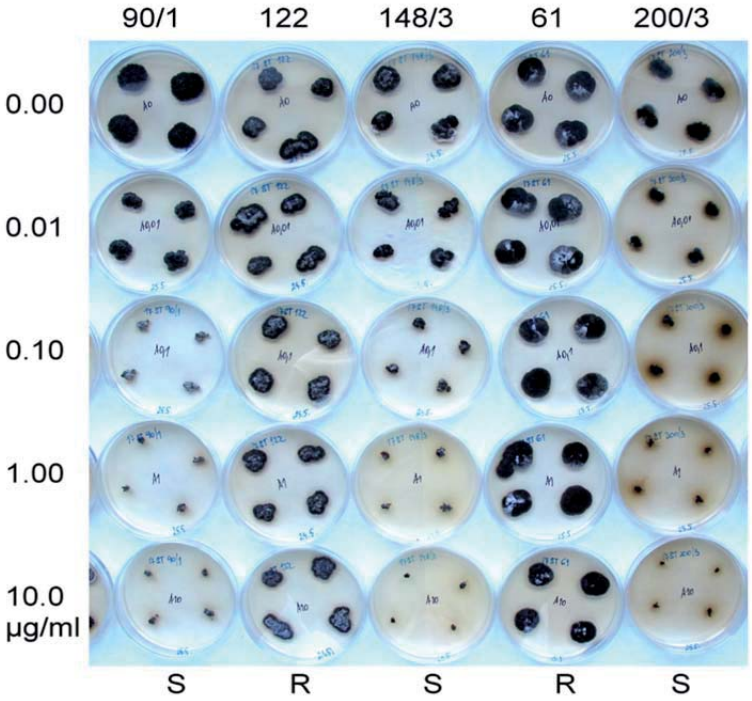
The leaves parts with *Z. tritici* lesions with pycnidia (Figure 1) were selected from those samples. After overnight incubation of symptomatic leaves in the wet chamber, pycnidium is releasing mucus mass called cirrus, containing pycnidiospores. Cirry were transferred to a Petri dishes with 3.9% potato dextrose agar (PDA) with the sterile needle and incubated at 20 °C for 5 days in the dark. Sixty-six monosporic isolates of *Z. tritici* were obtained from the collected leaves samples.

Figure 1 The leaf sample with black pycnidia, an asexual fructifications bodies

Biotest

When the mycelium was formed the agar was cut to 1.5 mm pieces with mycelium. Those cuttings were transferred on the PDA agar containing streptomycin and azoxystrobin. Serial dilutions of the azoxystrobin were 0.0, 0.01, 0.1, 1.0 and 10.0 µg/ml. Petri dishes with agar were incubated for 14 days in the dark at 20 °C. After those days diameter of each colony was measured (see Figure 2). Then the ED50 (µg/ml) value was calculated by probit analysis.

Figure 2 Growth of Z. tritici (R) resistant (122 and 61) and (S) sensitive (90/1, 148/3 and 200/3) isolates (in columns) on PDA (potato dextrose agar) with streptomycin and serial dilutions of azoxystrobin (in rows)



Molecular analyses

DNA extraction

Plant tissue and mycelia of *Z. tritici* isolates were ground to a fine powder in liquid nitrogen using a mortar and pestle. Homogenized, and total genomic DNA was extracted, using the DNeasy Plant Mini Kit (Qiagene, Germany), according to the manufacturer's instructions.

CAPS marker analysis

Cytochrome *b* sequences were selected in Gene Bank database (https://www.ncbi.nlm.nih.gov/) under accession number AY247413.1. Based on these sequences, a set of primers marked as STcytobF/R (Matušinsky et al., 2011) were designed. Firstly, part of cytochrome *b* was amplified and then by specified restriction endonuclease, this section was digested. A total reaction volume of PCR was 20 µl containing 0.2 mM of each nucleotide dNTP, 1U Taq polymerase, 2.5 mM $MgCl_2$, 1 x PCR buffer, 0.2 µM of each primer (STcytobF-TGAGGATTTGGAAGAGTCACC and STcytobR-GATTTCCTGAACCCGCTGTA) and 10 ng of DNA isolated from mycelium of monosporic isolate *Z. tritici*.

Reaction conditions

The reaction temperature is increased to 94 °C for 1 minute. Then 35 cycles of denaturation at 94 °C for 1 minute, annealing at 55 °C for 30 seconds and elongation at 72 °C for 40 seconds. Final incubation lasted 5 minutes under 72 °C.

In order to proceed the digestion, we mixed 5 µl of final PCR product together with 1 U of restriction endonuclease *Bse*XI and particular buffer. Total volume was 20 µl. Then we incubated a mixture at 37 °C for 16 hours. The final product of this analyses were fragments which are later on separated by the method of horizontal electrophoresis in 1.7% agarose gel.

qPCR

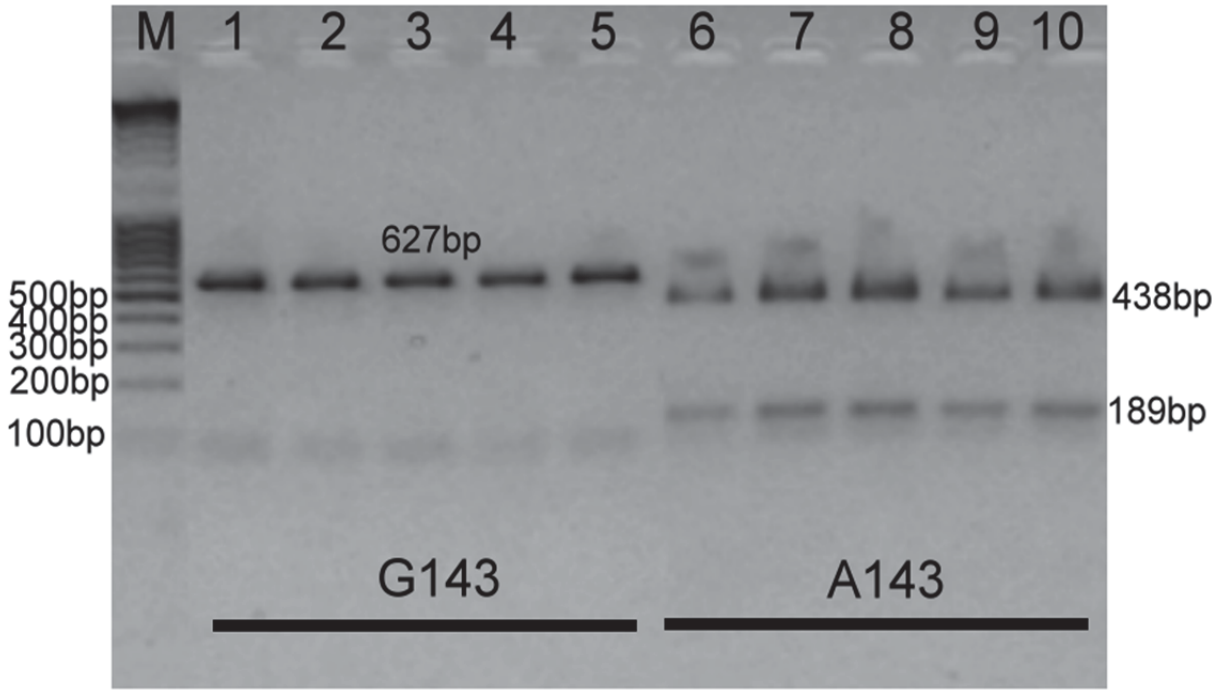
Infected leaf with typical symptoms was tested for the presence of the G143A mutation, conferring QoI resistance in *Z. tritici*. For the qPCR was used isolated DNA from that leaf sample, together with two other samples of *Z. tritici* (one sensitive and second resistant) as a control. DNAs of these two isolates were mixed 50:50 (see Figure 4).

For the singleplex SYBR Green qPCR assays was used Bio-Rad CFX Connect™ real time PCR detection system. For the wild type allele (G143) was used 5'-ACCTTATGGTCAAATGTCTTTATGATG-3' primer and for the mutant allele (A143) was used 5'-ACCTTATGGTCAAATGTCTTTATGATC-3'. The corresponding reverse primer was 5'-AGCAAAGAATCTGTTCAATGTTGC-3'. Cycling conditions were 10 min at 95 °C, followed by 40 cycles of 15 sec at 95 °C, 30 sec at 60 °C and 30 sec at 71 °C (FRAC 2015).

RESULTS AND DISCUSION

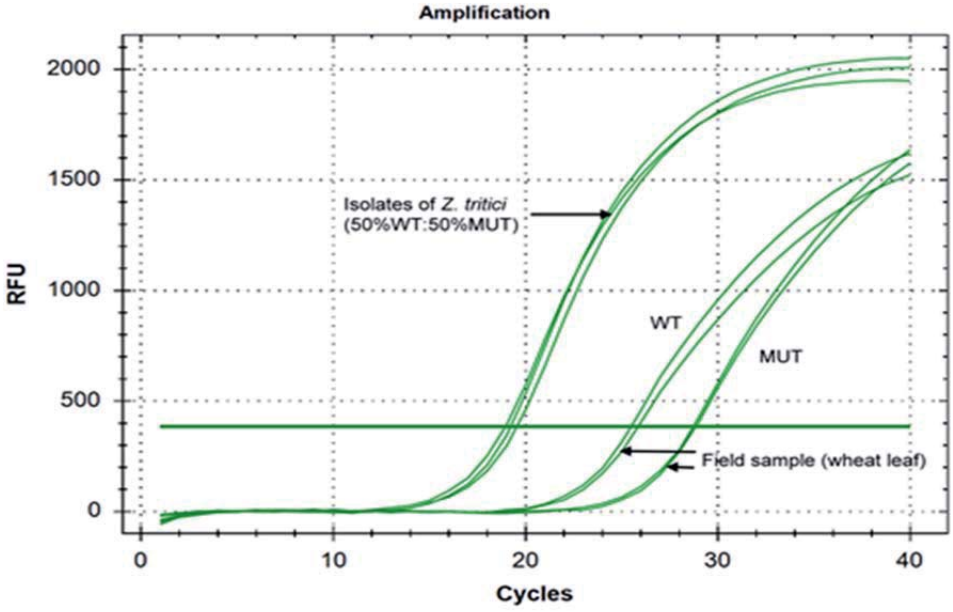
The biotest results showed that resistance was found in 54% of collected isolates. Selected resistant isolates in our test were undergone CAPS marker analysis (see Figure 3) and had G143A mutation in cytochrome *b*, associated with QoI resistance.

Figure 3 Separated fragments of selected Z. tritici isolates DNAs in 1.7% agarose gel. M is 100bp DNA Ladder, samples 1–5 were sensitive in biotest, samples 6–10 were resistant in biotest (DNA of samples 1–5 is not digested (one band 627bp) which corresponds to standard (wild) allele G143, DNA of samples 6–10 is digested to the two bands (438bp and 189bp) which corresponds to resistant (mutant) A143 allele of cytochrome b)



The primers for amplification of wild type allele (WT) and mutant allele (MUT) were used for distinguishing of sensitive and resistant cytochrome *b* allele in qPCR. The allele specific primers amplified WT or MUT allele. They were used for identification of presence G143 (WT) and A143 (MUT) alleles in field sample (wheat leaf infected by *Z. tritici*) (see Figure 4). The melting analysis showed that the used primers do not create any unspecific product, which could possibly interfere with the results of qPCR. In the past 20 years, damages caused by *Z. tritici* in the Czech Republic increased rapidly (Drabešová et al. 2013). The incidence and rapid increase in resistance to QoI fungicides have previously been reported from Western Europe (Fraaije et al. 2005). In our study, a significant proportion of studied isolates was found to be resistant to strobilurin azoxystrobin. If fungicides are used repeatedly, the number of resistant populations will continue to grow. In order to avoid resistance, it is of a high importance to follow anti-resistance strategies. Reducing of a number of the application during vegetation, as well as using mixtures of fungicides with different modes of action, will give good results. Good crop management and good host resistance are also essential.

Figure 4 Identification of wild type (WT) and mutant (MUT) allele of cytochrome b in the field sample by qPCR. Sensitive (WT) and resistant (MUT) DNA of Z. tritici isolates from mycelium mixed 50:50 were used as a control. The Y axis shows relative fluorescence units (RFU), the X axis shows the number of cycles



CONCLUSION

In 2017, resistance to strobilurin fungicides was confirmed in 54% of total sixty six analysed monosporic *Z. tritici* isolates. Molecular analysis confirmed the presence of G143A mutation in the cytochrome *b* gene of selected resistant isolates, which explains their resistance to strobilurins.

ACKNOWELEDGMENTS

The research was financially supported by the MZe NAZV QJ1530373.

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USING WASTEWATER AS IRRIGATION – INFLUENCE ON AVAILABILITY OF NINTROGEN IN SOIL AND SOIL HYDROPHOBICITY

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Abstract: Presented experiment was designed to determine the impact of using wastewater on soil hydrophobicity and availability of mineral nitrogen for soil microorganisms. This impact was studied by a pot experiment which was performed in control conditions of a greenhouse. The three types of waste water (treated and untreated greywater, yellow water) and deionized water (control) were used for irrigation of experimental containers (4.5 kg of soil) planted with *Zea mays* L. as an indicator. The measured results confirmed significant effect ($P < 0.05$) of wastewater application on formation of soil hydrophobicity and availability of mineral nitrogen for soil microorganisms due to composition of individual wastewater. The highest level of soil hydrophobicity was found in a variant where untreated greywater was applied, in comparison to control and other variants. Above all, application of yellow water significantly increased mineral nitrogen content in soil (by about 20%) and its availability for soil microorganisms in comparison with (un)treated greywater and control variant.

Key Words: yellow water, treated greywater, untreated greywater, fertilization, mineral nitrogen, soil hydrophobicity

INTRODUCTION

Over the last 20 years, drought has been the biggest "climatic threat" for farmers in the Czech Republic. The largest number of dry episodes was recorded in Žatecko, the Labska Plain and the South Moravian Region (Potop et al. 2012). Therefore, it can be assumed the impact of drought on agricultural ecosystems will grow in the near future not only in the Czech Republic but also globally (Potop et al. 2012, Wilhite et al. 2014). The decrease in soil fertility due to lack of water and nutrients in soil – main negative phenomenon of drought can be affected only by using new technologies and procedures in agriculture, such as changes in land management or new methods of irrigation (Bimüller et al. 2014, Geng et al. 2014). On the other hand, lack of irrigation resources represents the major limiting factor for the use of irrigation in arid area. Anyway, current sources can be replaced by wastewater with its benefits and also potential risks (Bimüller et al. 2014). The use of wastewater in agriculture represents new opportunity to the recycling of nutrients and improvement in biological and chemical properties of soil. The application of wastewater on arable land has to be monitored, because there are potential risks: contamination of soil by heavy metals, saturation of soil by nitrogen and leaching of nutrients from soil (Hanjra et al. 2012).

The main objectives of this experiment were as follows: (a) to describe and investigate the potential influence of wastewater irrigation on availability of mineral nitrogen in soil; and (b) to investigate the interconnection of fertilization and formation of soil hydrophobicity.

MATERIAL AND METHODS

Design of laboratory experiment

Laboratory experiment was used to investigate presented objectives from 29th January to 7th May 2014. Twelve experimental plastic (PVC) containers (height = 400 mm; width = 100 mm) were filled with arable soil (4.6 kg per one container) which was taken (upper layer; 0–30 cm) in the protective zone of underground source of drinking water "Březová nad Svitavou" (Lat: 49°39´52.9´´N; Long: 16°28´06.02´´E) according ISO 10 381-6. Above all, *Zea mays* L. was used as indicator plant (one per a container) and grown under controlled conditions in an experimental greenhouse (24 °C day temperature, 20 °C night temperature, air humidity 75% for all 24 h with a day length of 12 h with light intensity 4 000 lx).

Table 1 Overview of the laboratory experiment

Treatment	Designation	Characteristic
1	TG	Treated greywater
2	UG	Untreated greywater
3	YW	Yellow water
4	DW	Deionized water (control)

To demonstrate effect of wastewater irrigation on availability of mineral nitrogen (N_{min}) in soil and formation of soil hydrophobicity, four variants of experiment with different kinds of wastewater irrigation were established (see Table 1), each one in three repetitions. The individual samples of wastewater were taken from wastewater treatment plant (un/treated greywater) and public restroom (yellow water). The indicator plant was sown as a germinated seed and the first application of wastewater was performed after 12 days when the indicator plant had two leaves. Irrigation regime was selected on the basis of the actual soil moisture which was maintained at 70% of water holding capacity. Total irrigation dose for all variants and experimental container was 8 340 ml.

Chemical analyses of the used wastewater

The individual samples of wastewater (WW) were analysed according ISO 11905-1:1997 for the determination of nitrogen, ISO 10523 for potential pH measurement and ISO 15705:2002 was used for determination of the chemical oxygen demand index (COD). The measured results are presented in Table 2.

Table 2 The chemical analysis of used wastewater

Sample of water	pH	mg/kg					
		COD	N_{tot}	N_{inorg}	NH_4-N	NO_2-N	NO_3-N
TG	8.45	164	2.11	0.39	0.15	0.01	0.23
UG	8.49	143	5.25	2.26	0.13	1.56	0.56
YW	8.63	2 701	761	750	749	0.12	0.95

Water drop penetration time test – determination of soil hydrophobicity level

The Water Drop Penetration Time Test (WDPT) was used for the quantification of soil hydrophobicity at individual variants of experiment. WDPT was performed according Doerr (1998): five drops (one drop = 50 µl) of distilled water were applied onto soil surface using micropipette (Eppendorf, Germany). Subsequently, penetration time (PT) of individual drops was measured and recorded. Finally, only median of PT was used as an indicator of soil hydrophobicity or soil water repellence (SWR) for every replicate of all variants (for example TG a; TG b; TG c; etc.) and it was used for statistical analysis of whole variant (a, b, c → TG). Level of SWR was classified on the basis of Table 3. Individual terms of measurement were expressed by growth scale according to Berglund et al. (2014).

Table 3 Different classes of SWR on the basis of WDPT according different authors (Doerr 1998)

Classification	Adams et al. (1970)	Bisdom et al. (1993)	Doerr et al. (1996)
Hydrophilic	< 10	< 5	< 60
Slightly hydrophobic	10–60	5–60	–
Strongly hydrophobic	–	60–600	–
Severely hydrophobic	> 60	600–3600	–
Extremely hydrophobic	–	> 3600	> 3600

Determination of mineral nitrogen availability for soil microorganisms – Index of nitrogen availability

Mineral nitrogen (N_{min} consists of ammonium and nitrate N) availability in soil for microorganisms was measured as amount of N which was stored in the microbial biomass and therefore indicated availability of ammonium and nitrate N for microbes. Firstly, N_{min} availability was described by Bundy a Meisinger (1994). Authors used this method for example in studies Elbl et al. (2014): availability of N_{min} for soil microbes was determined on the basis of ammonium N production during 7 day waterlogged production. The analysis consists of two sub-steps: (a) determination of N_{min} content in soil samples before incubation and (b) determination of ammonium N which is released from microbial bodies (cells) after 7 day incubation.

First step (a): solution of 2M KCl was used for extraction of N_{min} from soil sample before incubation according to Bundy and Meisinger (1994). Subsequently, the concentration of released N_{min} in extraction solution was determined by distillation-titration method according to Peoples et al. (1989). Second step (b): a 20 g of soil sample and 50 cm^3 of distilled water were placed into airtight incubation bottle and placed into a thermostat (40 °C for 7 days). After incubation, only concentration of ammonium N was determined by the same method as total N_{min}, because concentration of nitrate N was unchanged.

Statistical analysis

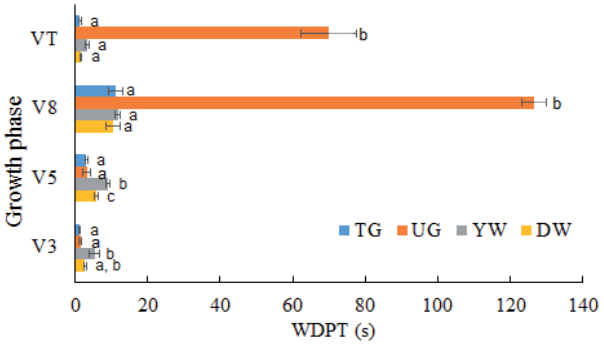
The one-way analysis of variance (ANOVA; $P < 0.05$) in combination with LSD Fischer test was used to determine significant differences in availability of N_{min} in soil and level of SWR between individual variants.

RESULTS AND DISCUSSION

Developed hydrophobicity

The values of WDPT were measured four times during indicator plant growth. Individual terms of measurement were expressed by vegetative corn growth scale according to Berglund et al. (2014). The complete overview of measured values is presented in a bar chart supplemented with results of statistical analysis (Figure 1).

Figure 1 Results of water drop penetration test (WDPT)



Legend: Different letters indicate significant differences at level of P < 0.05 separately for individual Vegetation Corn Growth (V3, V5, V8, and VT).

The measured values indicate potential impact of WW application on formation of SWR on soil surface. Results of the first measurement (V3 – start of experiment) showed only small differences between individual variants. These results showed the level of soil hydrophobicity was similar among individual variants TG, UG and YW. Only one significant difference ($P < 0.05$) was found between variant TG, UG and YW, anyway all results were smaller than 20 s which indicates low level of soil hydrophobicity (Doerr 1998) at the beginning of the experiment.

The significant differences between control and variant with WW application were found before the second measurement (V5 – WW was already applied). On the other hand, this data are not clear because WDPT was lower in variant TG, UG and higher in variant YW in comparison with control variant DW. Above all, measured values were lower than 60 s, which indicates low level of soil hydrophobicity according (Doerr 1998). Different situation was observed during the third (V8) and fourth (VT) measurement in comparison to the first two (V3 and V5). The application of UG had significant impact on formation of SWR because WDPT was higher than 120 s during the third measurement and then 60 s during the fourth one. This data, according Bisdom et al. (1993) and Doerr et al. (1996), indicate strongly hydrophobic soil. This situation was probably caused by organic (hydrophobic) matter, which was present in UG. The effect of organic compounds or hydrophobic components of organic matter was confirmed by Doerr (1998), Piccolo and Mbagwu (1999) and Tarchitzky et al. (2007) state the soil irrigated by fresh water was hydrophilic, and conversely soil irrigated by WW hydrophobic. The above authors also used the WDPT test. Moreover, Travis et al. (2010) compared three types of irrigation water (fresh water, treated and raw greywater) and their impact on SWR: the highest WDPT was seen on the surface of raw greywater irrigated soil versus soil irrigated with freshwater or treated greywater.

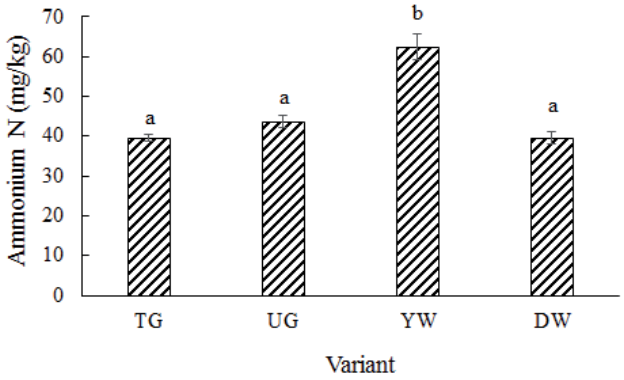
SWR represents an important parameter as it affects infiltration as well as stability of soil aggregates and retention of water in soil (Müller and Deurer 2011). The presented results indicated the application of UG can be used as remediation strategy for soil with low level of SWR. However, it is necessary to realize application of WW on the soil with high content of hydrophobic compounds can result in increase in level of SWR and subsequently some negative consequences may occur, for example decrease in ability to infiltrate and retain water.

Availability of mineral nitrogen for soil microorganisms

After end of the experiment, the concentration of N_{min} in soil of individual variants and its availability were measured. The following Figure 2 and Table 4 provide information on obtained results. The availability of N_{min} for soil microorganisms is expressed as the quantity of N which organisms were able to use and release from their cells. The measured results show that the application of TG or UG did not have effect on availability of N for soil microbes in comparison to control variant DW. Conversely, application of YW resulted in significant increase in availability of N for microorganisms in soil (Figure 2), by about 20% in comparison with other variants. This result clearly shows that composition of individual types of WW had direct impact on availability of N in soil and its content (consider Table 2 and 4). The YW contained more than five hundred times larger amount of nitrogen in comparison to other types of WW. The measured results are consistent with findings of Dimitriou and Aronsson (2004) and Sparling et al. (2005) who confirmed the application of WW affects content of nutrients in soil, especially N, depending on composition of individual types of WW.

Except the availability of N_{min} for soil microorganisms, the content of ammonium and nitrate N in soil samples after end of the experiment was measured as they represent the main two components of soil N_{min}. There were found significant differences in content of both, ammonium and nitrate N, between individual types of WW irrigation (Table 4). The highest content of individual N_{min} forms has always been found in variant where YW was applied. On the other hand, only small differences were found between variant, where TG or UG was applied, and the control variant (DW). This result indicates there is a relationship between composition of WW (see Table 2 – the highest content of N_{tot} was found in yellow water) and content of N_{min} in soil. The remaining nitrogen was washed away due to soil saturation (data not published).

Figure 2 Availability of N_{min} for soil microorganisms



Legend: The amount of ammonium N released from microbial cells after 7 day incubation in mg/kg of soil sample. Mean value ± standard deviation (presented by error bars) are shown. Different letters indicate significant differences at level of P < 0.05.

The effect of WW irrigation on content of N_{min} in soil is very important because there is a direct connection between presence of N in soil and soil fertility. Moreover, there is a potential risk of saturation of soil system by reactive N (Sutton 2011). Above all, Singh et al. (2012) confirmed the application of WW affects soil chemical properties depending on its composition.

Table 4 Content of ammonium (mg/kg) and nitrate N (mg/kg) in soil samples

Variants	Ammonium N	LSD	Nitrate N	LSD
TG	1.87 ± 0.48	a	2.37 ± 0.78	a
UG	1.59 ± 0.47	a	3.78 ± 0.43	a
YW	4.09 ± 0.64	b	51.08 ± 1.78	b
DW	2.97 ± 0.55	a,b	3.44 ± 0.54	a

Legend: The content of ammonium N and nitrate N in soil samples after end of the experiment, i.e. before 7 day incubation. Mean value ± standard deviation are presented. Different letters indicate significant differences at level of P < 0.05.

CONCLUSION

The presented experiment confirmed different effects of individual types of WW on SWR and availability of N_{min} for soil microorganisms. There is a big potential for the use of WW in agriculture, on the other hand presented results revealed the importance of monitoring the selected parameters of WW intended for irrigation. Irrigation by WW can affect content of N in soil, availability of N for soil microbes, and SWR which are important and necessary factors for soil fertility and quality in a case it contains necessary nutrients and substances.

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DETECTION OF CARBON CONTENT CHANGES AFTER BIOCHAR APPLICATION ON AGRICULTURAL FIELD EXPERIMENT USING BOTH METHOD WALKLEY-BLACK AND THERMOGRAVIMETRY

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Abstract: Introduction new methods for analysing samples is associated with comparison with standard methods. Thermogravimetry (TG) works on principle of monitoring thermal mass losses (TML) of samples in depended on temperature or on time during heating up, e.g. Determination of SOC content at 550 °C in muffle furnace during several hours. More detailed analyses of TML dynamics can provide more accurate and reliable data. Here are presented results of SOC content determined by TG (TML320–330 which correspondent temperature between 320 to 330 °C) compared with results determined by standard method (Walkley-Black, WB) on the samples from field experiment with biochar addition (0, 15, 30, 45 t/ha) in combination with mineral and organic fertilizer. The soil samples were sampled in 2 depths (0–0.1 and 0.1–0.2 m) in autumn 2016. The results determined by TG show significant underestimation of SOC content comparison with WB method. Biochar addition records TG at higher temperatures (for our samples by TML around 400 to 550 °C for both fertilizer management).

Key Words: soil organic carbon content, thermogravimetry, Walkley-Black method, mass loss ignition, mineral fertilizer, manure, biochar, field experiment

INTRODUCTION

Soil organic matter (SOM) is an important chemical soil property (Eleki et al. 2014) with positive impact on physical, hydrological, chemical, biochemical soil properties and crop yield (Bertora et al. 2009, Reijneveld et al. 2010). The SOM is assessed by soil organic carbon (SOC) content (Haynes and Naidu 1998).

The renewable energy sources is constantly increasing last years (Galvez et al. 2012). Among them pyrolysis which its by-product is biochar. Biochar is the solid material produced by thermochemical conversion of biomass including such as agricultural waste, animal manure or industrial wood by-products in an oxygen-limited environment etc. (Lehmann and Joseph 2015). It has an aromatic structure, which resulted in stable and highly resistant to chemical and biological degradation in soil (Atkinson et al. 2010). Atkinson et al. (2010) and Cayuela et al. (2014) point out its potential application for long-term C sequestration and climate change mitigation because biochar is

a C-rich material, primarily stabilized C (Ciais et al. 2013). Most studies focus on biochar impact on soil properties, which were conducted in pot experiments (Devereux et al. 2012, Castellini et al. 2015, Głab et al. 2016). In contrast, Sandhu et al. (2017) recommend testing across a broad spectrum of soil-crop combinations, biochar sources and different management aspects before largescale application.

The Walkley-Black method is one of the standard method for SOC determination citied in state methodologies and various modifications were generated according to the needs of users over time (Zbíral et al. 2011, Gelman et al. 2012, Mylavarapu 2014).

Thermogravimetry monitors mass losses (TML) of samples in dependency on temperature or on time during heating up. In soil science, this method is known from traditionally use for SOM determination via mass losses on ignition (MLI) of absolute dried samples by burning at 550 °C in muffle furnace during several hours (Peikert et al. 2015). More detailed analyses of TML dynamics in 10 °C temperature increase steps after standardized sample preparation provide more accurate and reliable data as content of organic carbon, total organic nitrogen, clay or carbonates. Most of these findings, however, are applicable to ordinary soils, not to those containing mixtures of organic and geological parent material (e.g. composts, gardening muds, technosols, and soils with high amount of fresh organic residues, debris, soot, cinder, or carbon from geological sources) (Siewert 2004).

The aim of this work is the detection of changes in SOC after adding different biochar amount (0, 15, 30, 45 t/ha) after first year field experiment using Walkley-Black and thermogravimetric method.

MATERIAL AND METHODS

The field experiment was established on the area of "Vyzkumna picninarska stanice Vatin" – Faculty of Agronomy, Mendel University in Brno (the Czech Republic) in the autumn of 2015 by plough of manure (depth 0.25 m). Vatin is located around 60 km NW of Brno (exactly Lat: 49° 31' 01.5" N and Long: 15° 58' 22.2" E) and the elevation of the research station is 540 m above the sea level. Soil type by soil classification FAO (2014) is Cambisol and soil texture by World Reference Base is sandy loam (content of clay 11.80%, silt 33.52% and sand 54.68%) (USDA 2017).

The field experiment compares influence of organic (30 t manure/ha) and mineral (200 kg superphosphate/ha) fertilizers in combination with different quantities of biochar application (0, 15, 30, and 45 t/ha). The total number of variants are 16 (2 types of fertilizers, 4 quantities of biochar, 2 depths of sampling). Each plot had 12 m^2 with each of 3 field repetitions (24 plots). Growing crop in year 2016 was corn (*Zea mays* L.).

Biochar comes from the Czech Republic, the company BIOUHEL.CZ s.r.o. based by Zlin. The input material for pyrolysis process was digestate (ca 80%) and cellulose fiber (ca 20%), the temperature of pyrolysis 450 °C. After application in spring 2016 the soil was cultivated at depth of 0.2 m as mixing by hand (shallow inserter).

The sampling and data for carbon determination based on sampling for physical properties. The sampling depths were 0–0.1 m and 0.1–0.2 m. The samples were taken from one (a) field repeating as disturbed samples in October 2016 in order to limit the impact of sampling on the field experiment. Next sampling will be carried out on other field repetitions in next years what should allow a series of sample collections over the time.

The soil samples were processed on the standard method with air drying and sieving across 2 mm mesh (Zbíral 2002). For SOC content determination with Walkley-Black (WB) method modified by Novák-Pelíšek (Klika et al. 1954) samples were subsequently grounded to size < 0.25 mm. The sample mass was 0.2 g with added 5 ml $K_2Cr_2O_7$, 10 ml H_2SO_4, 100 ml distilled H_2O and titrated with Mohr´s salt to constant red-brown colour (Jandák et al. 2013).

For SOC content determination by thermogravimetry (TG) samples were additionally stored at 76% relative air humidity to equilibrate the water content and analysed with thermoscale from Mettler-Toledo TGA/SDTA 851e. The sample mass was 0.8–1.0 g and the samples were heated up from 25 to 950 °C with heating rate of 5 °C per minute and a data capturing of 1 reading per 4 seconds (one value per 0.3 °C temperature increase). During the analytical procedure, the furnace with the sample was purged with a stream of air enriched by 76% relative humidity (related to 26 °C) with a flow rate of above 200 ml/min. The evaluation of results started with recalculation of mass losses

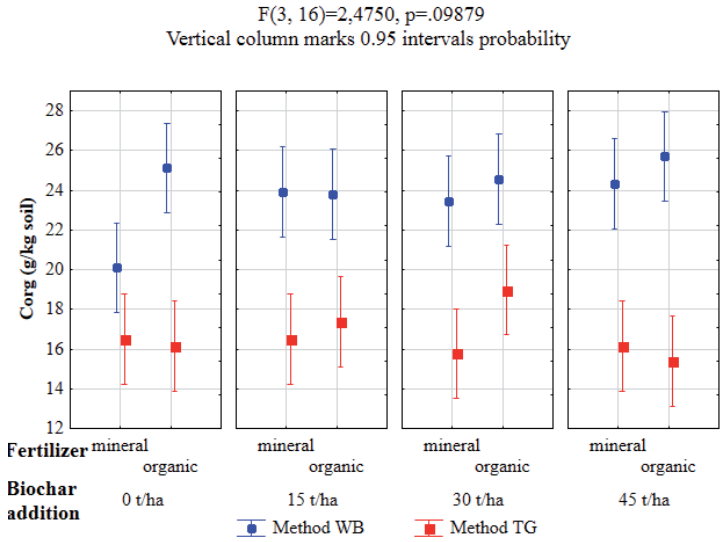
to 1 g of soil for comparability of analyses that were carried out with different sample weights. Subsequently, the number of data points was reduced to thermal mean mass losses measured in 10 °C temperature increase intervals. For SOC content determination, we used thermal mass losses from 320 to 330 °C only (Siewert 2004).

The results were processed by multiple analysis of variance (ANOVA; $P < 0.05$) in combination with Tukey test to determine significant differences between used laboratory methods for SOC determination (STATISTICA 12 program – StatSoft, USA). Once after first year the sampling was limited to one repetition, the statistical evaluation uses sampling from second layer as repeating.

RESULTS

Figure 1 describes the influence of fertilizers and biochar application on soil organic carbon (SOC) content determined by both WB and TG method. Statistical analysis did not reveal significant influence of different quantities of biochar and fertilizers on SOC content. However, differences between SOC content obtained from both WB and TG method are significant ($P < 0.001$) and higher by WB method.

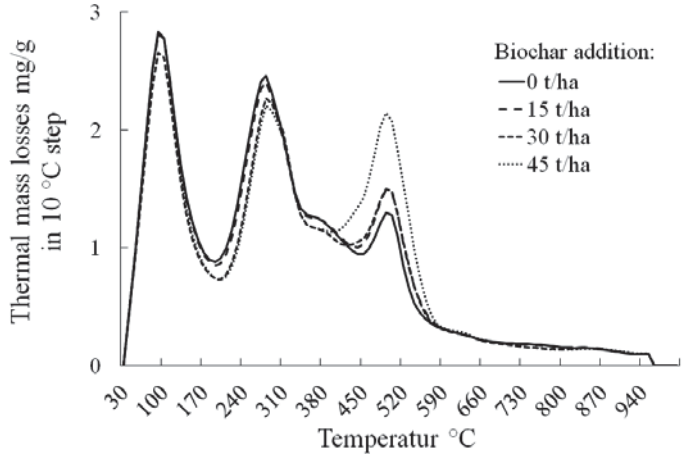
Figure 1 Comparison of organic carbon content determination with Walkley-Black method (WB) and thermogravimetry (TG)



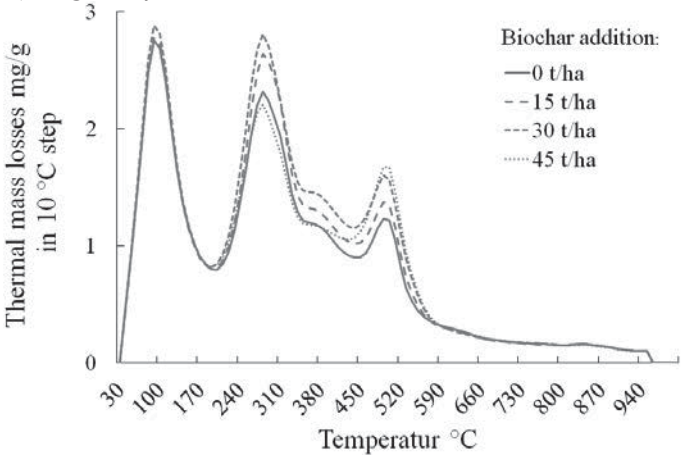
The Figure 2 shows the dynamics of thermal mass losses of plots with different quantities of biochar for plots with mineral (Figure 2A) and organic (Figure 2B) fertilizers.

Figure 2 Thermal mass losses of samples with the different amount of biochar for plots with mineral (A) and organic (B) fertilizers for soil samples from depth 0–0.1 m

A) Mineral fertilization



B) Organic fertilization



Results of statistical analyses reveal significant changes in TML ($P < 0.001$) around 400–550 °C with increasing quantity of added biochar in all plots with mineral fertilization. In case of organic fertilization the TML around 400–550 °C changes as well with increasing quantity of added biochar. In addition, plots with 15 and 30 t biochar/ha show significant increased mass losses ($P < 0.001$) at temperature around 250–450 °C. This area corresponds to addition of labile organic substances and SOC content. Plot with 45 t biochar/ha do not differ from plot without biochar addition in temperature range around 250–450 °C.

DISCUSSION

Many authors refer to influence of organic fertilizers on carbon content. The impact on carbon content depends from the amount of applied fertilizers, their type and sampling depth (Xie et al. 2014, Schlegel et al. 2017, Wei et al. 2017). In case of same amount of fertilizers, biochar has usually higher and long-term impact on C content (Wang et al. 2015, Moreno-Barriga et al. 2017) what can be explained by higher carbon content and with higher biological stability (Hilscher et al. 2009, Bruun et al. 2011, Ciais et al. 2013, Zhao et al. 2013, Wang et al. 2015, Moreno-Barriga et al. 2017).

Organic fertilizers frequently increase not only carbon content but variability of results as well what hampers evaluation due to significant differences (Körschens et al. 1994). It could be explained by possible errors during sampling and soil preparation for laboratory analysis. This could explain the limited ability to detect differences in carbon content in these experiments. Another reason could be the quality of field repeating. Here we used the second soil layer because it was mixed with the first layer after adding biochar and fertilizers. However, the impact of fertilizers could be different in first and second layer because of different conditions (density, oxygen availability, water content etc.). We aspect improvement of accuracy and significance with including all field repetition during next sampling what will allow layer as an impact factor on SOC content.

TG is known for limited ability to detect organic carbon content of extraneous sources such as fresh plant material, black carbon, biochar, ashes, cinder etc. (Siewert 2004). However, the dynamics of TML clearly reveal the qualitative changes on SOC influence of added biochar in plots with mineral and organic fertilizer (what confirms results of Tokarski et al. – submitted). These open new opportunities for detection of changes in SOM composition. For example, high quantity of biochar application seems to change the impact of organic fertilizers on thermal mass losses around 300 °C. This could be a result of increased microbiological decomposition of organic fertilizers caused by high quantity of biochar.

CONCLUSION

The limited ability of TG to capture the whole SOC content included extraneous carbon was confirmed and reveals in lower SOC content comparing with WB method. The disadvantage reverse to an advantage to detect different quality and quantity of extraneous carbon if consider dynamics of mass losses at higher temperature around 500 °C. Further experiments should show to which extend

the reliability and accuracy of such approach allows practical application and investigation of interaction between different organic fertilizers as an impact factor for carbon content regulation in soil.

ACKNOWLEDGEMENTS

The research was financially supported by the organisation Deutsche Bundesstiftung Umwelt, no. AZ 30017/718. Thanks to the LKS - Landwirtschaftliche Kommunikations- und Servicegesellschaft mbH for the assistance in laboratory experiments and the University of Applied Sciences Dresden for providing access to offices and laboratories.

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RESPONSE OF MILK THISTLE [*SILYBUM MARIANUM* L. (GAERTN.)] ON NITROGEN AND SULPHUR FERTILIZATION

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Abstract: The aim of this study was the determination of the effect of nitrogen and sulphur fertilization on plants height, maturation of inflorescence and seed production of milk thistle. The study was conducted as small-plot field experiment in experimental plots located in Šumperk (the Czech Republic). The following treatments influenced the experiment: 1) $N_{50}S_0$; 2) $N_{100}S_0$; 3) $N_{50}S_{25}$; 4) $N_{100}S_{50}$; 5) $N_{25}S_{12.5}$+$N_{25}S_{12.5}$ and 6) $N_{50}S_{25}$+$N_{50}S_{25}$ (dose in kg/ha). Fertilizers (calcium ammonium nitrate and ammonium sulphate nitrate) were applied in a single (treatments 1–4) and split doses (treatments 5 and 6). The positive effect of nitrogen fertilization on plants height was found. Significantly the highest plants height was found in the variant with application of 100 kg nitrogen before sowing. The highest number of flower heads was found in the variant with nitrogen fertilization without sulphur. Higher dose of nitrogen applied before sowing prolonged development of milk thistle maturing. Although no significant effect of nitrogen and sulphur fertilization on achenes production was found, the results of this experiment showed positive effect of application of nitrogen before sowing on growth and development of milk thistle plants.

Key Words: milk thistle, nitrogen, sulphur, height of plants, yield of achenes

INTODUCTION

Although milk thistle [*Silybum marianum* L. (Gaernt.)] is not regularly cultivated plant in the Czech Republic, it is the most often cultivated crop from the group of medicinal and aromatic plants and spices. The area of cultivation is approximately 5 000 hectares and the average yield of seed is 0.7 t/ha. The area is only estimated, because Czech Statistical Office does not monitor area of milk thistle (Přibylová 2014). The production of achenes is the main reason why milk thistle is cultivated. The achenes contain flavonolignan compounds silymarin which is used as medicinal substance (Pradhan, Girish 2006). Silymarin complex is a mixture of flavonolignans (silybin A and B, isosilybin A and B, silychristin and silydianin) and also flavonolignan taxifolin (Abbasi et al. 2010, Abenavoli et al. 2010, Elwekeel et al. 2013). The high-quality oil (approx. 25–30%) is also an important compound of achenes, it contains 43.5–64.4% linoleic acid, 20.8–29.8% oleic acid, 7.2–9.7% palmitic acid and 2.0–6.6% stearic acid (Khan et al. 2007, Afshar et al. 2014, Zhelev et al. 2014). Silymarin complex is used in human medicine, especially as a treatment of liver and spleen diseases (Cardile and Mbuy 2013).

One of macronutrients, that have important impact on milk thistle production, is nitrogen (N). It involves the growth and the height of vegetation (Omidbaigi and Nobakht 2001, Estaji et al. 2016), inflorescence development (Stancheva et al. 2008) and yield of achenes (Omer et al. 1993, Warren and Samsa 2011, Cwalina-Ambroziak et al. 2012, Estaji et al. 2016). Sulfur (S) is also a significant nutrient in plants nutrition, but the effect of sulphur on the production of milk thistle is not fully clarified. Also the effect of nitrogen and sulphur interaction on milk thistle growth and production has not been known yet. The literature only mentions the effect of S fertilization on fatty acid proportion in milk thistle oil (Wierzbowska et al. 2012), nitrogen utilization in achenes (Wierzbowska 2013) and length of phenological stages (Nasrabadi et al. 2014). The aim of this study was the evaluation

of the effect of combination of nitrogen and sulphur fertilization on milk thistle growth, inflorescence development and achenes production.

MATERIAL AND METHODS

The effect of nitrogen and sulphur fertilization on the growth of milk thistle was observed in the precise small-plot experiment. Experiment was established on the land of Agritec Research, Breeding and Services, Ltd. in Šumperk, field Bratrušov (GPS 49°59′20.486″N, 16°57′43.719″E). Milk thistle (variety Mirel, germination 93%, sowing rate 8 kg/ha) was seed on April 22, 2016. Fertilizers were applied in single doses (two days before sowing) and split doses (before sowing and during vegetation in the beginning of the elongation growth during elongation and branching of plants) according to the scheme in Table 1. All the variants were conducted in four repetitions.

Table 1 Diagram of small-plot experiment

Variant of fertilization	Dose of N (kg/ha)	Dose of S (kg/ha)	Fertilizer	Term of application
1 $N_{50}S_0$	50	0	CAN	20. 4. 2016
2 $N_{100}S_0$	100	0	CAN	20. 4. 2016
3 $N_{50}S_{25}$	50	25	DASA	20. 4. 2016
4 $N_{100}S_{50}$	100	50	DASA	20. 4. 2016
5 $N_{25}S_{12.5} + N_{25}S_{12.5}$	25 + 25	12.5 + 12.5	DASA	20. 4. + 24. 6. 2016
6 $N_{50}S_{25} + N_{50}S_{25}$	50 + 50	25 + 25	DASA	20. 4. + 24. 6. 2016

Legend: CAN - Calcium ammonium nitrate, DASA - ammonium sulphate nitrate.

Calcium ammonium nitrate (27% N) and ammonium sulphate nitrate (fertilizer DASA – 26% N and 13% S) were used as nitrogen and sulphur fertilizer. The height of plants, number of inflorescence (with mature achenes, with immature achenes, flowering), yield of mature achenes were determined in phase of terminal flower heads maturity (August 4, 2016) using 10 plants were randomly selected in each plot (40 plants per variant).

Statistica CZ 12 programme was used for the determination of the overall characteristics. To elaborate the significance of differences among the arithmetic means of each characteristic, we used the monofactor analysis of variance (ANOVA) followed by testing at a 95% ($p < 0.05$) level of significance using Fischer's LSD test.

RESULTS AND DISCUSSION

Nitrogen and sulphur fertilization significantly influenced milk thistle habit. Table 2 shows that height of plants was significantly increased after application higher doses of nitrogen compared to lower doses of nitrogen. Dose of 100 kg nitrogen increased the height of cover by 13.8 cm. The positive effect of nitrogen fertilization on plants height was also noticed by Omidbaigi and Nobakht (2001). The significantly highest height of plants was found after the application of 100 kg nitrogen, similar results are also reported by Estaji et al. (2016). The significant effect of mineral and organic fertilization on height of milk thistle plants was also presented by Hendawy et al. (2013).

Table 2 Height of milk thistle plants grown at different fertilization

Variant of fertilization	height of plants (cm)	SE (cm)	$p < 0.05$
1 $N_{50}S_0$	185.1	± 6.4	a
2 $N_{100}S_0$	198.9	± 0.2	b
3 $N_{50}S_{25}$	194.3	± 4.6	ab
4 $N_{100}S_{50}$	187.9	± 3.0	ab
5 $N_{25}S_{12.5} + N_{25}S_{12.5}$	193.3	± 3.3	ab
6 $N_{50}S_{25} + N_{50}S_{25}$	195.2	± 1.9	ab

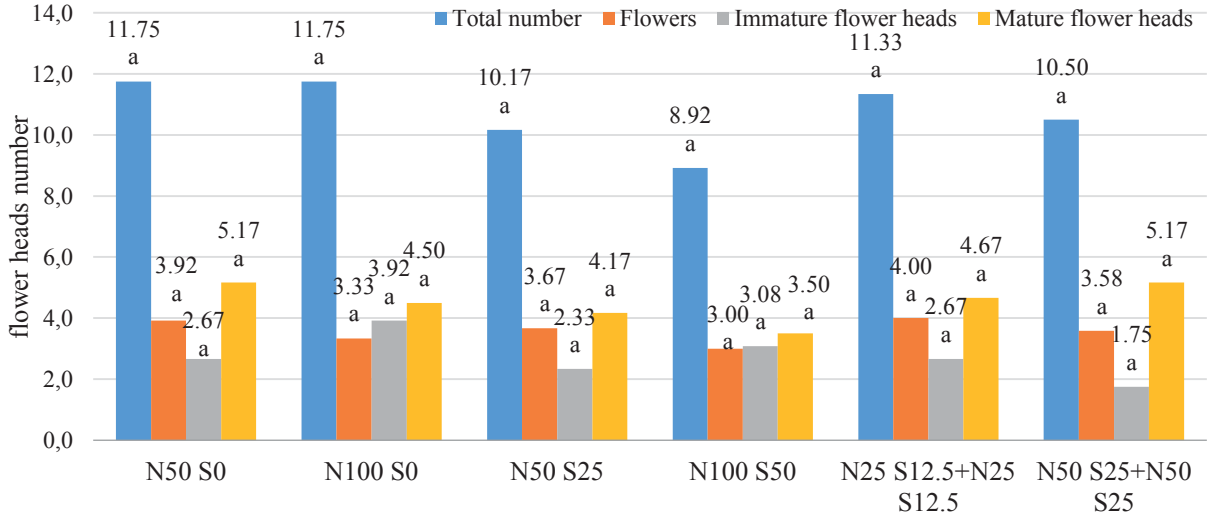
Legend: Means with same letter are not significantly different (P<0.05).

The effect of sulphur fertilization on plants height was different and it depends on nitrogen doses. The doses of 25 kg S/ha and 50 kg N/ha increased the height of plants almost about 5% in contrast to the variant only with nitrogen fertilization. Table 2 also shows that sulphur fertilization

in combination with 100 kg doses of nitrogen reduced the height of plants (187.9 cm) in contrast to only nitrogen fertilization (198.9 cm). Split nitrogen dose did not significantly influence the plants height. Likewise Omidbaigi and Nobakht (2001) did not note higher height of milk thistle plants after split doses of nitrogen.

The effect of nitrogen and sulphur fertilization on number of inflorescence was not proved in this experiment. The highest number of flower heads was found on nitrogen fertilized plants without sulphur application, while sulphur fertilization before sowing reduced the number of flower heads (Figure 1). Higher dose of nitrogen applied before sowing postponed milk thistle senescence. The number of mature flower heads was lower by 0.67 after the application of 100 kg N (var. 2) compared to the variant with 50 kg N/ha, but the number of immature flower heads was increased in the variant with higher dose of N. The reduction of mature flower heads caused by nitrogen application before sowing was bigger in the variants with sulphur fertilization (var. 3 and 4). Similarly in experiment of Nasrabadi et al. (2014) sulphur application postponed the start of elongating and flowering stages, which might be associated with the availability of higher N during crop growth. The opposite effect had split doses of nitrogen and sulphur. The higher doses of fertilizers increased the production of mature achenes and the production was similar to variant $N_{50}S_0$.

Figure 1 Number and type of inflorescence of milk thistle plants grown at different fertilization



Legend: Means with same letter are not significantly different (P<0.05).

The yield of achenes from mature flower heads presents Table 3. The results show that the effect of fertilization on achenes production (g/plant) was not significant. The reason of high variability of observed characteristics is probably lower uniformity of plant habit which could be caused by low breeding level of milk thistle variety. Even though the results in Table 3 are not significant, it is obvious that single and split doses of fertilizers with nitrogen and sulphur had positive effect on average yield of achenes. The results of achenes yield (g/mature heads) are not significant, nevertheless the variants with higher doses of fertilizers show positive response to the fertilization (Estaji et al. 2016).

Table 3 Yield of achenes of milk thistle mature heads/plants grown at different fertilization

Variant of fertilization	Yield of achenes (g/plant)	SE (g/plant)	p < 0.05	Yield of achenes (g/mature heads)	SE (g/heads)	p < 0.05
1 $N_{50}S_0$	9.28	± 0.61	a	1.84	± 0.27	a
2 $N_{100}S_0$	9.92	± 2.14	a	2.21	± 0.24	a
3 $N_{50}S_{25}$	10.55	± 1.78	a	2.50	± 0.30	a
4 $N_{100}S_{50}$	9.10	± 0.26	a	2.61	± 0.09	a
5 $N_{25}S_{12.5} + N_{25}S_{12.5}$	12.19	± 2.52	a	2.67	± 0.15	a
6 $N_{50}S_{25} + N_{50}S_{25}$	12.26	± 2.25	a	2.55	± 0.46	a

Legend: Means with same letter are not significantly different (P<0.05).

CONCLUSION

Although the nitrogen and sulphur fertilization significantly influenced only the height of plants, we could say that the application of nitrogen before sowing had positive effect on milk thistle habit and growth. The variants with nitrogen and sulphur fertilizers had higher positive effect on the achenes yield than the variants with nitrogen and without sulphur. When we observed inflorescence (immature and mature flower heads), the results showed that higher nitrogen and sulphur doses contributed to postpone senescence and the production of achenes was higher at the same time.

ACKNOWLEDGEMENTS

This study was supported by the IGA TP1/2016 (*New findings in the cultivation and use of milk thistle (Silybum marianum L.) in agriculture*).

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THE EFFECT OF INTERACTION BETWEEN DEFICIENT NUTRITION AND *PSEUDOMONAS SYRINGAE* PV. *TOMATO* INFECTION ON DEVELOPMENT OF TOMATO ROOT SYSTEM

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Abstract: The aim of this hydroponic experiment was the determination of the effect of deficient nutrition in combination with *Pseudomonas syringae* pv. *tomato* infection on tomato (*Solanum lycopersicum* L.) root system. Variants with deficient nutrition (nutrition solutions without nitrogen, phosphorus, potassium, calcium and magnesium) and one variant with complete nutrition (control variant) were observed. Plants were split in two groups, the first group was cultivated without inoculation, the second group was inoculated by bacteria *Pseudomonas syringae* pv. *tomato* which causes bacterial speck disease on tomato. The development and growth of root system were evaluated by using root electrical capacitance (REC) method which was measured by LCR meter. The highest REC of non inoculated group had plants with magnesium deficiency (0.766 nF). Inoculated plants with highest REC were plants from K-deficiency solution (0.406 nF). The length and area of root system were evaluated by program WinRhizo. According to these parameters, the biggest root system had plants with P deficiency from non inoculated group and plant cultivated without N from inoculated group. Bacterial infection caused the reduction of all observed parameters in contrast to the plants from non inoculated group, so the infection had negative effect on root growth and development.

Key Words: tomato, deficient nutrition, *Pseudomonas syringae* pv. *tomato*, root electrical capacitance

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is an important plant for human diet. The mineral nutrition of it is one of critical factors for plant development, growth and yield (Baligar et al. 1998). Tomato has different demands on nutrition during vegetation, but it belongs to the fruit vegetable with high demands on nutrients (Vaněk et al. 2012). Root system has important function for right nutrition and water uptake. The growth, size and activity of root system are involved by bioavailability of nutrients in the soil solution. Bioavailability of nutrients involves root morphology, density of lateral root and also number of root hair (Fageria 2013). Zhu et al. 2005, Wissuwa et al. 2005, Hawkesford et al. 2012, Kellermeier et al. 2013 present nitrogen (N), phosphorus (P) and potassium (K) as important nutrients for alter post–embryonic root developmental processes. Calcium (Ca) is an essential nutrient for growth of root system (Rahman and Punja 2009). Magnesium (Mg) has an important function in enzymatic reaction (Cowan 2002) and it has also an effect on initiation, density and length of root hair (Zhang et al. 2014).

The health of plant has also a great effect on growth and development of root system. Bacterium *Pseudomonas syringae* pv. *tomato (Pst*) causes bacterial speck disease and the main symptoms are small spots on the leaves. Spots are brown in the middle and they are surrounded by yellow rings. They could spread to the fruit in some cases (Goode and Sasser 1980). Varieties grown in the Czech Republic started to be susceptible to the pathogen of bacterial speck, so incidence of this disease increased (Kokošková and Pouvová 2012).

The aim of this work was to determine the effect of nutrients deficiency and infection caused by *Pseudomonas syringae* pv. *tomato* on growth and function of tomato root system in the earliest stages of development.

MATERIAL AND METHODS

Vegetation pots experiment was established in the form of an aqueous culture with tomato (*Solanum lycopersicum* L.) and it was placed into growth chambers of the Department of Agrochemistry, Soil Science, Microbiology and Plant Nutrition, Faculty of AgriSciences, Mendel University in Brno in 2016. Tomato bush variety Darinka was used as model crop. The seeds were sown into a nutrient–free substrate and when the plant roots reached approximate length 8 cm (14 days after beginning of germination), they were put into vegetation pots with complete nutrient solutions. After 14 days, plants were split into two groups, one of them was inoculated by the bacterial suspension (concentration 10^6 cfu/ml) which was composed of *Pst* isolates 111 and 214 from the bacterium collection in the Research Institute of Plant Production, Praha-Ruzyně. The second group was not inoculated and it was considered as control group. Both the groups of plants were put into solutions of different composition (Table 1). The solutions had been prepared by the method of Hoagland (Hoagland and Arnon 1938). The non-transparent plastic boxes (volume 45 l) were used as vegetation pots. The nutrient solutions were aerated at regular time terms (30 minutes in each 2 hours). The vegetation pots were situated in growth chambers (PlantMaster, CLF Plant Climatics GmbH, Germany) in controlled temperature, humidity and light mode (12 h day length, photosynthetic photo flux density of 350 µmol/m^2/s, temperature of 23/18 °C (day/night) and relative humidity of 55/70%). 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 the 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
Without Ca	–	1.518	–	0.136	–	–	0.241
Without Mg	0.821	0.506	0.436	0.136	–	–	–

The evaluation of monitored parameters (level of *Pst* infection, root electrical capacitance, length and area of root system) was performed 7 days after splitting into inoculated and non inoculated group. Plants cultivated in complete nutrient solution were considered as control plants.

First, the level of infection was visually evaluated by 4-level scale: 1st level – without symptoms, 2nd level – 3–5 spots on the leaves, 3rd level – ⅓ of leaf area with spots, 4th level – ¾ of leaf area with spots (Víchová 2004). Then the size of root system was detected by measuring root electrical capacitance (Chloupek 1977, Dalton 1995). REC was measured by LCR meter ELC–131D at frequency of 1 kHz in nanofarads (nF) in distilled water of constant composition (in Woulf bottle). One electrode was inserted in constant position at the bottom of the bottle and the second electrode was attached to plant hypocotyl. Due to position of electrodes, the electric circuit was created and the alternating current passes between the root system and water. Living parts of root are electric active because of polarization of living cells or membranes (Středa and Klimešová 2016). After determination of REC, the plants were divided into root and aboveground part. Root system was scanned and the length and area were analysed by program WinRHIZO, version Basic (Régent Instruments Inc., Quebec, Canada). These parameters were evaluated in 5 repetition and Statistica 12 CZ programme was used for statistical evaluation of these parameters. The effect of the deficient nutrient and *Pst* infection on leaves and development of the root system was evaluated by ANOVA

analysis of variance. Results are expressed as a mean ± standard error (SE). 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

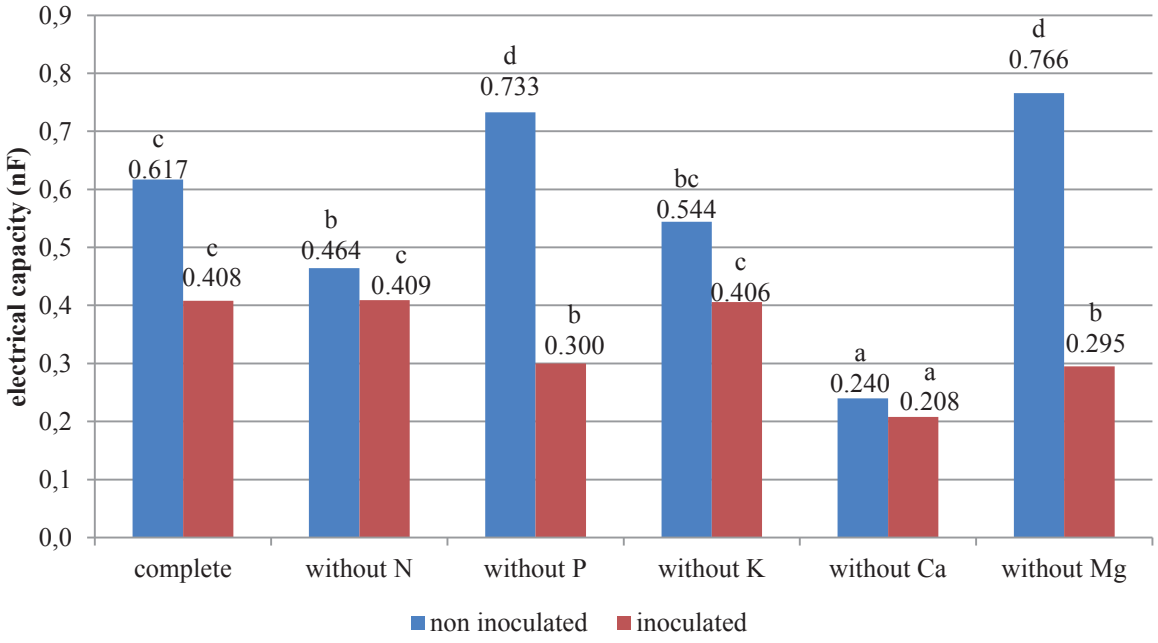
Deficient nutrition significantly involved the level of infection in variant with P, K and Ca deficiency (Table 2). The plants with P and Ca deficiency had significantly ($P<0.05$) higher level of infection in comparison with plants cultivated in complete nutrient solution. Calcium provides strength of cell wall (Huber et al. 2012) and inhibits enzymes which help pathogens to penetrate plant cells (Huber 1980), so the plants with the lack of Ca were less resistant to infection. Plants with K deficiency had also significantly ($P<0.05$) higher level of infection than control plants. Amtmann et al. (2008) present that potassium involves resistance to fungi and bacterial pathogens. Huber et al. (2012) also present that plants with K deficiency have not right protein synthesis and nitrogen compounds accumulate in cells. The pathogen uses these nitrogen compounds and it could cause higher level of infection.

Table 2 Determination of Pseudomonas syringae pv. tomato infection

Nutrient solution	Level of infection ± SE
Complete	$1.8^{a} \pm 0.24$
Without N	$2.2^{ab} \pm 0.37$
Without P	$3.4^{c} \pm 0.40$
Without K	$2.8^{bc} \pm 0.20$
Without Ca	$3.4^{c} \pm 0.40$
Without Mg	$2.4^{ab} \pm 0.20$

Legend: Means with same letter are not significantly different (P<0.05).

Figure 1 Electrical capacitance of tomato root system (nF). Electrical capacitance of non inoculated and inoculated group was statistically evaluated separately. Means with same letter are not significantly different (P<0.05).



From Figure 1 is obvious the difference in REC between non inoculated and inoculated plants. Plants with Mg deficiency show the highest difference between REC of non inoculated and inoculated

groups, electrical root capacitance of inoculated plant is lower by 61.5%. High difference between REC of non inoculated and inoculated plants was also found in the variant with P deficiency. Phosphorus contributes to resistance to infection (Prabhu et al. 2007), so plants with P deficiency were more attacked by *Pst* and this also could be reason for lower electrical capacitance of root. Zhu and Lynch (2004) and Dubrovsky (1997) reported when plants grow in low P conditions, the amount of root hair is increased. This change increased absorptive surface and it could cause higher electrical capacitance. This could be explanation for the fact that non inoculated plants with P deficiency had higher value of REC than control plants. The lowest difference between REC of non inoculated and inoculated plants was found in the variant with lack of calcium. These plants had also significantly the lowest electrical capacitance in contrast to control plants.

Table 3 The length (cm) of root system. Length and relative percent of non inoculated and inoculated group were statistically evaluated separately.

Nutrient solution	Non inoculated		Inoculated		LSD test between non inoculated and inoculated plants
	length cm	rel %	length cm	rel %	
Complete	$682.6^{b} \pm 6.55$	100.0	$412.0^{b} \pm 65.95$	100.0	b/a
Without N	$739.6^{b} \pm 167.83$	108.4	$567.8^{c} \pm 148.93$	137.8	a/a
Without P	$1564.5^{c} \pm 202.90$	229.2	$463.9^{bc} \pm 49.57$	112.6	b/a
Without K	$867.0^{b} \pm 88.46$	127.0	$505.4^{bc} \pm 58.68$	122.7	b/a
Without Ca	$893.4^{b} \pm 38.83$	130.9	$317.9^{ab} \pm 17.97$	77.2	b/a
Without Mg	$115.3^{a} \pm 17.94$	16.9	$124.1^{a} \pm 11.53$	30.1	a/a

Legend: Means with same letter are not significantly different (P<0.05).

Plants with P deficiency had the longest root system of non inoculated group, the length of these roots was significantly longer in comparison with control variant. The lack of phosphorus did not have negative effect on lenght and area of root system (Lopéz-Bucio et al. 2003). Roots of inoculated plants were significantly shorter in variants with P, K and Ca deficiency and also in control variant in contrast to non inoculated variants (Table 3). Plants with N deficiency had the significantly longest root system from inoculated group. When a plant suffers from nitrogen deficiency (especially nitrate), the lenght and area of root hair increases (Föhse and Jungk 1983) and the elongation of lateral root starts (Linkohr et al. 2002).

Table 4 The area (cm^2) of root system. Area and relative percent of non inoculated and inoculated group were statistically evaluated separately.

Nutrient solution	Non inoculated		Inoculated		LSD test between non inoculated and inoculated plants
	area cm^2	rel %	area cm^2	rel %	
Complete	$25.0^{b} \pm 1.52$	100.0	$14.4^{bc} \pm 2.42$	100.0	b/a
Without N	$25.5^{bc} \pm 5.82$	103.7	$18.6^{c} \pm 3.55$	129.1	a/a
Without P	$54.3^{d} \pm 6.00$	216.7	$16.1^{bc} \pm 1.95$	112.0	b/a
Without K	$32.5^{bc} \pm 3.30$	129.6	$16.6^{c} \pm 1.37$	115.3	b/a
Without Ca	$4.3^{a} \pm 0.47$	17.2	$4.1^{a} \pm 0.26$	28.5	a/a
Without Mg	$36.3^{c} \pm 1.78$	145.2	$10.7^{b} \pm 0.57$	74.3	b/a

Legend: Means with same letter are not significantly different (P<0.05).

Phosphorus deficient plants of non inoculated group had significantly the largest area of root in contrast to control. The lack of phosphorus could increase the area of root because the plant tries

to increase the possibility of phosphorus uptake (Hawkesford et al. 2012). The largest root area of *Pst* infected plants was found in plant with N deficiency. Table 4 shows that significantly the smallest area of root system was detected in plants with Ca deficiency (non inoculated and also inoculated). The *Pst* infection had also negative effect on the area, the inoculated variants (complete, without P, K and Mg) had significantly smaller area of root system than non inoculated plants.

CONCLUSION

The infection caused by *Pseudomonas syringae* pv. *tomato* and deficient nutrition had significant effect on root growth and development. Deficient nutrition had more negative effect on inoculated plant root system. Root electrical capacitance, length and area of root system were decreased in contrast to non inoculated plants. The highest level of *Pst* infection was found in the variants with P and Ca deficiency, control plants and N deficient plants had the least symptoms. Knowledge of interaction between infection and deficient nutrition can be useful for better health condition of plants which leads to higher production and better quality of tomatoes.

ACKNOWLEDGEMENTS

The research was financially supported by the non-project research of Faculty of AgriSciences, Mendel University in Brno.

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EVALUATION OF ROOT SYSTEM SIZE IN SELECTED *TRIFOLIUM VESICULOSUM* GENOTYPES

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Abstract: Drought is considered one of the most important factors influencing plant growth. The severity of drought is unpredictable because it depends on many factors (for example, occurrence and distribution of rainfall, temperature, evaporation and moisture storing capacity of soil). It is therefore important that the breeding of new crop varieties aims to achieve greater drought tolerance. One of the parameters contributing to greater drought tolerance is larger root system size. The root system plays an important role in nutrients uptake, which influences crop quality and yield. In this experiment, root system size was determined using an LCR meter to measure roots' electrical capacity. The electrical capacity gradually increased over time for all genotypes of the species *Trifolium vesiculosum* (No. 132, 99–48, 90–113, Tifclo-1) in relation to gradual development of the root system. Parental components which had mean root system size in all measuring intervals greater than 0.6 nF were selected within each genotype. Plants with the greatest root system electrical capacities were further crossed, and the resulting F1 generation will be similarly selected

Key Words: drought, electrical capacity, *Trifolium vesiculosum*, root system size

INTRODUCTION

An increase in the frequency of extreme weather events may be expected in connection with climate change (Středa et al. 2013). Drought is the most prevalent such extreme manifestation (Basu et al. 2016). It is defined as a recurring extreme weather event over land characterized by below-normal precipitation over a period of months to years (Dai 2010). Drought significantly affects the yield and quality of crops. The breeding of new varieties, therefore, focuses on increasing tolerance to drought. One of the parameters contributing to stronger drought tolerance is larger plant root systems.

One method of determining root system size (RSS) is that of measuring the electrical capacity of the roots. An advantage of this method is that it is an easy, rapid and non-destructive approach to estimating root system size based on a correlation between root mass and electrical capacitance of the whole root system (Aulen and Shipley 2012, Chloupek 1977, Dalton 1995). This method makes it possible to identify only the active (live) parts of a root system because the polarization of live membranes or cells is occurring there, and that means the live parts are electrically active (Školníková and Škarpa 2016, Vintrlíková and Středa 2014). The method for measuring the electrical capacity of roots has been used with many plants, including tomato (van Beem et al. 1998), poplar (Preston 2004), maize (McBride et al. 2008) and bean (Ellis et al. 2013).

The aim of this work was to evaluate root system size for various genotypes of the species *Trifolium vesiculosum*. Based on the results, plants with higher electrical capacity values will be selected and these plants will be used for crossing and creating a new variety with stronger drought tolerance.

MATERIAL AND METHODS

Experimental design, measurement method and genotype selection

Genotypes (No. 132, 99–48, 90–113, Tifclo-1) of the species *Trifolium vesiculosum* (Figure 1) were studied in a pot experiment. The genotypes were obtained from Plant Genetic Resources

Conservation Unit, Griffin, Georgia, United States of America. The pot experiment was commenced under greenhouse conditions in the spring of 2016. Plants were grown in pots with horticultural substrate. Five plants were measured from each genotype.

Root system size was determined using a method that measures electrical capacity (Chloupek 1972). Electrical capacity was measured using a VOLTCRAFT LCR 4080 instrument at a frequency of 1 kHz in nanofarads (nF). One electrode (clamp – cathode) was attached to the plant hypocotyl and the other electrode (needle – anode) was inserted into the soil. The electrical capacity was measured in the electrical circuit through which the alternating current passes between the root system and soil. Seven RSS measurements were taken overall in 7-day intervals. The measurement was in progress at the BBCH 19–49 developmental stage. The data thus obtained were evaluated as arithmetic mean and standard deviation. Data were processed in Microsoft Excel.

Figure 1 Photographs of the genotypes of species Trifolium vesiculosum: No. 132, 99–48, 90–113, Tifclo-1

RESULTS AND DISCUSSION

Mean electrical capacities of root systems of *Trifolium vesiculosum* genotypes

Table 1 Mean root system size as measured by electrical capacity (nF) of the root systems for genotypes No. 132, 99–48, 90–113 and Tifclo-1 in 7-day measurement intervals

Genotype	Parameter	Measuring date						
		1st week	2nd week	3rd week	4th week	5th week	6th week	7th week
No. 132	Mean RSS	0.622	0.867	0.664	0.983	0.966	1.203	0.947
	SD	0.116	0.096	0.273	0.255	0.193	0.128	0.183
99–48	Mean RSS	0.417	0.523	0.551	0.876	0.763	0.888	0.751
	SD	0.096	0.322	0.154	0.364	0.322	0.207	0.173
90–113	Mean RSS	0.670	0.833	0.514	0.598	0.541	0.730	0.675
	SD	0.269	0.327	0.166	0.151	0.161	0.274	0.282
Tifclo-1	Mean RSS	0.927	0.980	0.604	0.760	0.807	0.811	0.680
	SD	0.412	0.261	0.215	0.199	0.314	0.281	0.226

Legend: SD – standard deviation, RSS – root system size

Table 1 shows the mean and standard deviation values for the electrical capacities (nF) of root systems for the genotypes No. 132, 99–48, 90–113 and Tifclo-1 in 7-day measurement intervals.

The values in the table show that electrical capacity in 7-day measurement intervals gradually increased over time. The increased electrical capacity is related to the progressive development of the root system. Kendall el al. (1982) also found in red clover and alfalfa that electrical capacity grew with the development of the root system.

Figure 2 shows a graph of the mean values for electrical capacity (nF) of root systems for the four genotypes. The graph shows that mean values of electrical capacity differed among genotypes. Genotype No. 132 reached the highest electrical capacity values. Genotype 90–113, on the other hand, exhibited the lowest values. Significant differences between plants within a genotype were also observed, wherein some plants had electrical capacities significantly greater or lesser than the mean genotype. Chloupek et al. (1999) had previously found that alfalfa plants had electrical capacities significantly larger or smaller than the mean genotype. In the third week was decreased in the electrical capacity due to the high temperature period. High temperature period reduces root growth, number, and mass (Huang et al. 2012). Subsequent regeneration processes in the roots have led to an increase in RSS. Significant differences in electrical capacity between plants within a genotype are associated with higher standard deviations (Table 1).

Figure 2 Graph of mean values of electrical capacity (nF) of root systems for genotypes No. 132, 99–48, 90–113 and Tifclo-1 in 7-day measurement intervals

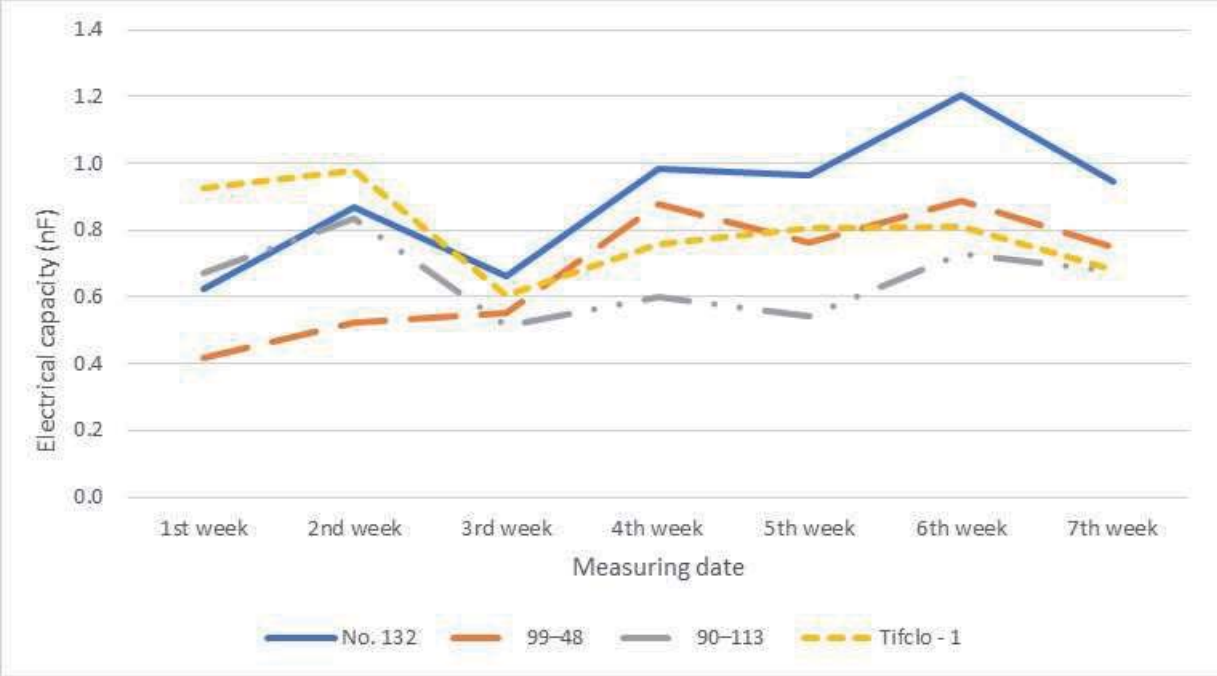

Table 2 shows mean electrical capacity (nF) values for the root systems of individual plants of genotypes No. 132, 99–48, 90–113 and Tifclo-1 for all measurement intervals. The table shows that electrical capacity differed even among plants within a single genotype. On average across plants 1–5 for each genotype, the best (highest) values were found in plants of genotypes No. 132 and Tifclo-1.

Table 2 Mean values of electrical capacity (nF) of the root systems of individual plants of genotypes No. 132, 99–48, 90–113 and Tifclo-1 for all measurement intervals

Plant number	Genotypes			
	No. 132	Tifclo-1	90–113	99–48
1	0.825	0.968	0.744	0.897
2	0.936	0.313	0.545	0.933
3	0.846	0.907	0.384	0.611
4	1.086	0.676	0.677	0.453
5	0.773	0.798	0.907	0.511
Column average	0.8932	0.7324	0.6514	0.681

CONCLUSION

Root system size was determined by measuring electrical capacity for genotypes No. 132, 99–48, 90–113 and Tifclo-1. The results were used as a guide for negative selection, and parental components suitable for further crossing were selected.

Parental components having mean root system size in all measuring intervals greater than 0.6 nF were selected within each genotype. For genotype No. 132, all plants exhibited mean electrical capacity greater than 0.6 nF. For genotype 90–113, only plants 1, 4 and 5 had mean electrical capacity greater than 0.6 nF. For genotype Tifclo-1, plants 1, 3, 4 and 5 exhibited mean electrical capacity greater than 0.6 nF. For genotype 99–48, only plants 1, 2 and 3 had mean electrical capacity greater than 0.6 nF.

Plants with the greatest root system electrical capacities were further crossed, and the resulting F1 generation will be selected for its larger root system.

ACKNOWLEDGEMENTS

Financial support for this research was based on the long-term conceptual development of the research organization Agricultural Research, Ltd.

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RELATIONSHIP BETWEEN BARLEY YIELD AND ANNUAL PRECIPITATION CONDITIONS

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Abstract: Barley grain yields and values of the effective drought index (EDI) in the critical period in terms of yield formation of spring barley in the Czech Republic were evaluated for the period 1975–2015. The EDI was calculated for four different experimental localities. The EDI is based on the calculation of effective precipitation using only daily precipitation. Both kind of the years with unfavourable precipitation conditions: 1976, 1984, 2004 and 2015, and the years with favourable precipitation conditions: 1975, 1979, 2006 and 2010 were determined. The historical grain yield range from the experimental localities for the barley was correlated with the values of the EDI at each locality for each decade (ten days). At most areas there were statistically significant relationships between grain yield and the EDI in different stages of vegetation; at some locations highly significant relationship. The statistically significant correlations ($\alpha = 0.01$, $\alpha = 0.05$) were found in 2nd, 4th, 6th, and 7th decade of vegetation.

Key Words: barley, yield, drought, the effective drought index

INTRODUCTION

One of the most common limiting factors affecting plant yield is the drought stress, which causes a number of metabolic as well as mechanical changes and initiates physiological, biochemical and molecular reactions. Based on the intensity and period of drought, with the interaction of other stress factors and considering the phase of development and genotype, these reactions ensure the adaptability to limited environmental conditions (Kalefetoglu and Ekmekci 2005).

Drought is a period of constant hydrometeorological imbalance caused by a cumulative occurrence of dry periods with low or non-measurable precipitation. Generally, drought is classified from meteorological, hydrological, agricultural and social-economical aspect (Botterill and Wilhite 2006). According to Palmer (1965), drought is a significant deviation from normal hydrological conditions of a particular area. However, drought is a result of many factors and meteorological elements, and as a temporary climatic anomaly can occur in all climatic zones.

The lack of water is one of the world's most important stress factors for plant production and can have a significant impact not only on volume, but also on the quality of the produce. Farooq et al. (2014) claims that during the vegetation plants need different amounts of water. Majority of plant species is very sensitive to the lack of water in the flowering stage and at the time when the growth of vegetative organs is the most intensive. The impact of individual episodes of meteorological drought in the form of the decrease of crop yield, is affected not only by the length and intensity of the meteorological drought, but also by the time of the occurrence (in the key phenological phases of the crops or when the water demand is higher). Therefore, the course and effects of each drought episode are unique (Brázdil and Kirchner 2007).

MATERIAL AND METHODS

The aim of this work was to quantify the relationship between the EDI and barley grain yield. As a model crop for characterization of the relationship the spring variety of barley (*Hordeum vulgare* L.) was selected. Relationship was found between the EDI (the effective drought index) and barley grain yield at 4 experimental localities of Central Institute for Supervising and Testing in Agriculture (CISTA) in the period between 1975–2015, while the years 1977, 1991, 1992, 1993 and 1994 were not concerned due to the absence of yield data.

The experimental localities are located in different agro-climatic areas in the altitude between 295 and 465 meters above sea level with the average annual temperature between 7.4 and 8.3 °C and total annual precipitation between 537 and 616 mm (Table 1 and Table 2). Unified agricultural engineering was applied at the monitored localities (pre-crop, fertilization), and the varietal composition for the CISTA experiments in the particular year was similar. Only the yields after a good pre-crop (mostly legume) from the growing system at the optimum fertilization intensity level and plant protection following CISTA method for experiment management were evaluated.

Table 1 Basic CISTA experimental localities characteristics

Locality	Altitude (m)	Long-term average temperature t_{30} (°C)	Long-term average precipitation p_{30} (mm)	Soil type (Němeček et al. 2011)
Hradec n. Svitavou	465	7.4	616	HMl–jh
Chrastava	345	8.0	738	HMl–ph
Pusté Jakartice	295	8.3	584	HMl–h
Staňkov	370	8.1	537	HMm–h

Legend: HMm – orthic luvisol, HMl – illuviated brown soil (no FAO term), h – loamy soil (medium), jh – clayey-loam (heavy), ph – sandy-loam (medium), t_{30} and p_{30} – the long-term average temperature t_{30} and the long-term average precipitation p_{30} (1971–2000)

Table 2 Agro-climatic characteristics of CISTA experimental localities (Kurpelová et al. 1975)

Station	Agroclimatological macroarea	Agroclimatological area	Agroclimatological subarea
Hradec n. Svitavou	mildly warm	slightly warm	mildly humid
Chrastava	mildly warm	slightly warm	mildly humid
Pusté Jakartice	warm	mildly warm	mildly dry
Staňkov	mildly warm	relatively mildly warm	mildly dry

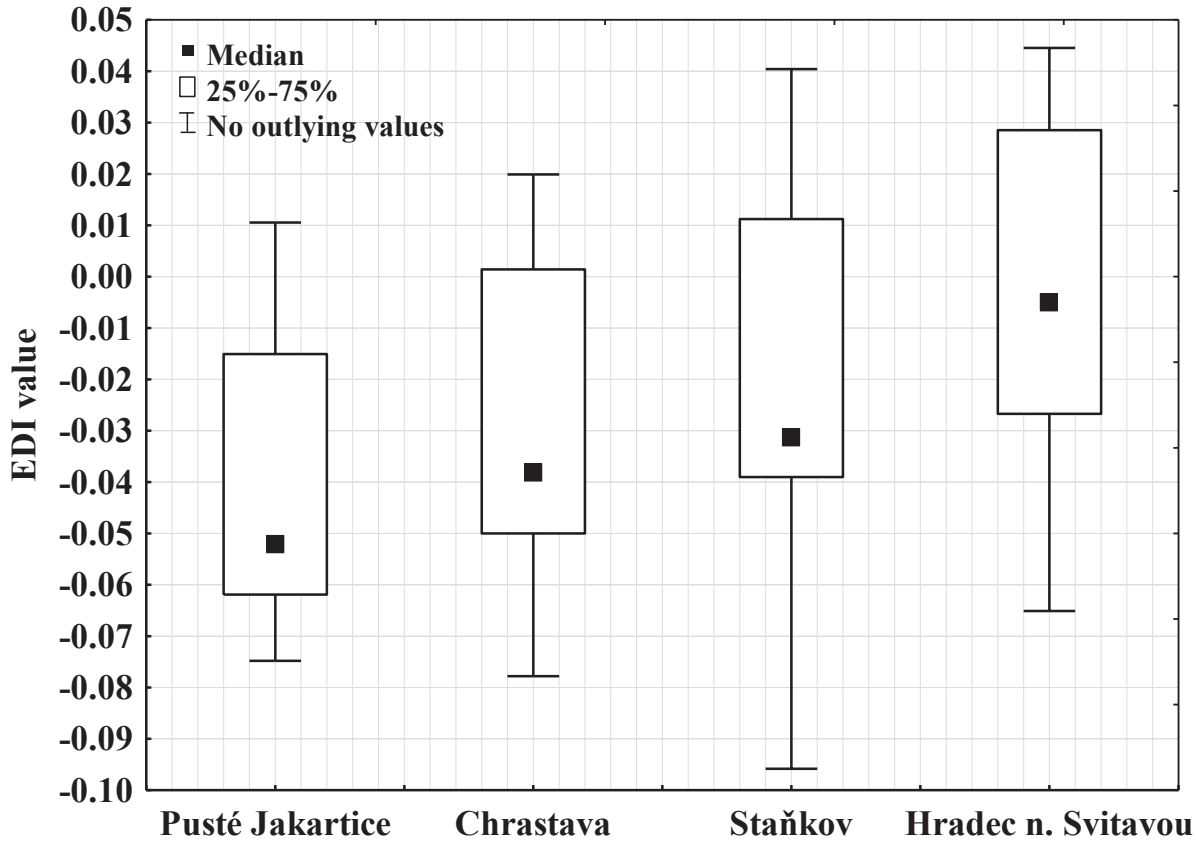
In this work, a sophisticated method of effective precipitation by Byun and Wilhite (1999) was used, which shows a number of drought indicators including the standardized EDI. The final EDI value is represented by a non-dimensional number. It is a standardized value of currently usable water sources, which allows a comparison of different locations regardless of their climatic characteristics. The EDI allows us to determine the beginning and end of the dry periods, since it uses the daily precipitation data of the last 365 days. But the EDI is calculated for the whole precipitation period and monitors the continuity of the drought period during all the calculating process. Thus it is different from all other drought indexes, which only offer the calculation for a limited drought period (for example 12 months), and it is also able to diagnose long-term drought periods, which may even last a few years. Another advantage of this index is its low demand of entry data. It only needs a long time line of precipitation totals, which is more available than other meteorological data.

Barley grain yields were confronted with the EDI values of days 111 to 180 of the year in the monitored period 1975–2015. The relationship was expressed via correlation coefficient.

RESULTS AND DISCUSSION

On the basis of the effective precipitation method, the EDI was calculated in the individual decades (ten-day periods) of the monitored period 1975–2015 for each of the experimental localities. The EDI values below 0 mark unfavourable precipitation conditions, while values above 0 mark favourable precipitation conditions. Average seasonal EDI value of the period 1975–2015 at 4 selected localities oscillates between -0.004 and -0.041. The most favourable long-term EDI value for all decades was measured for Hradec nad Svitavou locality (-0.004) at higher altitude, while the less favourable average value was found out for Pusté Jakartice locality (-0.041), which is situated at lower altitude.

Figure 1 Average values of EDI at CISTA localities in 1975–2015



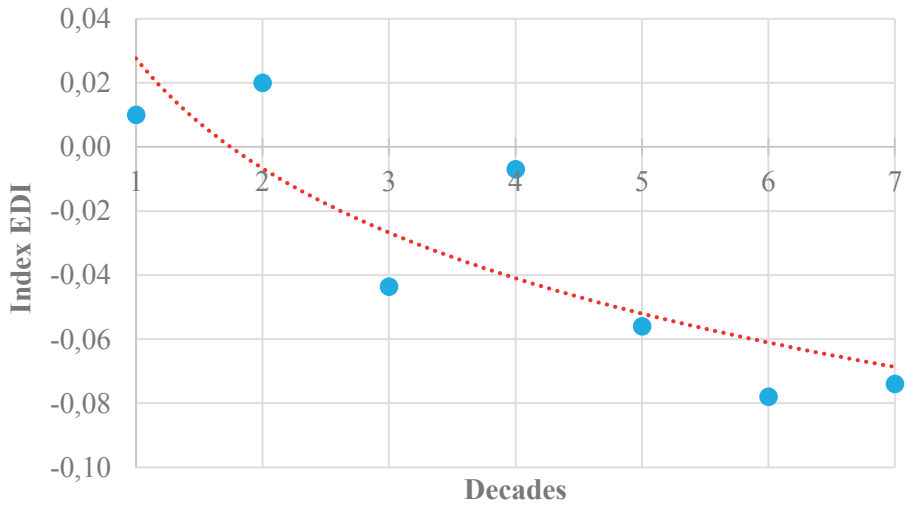
Maximum average EDI value of the monitored period 1975–2015 was reached in the decade between days 71 and 80 at Hradec nad Svitavou locality (0.045) and the lowest average EDI value was reached at Staňkov locality (-0.096) in the decade between days 171 and 180 (see Figure 1). With the help of the EDI all the years in all the stations were evaluated. Very poor precipitation conditions were recorded in 1976, 1984, 2004, and 2015, when the average annual EDI value of all decades was below -0.2. The highest EDI values (above 0.1) were reached in 1975, 1979, 2006, and 2010, so these years were rich in precipitation.

Concerning the grain yield (Table 3), the year 2014 appears to be the best, for at 2 localities the highest yield was recorded, despite the average annual EDI value was only (-0.065). The yield in 2008 was the poorest, for at 2 localities the lowest yield was recorded. Low average annual EDI value was measured in this year (-0.018). The highest average yield between 1975 and 2015 was reached at Pusté Jakartice locality (6.73 t/ha). The lowest average yield was recorded at Chrastava locality (5.81 t/ha), where the average EDI values of the decades were mostly negative, especially in the last decades of the vegetation period (days from 131 to 180; see Figure 2), including flowering phase – i.e. the most critical period for the grain production (Klimešová et al. 2017). The lowest barley yield was again recorded at Chrastava locality (3.53 t/ha) in 2008. The highest yield was recorded at Pusté Jakartice locality (9.35 t/ha) in 2014, which is located at lower altitude.

Table 3 Average, highest and lowest yields of spring barley grain at selected localities in 1975–2015 (t/ha)

Stations	Altitude (m)	MAX	Year	MIN	Year	Average
Hradec n. Svitavou	465	8.47	2003	4.70	2008	5.97
Chrastava	345	8.03	2015	3.53	2008	5.81
Pusté Jakartice	295	9.35	2014	4.45	1981	6.73
Staňkov	370	9.07	2014	4.65	1983	6.50

Figure 2 Average ten-day values of EDI index at Chrastava for the period 1975–2015



In this analysis the relationship of the EDI values of all decades (days 111–180) and the grain yield was evaluated within the period 1975–2015 (Table 4). The relationship was characterized by the correlation coefficient. Statistically significant (α = 0.05) or statistically highly significant (α = 0.01relationship was recorded in 2nd,4th, 6th, and 7th decade of the vegetation period. Highly significant relationship (α = 0.01, n = 36) was recorded at Pusté Jakartice and Staňkov localities in the 4th decade of vegetation (days 141–150), and at Chrastava locality in the 7th decade of vegetation (days 171–180). Statistically significant relationship (α = 0.05) was recorded at Hradec nad Svitavou, Pusté Jakartice, and Staňkov localities in 2nd and 6th decade of vegetation period.

Table 4 Relationship between EDI and barley grain yield expressed by the correlation coefficient at selected localities during 1975–2015

Decade (ten days)	HRA	CHT	PJA	STV
111–120	0.271	0.144	0.043	-0.315
121–130	*0.410	0.260	-0.172	*-0.354
131–140	0.054	-0.134	0.111	0.121
141–150	-0.303	-0.061	**0.513	**0.457
151–160	-0.180	-0.223	-0.133	-0.233
161–170	-0.106	0.118	*-0.350	*-0.400
171–180	-0.294	**-0.502	-0.067	-0.007

*Legend: ** statistically highly significant relationship, * statistically significant relationship, HRA – Hradec n. Svitavou, CHT – Chrastava, PJA – Pusté Jakartice, STV – Staňkov*

Spring cereal varieties are known to be less tolerant to the lack of water, because the winter varieties grow a larger root system. According to Haberle et al. (2008) the occurrence of drought during seeding and the vegetative growth stages affects the herbage emergence and the reduction of the offshoot. Drought in some growth stages can have a positive effect on the development

of the root system and consequently on the grain yield (Svačina et al. 2014). Drought occurring during the generative growth phases affects on the reduction of number of spikelets and grains. Flowering is a critical stage, because a lack of water during this stage has a worse impact than during other growth stages. Another critical period is the beginning of the grain formation stage, which determines the number of cells in the endosperm (Haberle et al. 2008).

CONCLUSION

For four selected CISTA experimental localities average, maximum, and minimum yields of spring barley grain were evaluated for the period 1975–2015. Oscillation of the EDI values in individual decades of the critical period for development and yield formation of spring barley was also evaluated (days 111–180) in the Czech Republic. Significant variability of precipitation conditions at individual localities during the vegetation period of the observed years was recorded. With the EDI value < 0, the years 1976, 1984, 2015 and 2004 (sorted from the driest) are considered dry. The years rich in precipitation were (sorted from the moistest) 1979, 2010, 1975 and 2006.

The relationship between the course of weather and yield was proven on the basis of correlation of the EDI values with yields of the spring barley per ha. Statistically significant relationships were recorded at all the experimental localities with 99% or 95% probability in certain stages of the vegetation period (2nd, 4th, 6th, and 7th decade of the vegetation). This implies the use of the EDI for modeling of the impact of weather on the yield formation in a significant area of the Czech Republic and the possibility to use it for modeling yields on the basis of scenario data.

ACKNOWLEDGEMENTS

This contribution was written at Mendel University in Brno as a part of the project IGA FA MENDELU no. IP_1/2017 with the support of the Specific University Research Grant, provided by the Ministry of Education, Youth and Sports of the Czech Republic in the year of 2017.

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SPECIES COMPOSITION OF VEGETATION IN WINE VILLAGE BRATČICE AND SYROVICE

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Abstract: The purpose of this work is to compile a list of species growing in vineyards of two wine villages Bratčice and Syrovice, Znojmo wine subregion. Within the wine village of Bratčice, vine line Široké klinky. Within the wine village of Syrovice there were evaluated 2 vine lines: Stará hora and Nad mlýnem. In Široké klínky (wine village of Bratčice) was the most species rich vegetation within evaluated vine lines, 46 plant species were found. Whereas 40 plant species were found in Stará hora vine line (wine village of Syrovice) and only 17 species were found in Nad mlýnem vine line (wine village of Syrovice). The most frequently occurred species were grasses as: *Lolium perenne, Festuca rubra, Festuca pratensis, Arrhenatherum elatius*.

Key Words: vegetation, vineyards, plant species, Bratčice, Syrovice

INTRODUCTION

The grapevine cultivation in our country is known since the 3rd century AD. The roman emperor Mark Aurelius had planted the vineyards near the village of Mušov, now no longer exist, under the Pálava hills. From the first vineyards the vine spread all over South Moravia (Pátek 1998). In 2015, the total vineyard area amounted to 17.700 ha (Bublíková 2016). Of this total, the organically farmed 1 025 hectares (Ekologické zemědělství v ČR 2016).

The basic nature of viticulture in the 21st century is to maintain and above all the increase in the natural fertility of the soil. Soil fertility is determined by the positive interaction of matter rock, humus, soil organisms and organic matter. Soil fertility is the soil's ability to supply higher plants enough of air, water, nutrients and they create an environment to fixing roots. The complete exclusion or minimal application of pesticides that are toxic to individual components of soil edaphone and the greening of vineyard interconnections are very important elements of soil care (Pavloušek 2007). The greening of the vineyards is closely related to the expansion of organic vine farming and expanding integrated production (Hrabalová 2016).

According to Pavloušek (2011) we use a wide spectrum of plant species to plant vineyards. Appropriate species used for planting the vineyard are sought according to pH, soil structure, humus, exposition, planting space, vine varieties, cutting, planting time and passage frequency (Sedlo 1994).

The structure of the soil in the vineyards is permanently distorted one-sided treatment of the soil in the long term and leads to soil compaction. Significant problems are caused by the lack of humus. Looking at these aspects, new greenhouse compounds are mixed. It is contain a rich representation of individual families. These are families of *Brassicaceae*, *Fabaceae*, *Poaceae*) and other dicotyledonous flowering herbs (Ziegler 2004).

The main principles of the seed mixtures include representations of at least three kinds of different genera. Another principle is that grass species can not be dominant (less than 50%). At least 33% must be leguminous plants. The mixture must also appear 1–2% deep rooting plants. Sufficient space must be left with natural flora (Ziegler 2004).

The purpose of this work is to compile a list of species growing in vineyards of the wine villages Bratčice and Syrovice. Other purpose of this work is to evaluate the importance of plant species in terms of vine growing and ecosystem.

MATERIAL AND METHODS

Characteristics of the interest territory Bratčice

The cadastral area of Bratčice is located in the South Moravian Region, about 20 km south of Brno. The altitude is approximately 215 m a.s.l. The area falls into a very warm and dry climatic region within the Czech Republic.

The total area of the Bratčice cadastral area is 616.9 ha, of which the agricultural land is 505.9 ha. Within agricultural land, arable land is 484.8 ha, meadows and pastures 8.1 ha, orchards 0.3 ha and vineyards 1.9 ha.

The Bratčice village is governed by the wine law as a wine village belonging to the Znojmo wine subregion and the wine region of Moravia. Within the wine village there are 2 vine lines of the Staré hory and Široké klinky. Staré hory is not currently planted with vineyards and therefore have not been evaluated.

Characteristics of the interest territory Syrovice

The cadastral area of Syrovice is located in the South Moravian Region, about 20 km south of Brno. The altitude is approximately 202 m a.s.l. The area falls into a very warm and dry climatic region within the Czech Republic.

The total area of the Syrovice cadastral area is 881.5 ha, of which the agricultural land is 707.9 ha. Within agricultural land, arable land is 673.2 ha, meadows and pastures 1.1 ha, orchards 1.6 ha and vineyards 11.8 ha.

The Syrovice village is governed by the wine law as a wine village belonging to the wine region of Moravia and the Znojmo wine subregion. Within the wine village there are 2 vine lines of the Stará hora and Nad mlýnem.

Methodology of evaluation of vegetation species composition

Evaluation of vegetation was made using a floristic list of the found species. Evaluation was made in 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 wine lines. The found species were registered during the 3 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 rated vineyards

The first evaluated area was the vine lines Široké klínky (wine village Bratčice). Most of the line area is consist of arable land and there are small parts of vineyards. A similar cultivation method is applied to the entire vineyard. On this plot is used alternating cultivated and grassed inter-rows. During the monitoring, 46 plant species were found on this track.

Among the species with very abundant occurrence on this track belong (level 3 according to the scale): *Achillea millefolium*, *Amaranthus retroflexus*, *Convolvulus arvensis*, *Conyza canadensis*, *Hordeum murinum*, *Lolium perenne*, *Mercurialis annua* and *Trifolium repens.*

The common occurrences on this track were (level 2 according to the scale): *Atriplex patula*, *Bromus hordeaceus*, *Cirsium arvense*, *Consolida regalis*, *Dactylis glomerata*, *Echinochloa crus-galli*, *Euphorbia helioscopia*, *Festuca pratensis*, *Festuca rubra*, *Chenopodium album*, *Lamium album*, *Lathyrus tuberosus*, *Malva neglecta*, *Setaria viridis*, *Stellaria media* and *Tripleurospermum inodorum.*

Species with a rare or rare occurrence on this track were (level 1 according to the scale): *Avena fatua*, *Bromus tectorum*, *Carduus acanthoides*, *Carex hirta*, *Elytrigia repens*, *Galium aparine*, *Geum urbanum*, *Humulus lupulus*, *Panicum miliaceum*, *Papaver rhoeas*, *Pastinaca sativa*, *Plantago lanceolata*, *Plantago major*, *Reseda lutea*, *Robinia pseudacacia*, *Senecio vulgaris*, *Taraxacum* sect. *Ruderalia*, *Tragopogon orientalis*, *Triticum aestivum*, *Urtica dioica*, *Urtica urens* and *Veronica persica.*

The second evaluated area was the vine lines Stará hora (wine village Syrovice). On the territory of this line, the vineyards are also only partially and most of the area is used in a different way. These are mainly vineyards that are part of gardens or small plots and are mostly managed by small breeders. On this plot is used cultivated inter-rows with intensive regulation of vegetation. During the monitoring, 40 plant species were found on this track.

Among the species with very abundant occurrence on this track belong (level 3 according to the scale): *Amaranthus retroflexus*, *Cirsium arvense*, *Convolvulus arvensis*, *Elytrigia repens*, *Festuca pratensis*, *Festuca rubra* and *Lolium perenne.*

The common occurrences on this track were (level 2 according to the scale): *Agrostis capillaris*, *Apera spica-venti*, *Artemisia vulgaris*, *Bromus tectorum*, *Conyza canadensis*, *Erigeron annuus*, *Chenopodium album*, *Plantago lanceolata*, *Setaria viridis*, *Securigera varia*, *Trifolium repens* and *Tripleurospermum inodorum.*

Species with a rare or rare occurrence on this track were (level 1 according to the scale): *Achillea millefolium*, *Arrhenatherum elatius*, *Carduus acanthoides*, *Cornus sanguinea*, *Echinochloa crus-galli*, *Echinops sphaerocephalus*, *Eryngium campestre*, *Galeopsis tetrahit*, *Geranium pusillum*, *Chenopodium strictum*, *Juglans regia*, *Lamium purpureum*, *Lappula squarrosa*, *Mercurialis annua*, *Rosa canina*, *Silene vulgaris*, *Taraxacum sect. Ruderalia*, *Thlaspi arvense*, *Trisetum flavescens*, *Urtica dioica* and *Verbascum austriacum.*

The third evaluated area was the vine lines Nad mlýnem (wine village Syrovice). Most of the line area is consist of arable land, orchards, gardens and vineyards. During the monitoring, 17 plant species were found on this track.

Among the species with very abundant occurrence on this track belong (level 3 according to the scale): *Arrhenatherum elatius*, *Bromus tectorum*, *Dactylis glomerata*, *Festuca pratensis*, *Festuca rubra* and *Trisetum flavescens.*

The common occurrences on this track were (level 2 according to the scale): *Aethusa cynapium*, *Achillea millefolium*, *Erigeron annuus*, *Fragaria moschata*, *Medicago lupulina*, *Reseda lutea*, *Robinia pseudacacia* and *Trifolium repens.*

Species with a rare or rare occurrence on this track were (level 1 according to the scale): *Cirsium arvense*, *Erigeron acris* and *Medicago minima.*

Evaluation of plant species occurrence in monitored vine lines

Of the plant species found, some effectively prevent erosion. Such species belong *Lolium perenne*, *Festuca rubra*, *Festuca pratensis*, *Elytrigia repens*, *Securigera varia*, *Trifolium repens*, *Agrostis capillaris*, *Arrhenatherum elatius*, *Achillea millefolium*, *Dactylis glomerata* and *Trisetum flavescens*.

Some species bind atmospheric nitrogen and thus enrich the soil. Such species belong *Trifolium repens*, *Securigera varia*, *Lathyrus tuberosus*, *Robinia pseudacacia*, *Medicago minima* and *Medicago lupulina.*

Some species are very attractive to insects as a food source. Such species belong *Securigera varia*, *Tripleurospermum inodorum*, *Trifolium repens*, *Plantago lanceolata*, *Plantago major*, *Artemisia vulgaris*, *Erigeron annuus*, *Erigeron acris*, *Conyza canadensis*, *Echinops sphaerocephalus*, *Carduus acanthoides*, *Verbascum austriacum*, *Lamium album*, *Lamium purpureum*, *Geranium pusillum*, *Galeopsis tetrahit*, *Eryngium campestre*, *Taraxacum* sect. *Ruderalia*, *Rosa canina*, *Achillea*

millefolium, *Silene vulgaris*, *Lappula squarrosa*, *Fragaria moschata*, *Reseda lutea*, *Medicago lupulina*, *Medicago minima*, *Robinia pseudacacia*, *Cirsium arvense*, *Lathyrus tuberosus*, *Consolida regalis*, *Stellaria media*, *Tragopogon orientalis*, *Papaver rhoeas*, *Aethusa cynapium*, *Pastinaca sativa*, *Veronica persica* and *Senecio vulgaris*.

Some species have very deep roots and can compete with grapevine. Such species belong *Convolvulus arvensis*, *Securigera varia*, *Humulus lupulus*, *Juglans regia*, *Cirsium arvense*, *Artemisia vulgaris*, *Robinia pseudacacia*, *Taraxacum* sect. *Ruderalia*, *Rosa canina*, *Lathyrus tuberosu* and *Pastinaca sativa.*

Some species quickly produce a large amount of biomass, which complicates the work in the vineyard. Such species belong *Hordeum murinum*, *Amaranthus retroflexus*, *Setaria viridis*, *Tripleurospermum inodorum*, *Echinochloa crus-galli*, *Atriplex patula*, *Chenopodium album*, *Chenopodium strictum*, *Bromus tectorum*, *Bromus hordeaceus*, *Avena fatua* a *Galium aparine.*

CONCLUSION

During the monitoring of vineyard vegetation of selected wine villages, most species were found in vine line of Široké klínky (wine village of Bratčice). It was found 46 plant species. In vine line of Stará hora were found 40 plant species. The least species was found on the Nad mlýnem vine line (wine village of Syrovice). It was found 17 plant species.

The most frequently occurring species of weed were: *Lolium perenne*, *Festuca rubra*, *Festuca pratensis*, *Arrhenatherum elatius*, *Trisetum flavescens*, *Elytrigia repens*, *Hordeum murinum*, *Dactylis glomerata*, *Bromus tectorum*. The other most frequently occurring species of herbaceous perennial plant were: *Trifolium repens*, *Achillea millefolium*, *Cirsium arvense*, *Convolvulus arvensis*, *Conyza canadensis*. And from annual herbs were the most frequently occurring species: *Mercurialis annua* and *Amaranthus retroflexus*.

At present, the vineyards are perceived as a plant association, where other plant species grow next to the grapevine. Occurrence of many species of plants prevent erosion, provide food insects and vertebrates, and enriches the soil with nitrogen.

ACKNOWLEDGEMENTS

This work was supported by a Programme of applied research and development of national and cultural identity, project DG16P02R017 "Viticulture and wine for preservation and restoration of cultural identity of wine regions in Moravia ".

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THE SPECIES COMPOSITION OF VEGETATION GROWING ON THE RECULTIVATED PARTS OF MUNICIPAL WASTE LANDFILLS IN NĚTČICE

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Abstract: The vegetation at a recultivated landfill create a continuous succession of vegetation in order to prevent the erosion of the soil brought. And the roots of the vegetation must not grow into the body of the landfill itself and should form a limited amount of biomass so that it is not demanding in terms of maintenance. The aim of the thesis was to determine the species composition of plants that are able to sustain themselves in an active landfill in Nětčice (sites are located in Zlín Region, Czech Republic). Three habitats were selected on the land with the recultivated part of the landfill. The landfill was recultivated in 2010, 2011or 2012. The evaluation of the vegetation was carried out using the recording phytosociological methods. Coverage of the species found in the selected sites. Altogether 50 plant species were found. The data were performed using a redundancy analysis (RDA). The plant species found whose roots can grow into the body of the landfill are mainly *Convolvulus arvensis, Cirsium arvense, Melilotus albus, Melilotus officinalis, Securigera varia*. The species *Calamagrostis epigejos* is a useful component of the recultivated habitats. It forms a continuous string of dense vegetation that prevents erosion and inhibits the growth of other plant species and is therefore a welcome component of these habitats.

Key Words: vegetation, landfill, landfill recultivation, *Calamagrostis epigejos*

INTRODUCTION

Vegetation is one of the most important components of ecosystems. It is important both in terms of how the ecosystem functions and in terms of animal life preservation. Plants serve as a shelter and a source of food for creatures ranging from insects to vertebrates (Laflamme 2007).

A change in the vegetation at a certain place over a period of time is called succession. A process called secondary succession takes place in locations where vegetation was already present but was partially or completely eliminated as a result of anthropogenic activities (the extraction of mineral resources, forest felling, etc.) (Jehlík 1998). According to Connela and Slatyer (1977), the process of succession is based on the relationships between the plant populations in the given habitat. According to Bastl et al. (1997), the earlier succession stages are generally more prone to disruption than the later stages. The period for which dominant non-native species survive in specific habitats may be very long. The succession stages are generally less affected for habitats in stress conditions. At later succession stages, the involvement of invasive communities is restricted by the competition of a canopy of plants that are already growing on the place.

We can see the process of succession in municipal waste landfills as well. Landfills or parts of landfills that are already closed (no further waste is brought) are recultivated. Their surface is covered with special foils followed by a layer of soil, and vegetation is then planted. The task of the vegetation is to create a continuous stand so as to prevent the erosion of the soil brought

to the landfill. Furthermore, the roots of the vegetation must not grow into the body of the landfill itself and should form a limited amount of biomass so that it is not demanding in terms of maintenance.

MATERIAL AND METHODS

Characterization of growing locality

The work was conducted in the cadastral area Nětčice (Zlín region). It is a sanitary landfill incorporated with multilayer composite bottom liner, leachate and landfill gas collection system, and a final cover system. In terms of maintenance, the landfill is classified in the S-category - other waste, sub-category S-OO3. Up to now, Stage I of 19 200 m^2 has been constructed together with parts of Stage II (5 500 m^2) and Stage III (7 500 m^3). The facility receives waste (category of other waste) from a catchments area with the population of ca. 75 000 residents. The approved landfill sector for waste of sub-category S-OO1 has not been opened yet. The sector will be intended for the disposal of waste (category of other waste) with the low content of organic biologically degradable substances. A sector of the landfill will be intended largely for the disposal of asbestos-containing wastes, gypsum-based waste, stabilized waste, waste with the high sulphur content and waste with the increased content of metals. Waste with the substantial content of organic biologically degradable substances must not be stored in that sector.

The area belongs in the Kojetín bioregion (Culek 1996) situated in central Moravia and occupying the geomorphological subunit of Central Moravia Floodplain. The bioregion is formed by a broad alluvial plain with regulated rivers. Biota is of azonal character and dominated by agrocoenoses, preserved floodplain forests, remainders of meadows and ponds with abundant fauna (Vaverková et al. 2012).

According to Quitt (1971), the entire region lies in the warm zone T2. Weather is warm, poor to precipitation.

Methodology for evaluating vegetation and processing statistics

Three habitats that differ in terms of the time at which the recultivation was carried out were selected on the land with the recultivated part of the landfill. The landfill was recultivated in 2010 at the site of the first habitat, in 2011 at the site of the second habitat and in 2012 at the site of the third habitat.

The evaluation of the vegetation was carried out using the phytosociological plots of size 20 m^2. The coverage was estimated as a percentage. The monitoring took place in July 2017. Eight phytosociological images were recorded at each habitat (together twenty four). The scientific names of each weed species were used according to Kubát (Kubát et al. 2002).

The evaluation of the coverage of the species found at the selected habitats with different waste was carried out by means of multidimensional analyses of ecological data. A redundancy analysis (RDA) based on the linear response model was finally used.

RESULTS AND DISCUSSION

Altogether 50 plant species were found. The average coverage of species found in the monitored habitats with different recultivation periods is specified in Table 1.

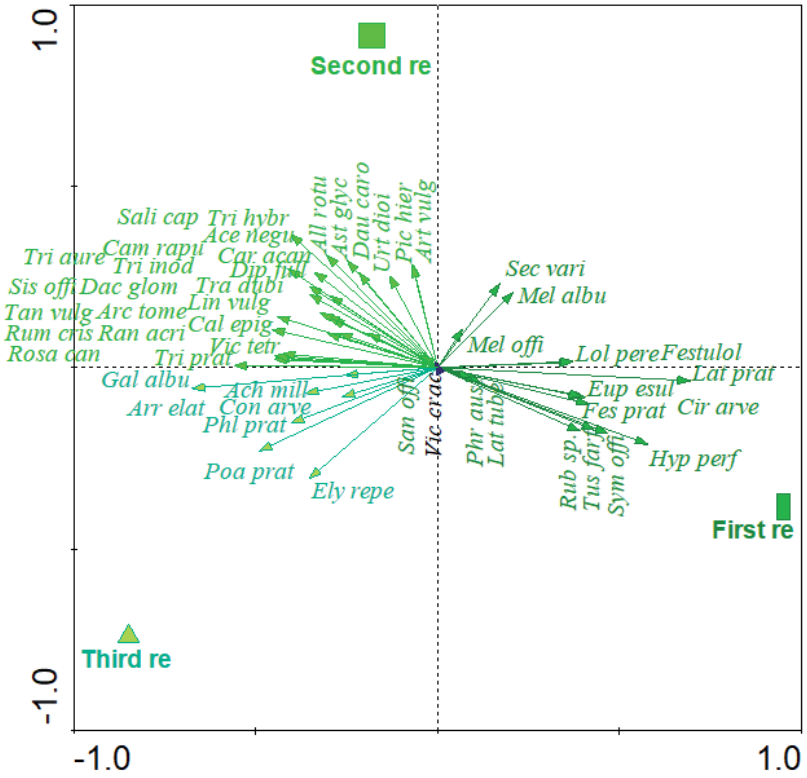
The results of the RDA analysis, which evaluated the relationship of the habitat to the different types of waste and plant species, are significant for first canonical axes at the significance level $\alpha = 0.011$ and are therefore statistically conclusive. The ordination diagram (Figure 1) represents the graphical visualisation. Based on the results, the species can be divided into four groups.

Table 1 The average coverage of species on the observed habitats with different waste

Species	Abbreviations	Habitats (average coverage in %)		
		Recultivation in 2010	Recultivation in 2011	Recultivation in 2012
Acer negundo	*Ace negu*	0.3		
Achillea millefolium	*Ach mill*	0.1	0.3	0.7
Allium rotundum	*All rotu*		0.3	
Arctium tomentosum	*Arc tome*	0.1		
Arrhenatherum elatius	*Arr elat*	13.8	23.1	27.9
Artemisia vulgaris	*Art vulg*	0.8	1.2	0.3
Astragalus glycyphyllos	*Ast glyc*		0.6	
Calamagrostis epigejos	*Cal epig*	2.4	14.2	14.0
Campanula rapunculoides	*Cam rapu*	0.1	0.1	
Carduus acanthoides	*Car acan*	0.1	0.8	0.4
Cirsium arvense	*Cir arve*	13.9	5.9	4.0
Convolvulus arvensis	*Con arve*	0.4	1.0	2.1
Dactylis glomerata	*Dac glom*	0.4	2.7	3.0
Daucus carota	*Dau caro*	0.3	0.2	
Dipsacus fullonum	*Dip full*		0.3	0.1
Elytrigia repens	*Ely repe*	1.3	1.1	5.7
Euphorbia esula	*Eup esul*	2.1	0.3	
Festuca pratensis	*Fes prat*	13.5	5.6	2.9
Festulolium	*Festuloli*	21.9	15.9	9.3
Galium album	*Gal albu*	2.1	14.1	20.0
Hypericum perforatum	*Hyp perf*	4.6	0.2	
Lathyrus pratensis	*Lat prat*	24.0	7.0	0.1
Lathyrus tuberosus	*Lat tube*	0.6		
Linaria vulgaris	*Lin vulg*	0.1		
Lolium perenne	*Lol pere*	9.1	5.6	2.9
Melilotus albus	*Mel albu*	5.6	7.8	
Melilotus officinalis	*Mel offi*	0.6	0.6	
Phleum pratense	*Phl prat*		1.0	3.6
Phragmites australis	*Phr aust*	0.6		
Picris hieracioides	*Pic hier*	0.4	0.7	
Poa pratensis	*Poa prat*		2.6	10.0
Ranunculus acris	*Ran acri*	0.1		
Rosa canina	*Rosa canin*		0.1	0.4
Rubus sp.	*Rub sp.*	3.5	0.6	0.7
Rumex crispus	*Rum crisp*			0.3
Salix caprea	*Sali capr*	0.1		
Sanguisorba officinalis	*San offi*	0.4		
Securigera varia	*Sec vari*	6.0	8.3	0.9
Sisymbrium officinale	*Sis offi*			0.1

Symphytum officinale	*Sym offi*	1.9		
Tanacetum vulgare	*Tan vulg*			0.3
Tragopogon dubius	*Tra dubi*	0.1	1.1	1.0
Trifolium aureum	*Tri aure*		2.3	1.4
Trifolium hybridum	*Tri hybr*		0.6	0.1
Trifolium pratense	*Tri prat*			0.3
Tripleurospermum inodorum	*Tri inod*		0.1	
Tussilago farfara	*Tus farf*	5.0		
Urtica dioica	*Urt dioi*	0.3	0.6	
Vicia cracca	*Vic crac*	0.4		
Vicia tetrasperma	*Vic tetr*	0.1	3.7	5.7

Figure 1 Ordination diagram (RDA) expressing the relationship of the plant species found and different recultivation habitats



*Legend: "*First re*" habitat with recultivation that took place in 2010. "*Second re*" habitat with recultivation that took place in 2011. "*Third re*" habitat with recultivation that took place in 2012.*

The first group of species was more common in the first habitat, where recultivation is the most recent. The group included the following species: *Cirsium arvense, Convolvulus arvensis, Elytrigia repens, Euphorbia esula, Festuca pratensis, Festulolium, Hypericum perforatum, Lathyrus pratensis, Lathyrus tuberosus, Lolium perenne, Melilotus albus, Melilotus officinalis, Phragmites australis, Rubus sp., Securigera varia, Symphytum officinale* and *Tussilago farfara*. Twenty-eight species were found in this habitat.

The second group of species was more common in the second habitat, where the recultivation was carried out in 2011. The group included the following species: *Acer negundo, Allium rotundum, Arctium tomentosum, Artemisia vulgaris, Astragalus glycyphyllos, Calamagrostis epigejos, Campanula rapunculoides, Carduus acanthoides, Dactylis glomerata, Daucus carota, Dipsacus fullonum, Linaria vulgaris, Picris hieracioides, Ranunculus acris, Rosa canina, Rumex crispus, Salix caprea, Sisymbrium officinale, Tanacetum vulgare, Tragopogon dubius, Trifolium aureum, Trifolium*

hybridum, Trifolium pratense Tripleurospermum inodorum, Urtica dioica and *Vicia cracca.* Thirty-five species were found in this habitat.

The third group of species was more common in the third habitat, where the first recultivation took place. The group included the following species: *Achillea millefolium, Arrhenatherum elatius, Galium album, Phleum pratense, Poa pratensis* and *Sanguisorba officinalis.* Thirty-five species were found in this habitat.

CONCLUSION

In the habitat where recultivation took place the most recently, there is a predominance of species that can be considered as field weeds (*Elytrigia repens, Convolvulus arvensis*) and species that expand (*Arrhenatherum elatius, Calamagrostis epigejos*). In habitats recultivated in 2010, field weed species and species that expand are slowly disappearing and local species are starting to appear. Local species (*Lathyrus pratensis, Melilotus albus*) or sown species (Festulolium, *Festuca pratensis, Lolium perenne*) are predominant in the habitats that have been recultivated the earliest.

Plant species with deep roots pose a certain risk. Their roots can grow into into the actual body of the landfill and receive substances that they can then transport to the parts above the ground and thereby release them into the food chain. Furthermore, the roots can disrupt the protective layer of the landfill's body and thus release dangerous substances and contaminate groundwater. Especially the species *Convolvulus arvense, Cirsium arvensis, Melilotus albus, Melilotus officinalis and Securigera varia* can be problematic in this regard. The species *Calamagrostis epigejos* is a usefull component of the recultivated habitats. It forms a continuous string of dense vegetation that prevents erosion and inhibits the growth of other plant species and is therefore a welcome component of these habitats.

Recultivated landfills are an interesting ecosystem where succession takes place. It is, therefore, necessary to monitor the changes in vegetation and influence this development if necessary.

ACKNOWLEDGEMENTS

This work was created with the financial support of project no. TP 5/2017 of the Internal Grant Agency of the Faculty of AgriSciences at the Mendel University in Brno.

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HERBICIDE PROTECTION OF MILK THISTLE (*SILYBUM MARIANUM* L. GAERTN.) STANDS

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Abstract: Milk thistle [*Silybum marianum* (L.) Gaertn.] is currently a sought-after plant commodity, but the problem of herbicide protection, which is a limiting factor in its cultivation, has not been solved yet. Milk thistle achenes can be used in a variety of ways (as dietary supplements, livestock feeding etc.), but above all they are an important raw material for processing in the pharmaceutical industry. The aim of this work was to evaluate the phytotoxicity and the suitability of selected herbicides in small-scale experiments during the years 2014, 2015 and 2016. The results indicate that the preparations used in the study have sufficient selectivity for the milk thistle plants. According to the results, the preparations TARGA SUPER 5 EC and GALLANT SUPER can be recommended during the cultivation.

Key Words: milk thistle, herbicide, weeds

INTRODUCTION

Milk thistle [*Silybum marianum* (L.) Gaertn.] is an annual cultural crop belonging to the *Asteraceae* family, originating from the Mediterranean. Since the ancient times it has been known for its positive effect in the treatment and prevention of hepatic tissue diseases (Kroll et al. 2007, Abenavoli et al. 2010, Habán et al. 2010, Calani et al. 2012). In the Czech Republic, the milk thistle takes the first place as for the area of cultivation of all the MAPs (medicinal, aromatic and spice plants) (Přibylová et al. 2014). Milk thistle achenes are the product of particular interest, because they contain the silymarin complex, which is a mixture of flavolignans, including silybin A, B (50–60%), isosilybin A, B (5%), silydianin (10%), silychristin (20%) a flavonoid taxifolin (Stancheva et al. 2008, Abbasi et al. 2010, Çağdaş et al. 2011, AbouZid 2012). The total content of the silymarin complex in achene dry matter varies in the range of 1–3% (Andrzejewska et al. 2011, Katar et al. 2013). According to Nasrabadi et al. (2014) the silymarin complex is contained in the whole plant, but it is only advantageous to isolate it from the achenes. The milk thistle achenes contain also high quality oil (15–30%), its main components being linoleic acid (60%), oleic acid (30%) and saturated palmitic acid (about 9%). The achenes contain also proteins (30%), carbohydrates (mostly arabinose, glucose, xylose and rhamnose), tocopherol (0.038%), sterols (0.063%) and last but not least, flavonoids (quercetin, taxifolin) (Abenavoli et al. 2010).

The optimum growth conditions, especially in the phase of flowering and ripening, affect the yield enhancement (Stancheva et al. 2008). From an agronomic point of view, milk thistle is considered to be an undemanding crop adaptable to the different conditions. It can be grown on sandy soils, but also on heavy and clay soils (Vereš and Týr 2012, Afshar et al. 2014).

One of the limiting factors during the milk thistle cultivation is the weeds infestation and interference. From the crop rotation point of view, the milk thistle is a suitable precursor for corn in a sustainable farming system (Týr and Vereš 2011, Karkanis et al. 2011). According to Zheljazkova et al. (2006) milk thistle is sensitive to a wide range of herbicides used for other

cultural crops. Defining capability is an important agronomic characteristic of milk thistle thanks to the rapid creation of leaf rosette and a large amount of leaf matter. However, at the beginning of the vegetation period, especially in the germination stage and during the extensive growth, the competitiveness of milk thistle to weeds is relatively low (Delchev 2016).

Most common weeds found in milk thistle stands are: *Sonchus arvensis* L., *Agropyron repens* Beauv., *Cirsium arvense* Scop., *Convovulus arvensis* L., *Raphanus raphanistrum* L., *Sinapis arvensis* L., *Galium aparine* L., *Chenopodium album* L., *Mentha crispa* L., *Stachys arvensis* L. and *Atriplex patula* L. (Delchev 2016). In Bulgaria, in the Plovdiv region, where Zheljazkov et al. (2006) carried out their research during the years 1995 and 1996, typical weed representatives were *Amaranthus retroflexus* (L.), *Setaria viridis* (L.) Beauv., *Cynodon dactylon* (L.) Pers., as well as *Amaranthus blitoides* S. Wats., *Chenopodium album* L., *Datura stramonium* L., *Polygonum convolvulus* L., *Polygonum aviculare* L., *Solanum nigrum* L., *Xanthium strumarium* L., *Abutilon theophrasti* Medik., *Digitaria sanguinalis* L., from the biennial species also *Convolvulus arvensis* L., *Leonurus cardiata* L., *Cirsium arvense* L. and *Sorghum halepense* (L.) Pers.

MATERIAL AND METHODS

Characterization of growing locality, experimental design

The herbicidal experiments were established in the form of randomized blocks, each in 3 replicates on experimental plots of the Agritec Plant Research, Ltd., in the years 2014, 2015 and 2016. All variants and their designations are given in the Table 1. The size of the plots was 12.5 m^2 of which 10 m^2 were harvested. Application of the herbicide was carried out on June 23rd, 2014; June 4th, 2015 and June 19th, 2016. A small-scale sprayer HEGE 32 was used for the application.

In all experimental years were found following annual dicotyledonous weeds in the trials: THLAR (*Thlaspi arvense* L.), CAPBP (*Capsella bursa-pastoris* (L.) MED.), VERHE (*Veronica hederifolia* L.), LAMPU (*Lamium purpurea* L.), MATMA (*Matricaria discoidea* DC.), MATCH (*Matricaria recutita* L.), VIOAR (*Viola arvensis* MURRAY), CHEAL (*Chenopodium album* L.), and others. From monocotyledons then: ECHCG (*Echinochloa crus-galli* (L.) P.B.) a AGRRE (*Elytrigia repens* (L.) NEVSKI) – persistent weed. In 2014, 2015 also annual weed AVEFA (*Avena fatua* L.).

Evaluation of the phytotoxicity of individual preparations was carried out in three terms (7, 14 and 28 DPA - days after application) according to the Methodology for the determination of the phytotoxicity of preparations [EPPO No. 135/1988].

The small-scale combine harvester SAMPO was used for the harvest. The experimental plots were harvested on October 15th, 2014, August 1st, 2015 and September 7th, 2016. After the harvest, the yield from the area unit was determined.

The evaluation of results was performed by the analysis of variance (ANOVA) using the statistical program STATISTICA (data analysis software system), StatSoft, Inc. (2013), version 12. Fisher's test at a significance level of $P=0.05$ was chosen for subsequent testing.

Table 1 Overview of the herbicide treatment variants in the years 2014, 2015 and 2016

No.	**Preparation**	**Active compound**	**Amount**
1	CONTROL	-	-
2	STOMP 400 SC	pendimethalin	2.5 l
3	BETANAL MAXX PRO	desmedipham+ethofumesate+phenmedipham+lenacil	1.5 l
4	REFINE 50 SX	thifensulfuron-methyl	20 g
5	BUTISAN STAR	qinmerac+metazachlor	1.5 l
6	TARGA SUPER 5 EC	quizalofop-P-ethyl	2.0 l
7	GALLANT SUPER	haloxyfop-R methylester	1.0 l
8	GLEAN 75 WG	chlorsulfuron	10 g
9	STARANE 250 EC	fluroxypyr	0.5 l

RESULTS AND DISCUSSION

In all experimental years, the phytotoxicity of the herbicide preparations used for milk thistle was evaluated in three terms.

The phytotoxicity evaluation in the year 2014

7 DPA (BBCH 16–19)

STOMP 400; BETANAL MAXX PRO; REFINE 50 SX - Phytotoxicity > 5%. Slight growth delay compared to control, mild necrosis of the leaves that had already been developed when applying the herbicide.

BUTISAN STAR - Phytotoxicity 5–10%. Growth delay, mild necrosis of the leaves that had already been developed when applying the herbicide.

TARGA SUPER 5 EC; GALLANT SUPER - Phytotoxicity 0%. Herbicidal effect only on monocotyledonous and perennial weeds.

GLEAN 75 WG, STARANE 250 EC - Phytotoxicity 20–30%. Compared to untreated control, chlorotic plants were observed, retardation and slower growth. Necrotic stains on leaves. Death of plants, especially the weaker ones.

14 DPA (BBCH 19–32)

STOMP 400 SC; BETANAL MAXX PRO; REFINE 50 SX - Phytotoxicity 0%. Phytotoxicity has subsided.

BUTISAN STAR - Phytotoxicity 5%. Growth inhibition, with no noticeable necrosis or color changes on leaves.

TARGA SUPER 5 EC; GALLANT SUPER - Phytotoxicity 0%. Herbicidal effect only on monocotyledonous and perennial weeds.

GLEAN 75 WG; STARANE 250 EC - Phytotoxicity 20 %. Necrotic stains on newly growing leaves are no longer observed. Growth retardation of milk thistle plants, i.e. the delay of the onset of the individual growth phases compared to the untreated control. Death of plants observed, especially the weaker ones.

28 DPA (BBCH 37–61)

STOMP 400 SC; BETANAL MAXX PRO; REFINE 50 SX; BUTISAN STAR - Phytotoxicity 0%. Phytotoxicity has subsided.

TARGA SUPER 5 EC; GALLANT SUPER - Phytotoxicity 0%. Herbicidal effect only on monocotyledonous and perennial weeds.

GLEAN 75 WG - Phytotoxicity 15%. Necrotic stains on newly growing leaves are no longer observed. Growth retardation of milk thistle plants, i.e. the delay of the onset of the individual growth phases compared to the untreated control.

STARANE 250 EC - Phytotoxicity 10%. Necrotic stains on newly growing leaves are no longer observed. Growth retardation of milk thistle plants, i.e. the delay of the onset of the individual growth phases compared to the untreated control.

The phytotoxicity evaluation in the year 2015

7 DPA (BBCH 15–18)

STOMP 400 SC; BETANAL MAXX PRO; REFINE 50 SX - Phytotoxicity to 5%. Slightly delayed growth, slight necrosis of leaves that were already developed at the time of application.

BUTISAN STAR - Phytotoxicity 5%. Delayed growth, slight necrosis of leaves that were already developed at the time of application.

TARGA SUPER 5 EC; GALLANT SUPER - Phytotoxicity 0%. Herbicidal effect only on monocotyledonous weeds.

GLEAN 75 WG - Phytotoxicity 10%. Compared with the untreated variant, chlorotic plants were observed, as well as retardation and slower growth. Necrotic stains on leaves. Minimal mortality, especially of weaker plants.

STARANE 250 EC - Phytotoxicity 5%. Delayed growth, slight necrosis of leaves that were already developed at the time of application.

14 DPA (BBCH 18–31)

STOMP 400 SC; BETANAL MAXX PRO; REFINE 50 SX - Phytotoxicity 0%. Phytotoxicity has subsided.

BUTISAN STAR – Phytotoxicity 3% - Growth inhibition, with no apparent necrosis or color changes.

TARGA SUPER 5 EC; GALLANT SUPER - Phytotoxicity 0%. Herbicidal effect only on monocotyledonous and perennial weeds.

GLEAN 75 WG - Phytotoxicity 5%. Necrotic stains on newly growing leaves are no longer observed. Growth retardation of milk thistle plants, i.e. the delay of the onset of the individual growth phases compared to the untreated control. Death of plants observed, especially the weaker ones.

STARANE 250 EC - Phytotoxicity 3%. Growth inhibition, with no apparent necrosis or color changes.

28 DPA (BBCH 37–59)

STOMP 400 SC; BETANAL MAXX PRO; REFINE 50 SX; GLEAN 75 WG; STARANE 250 EC; BUTISAN STAR - Phytotoxicity 0%. Phytotoxicity has subsided.

TARGA SUPER 5 EC; GALLANT SUPER - Phytotoxicity 0%. Herbicidal effect only on monocotyledonous and perennial weeds.

The phytotoxicity evaluation in the year 2016

7 DPA (BBCH 16–31)

STOMP 400 SC; BETANAL MAXX PRO; REFINE 50 SX - Phytotoxicity > 5%. Slightly delayed growth, slight necrosis of leaves that were already developed at the time of application.

TARGA SUPER 5 EC; GALLANT SUPER - Phytotoxicity 0%. Herbicidal effect only on monocotyledonous and perennial weeds.

GLEAN 75 WG - Phytotoxicity 7%. Compared with the untreated variant, chlorotic plants were observed, a well as retardation and slower growth. Necrotic stains on leaves. Minimal mortality, especially of weaker plants.

STARANE 250 EC - Phytotoxicity 5%. Delayed growth, slight necrosis of leaves that were already developed at the time of application.

14 DPA (BBCH 31–35)

STOMP 400 SC; BETANAL MAXX PRO; REFINE 50 SX - Phytotoxicity 0%. Phytotoxicity has subsided.

TARGA SUPER 5 EC; GALLANT SUPER - Phytotoxicity 0%. Herbicidal effect only on monocotyledonous and perennial weeds.

GLEAN 75 WG - Phytotoxicity 3%. Necrotic stains on newly growing leaves are no longer observed. Growth retardation of milk thistle plants, i.e. the delay of the onset of the individual growth phases compared to the untreated control.

STARANE 250 EC - Phytotoxicity 3%. Growth inhibition, with no apparent necrosis or color changes.

28 DPA (BBCH 39–67)

STOMP 400 SC; BETANAL MAXX PRO; REFINE 50 SX; - Phytotoxicity 0%. Phytotoxicity has subsided.

TARGA SUPER 5 EC; GALLANT SUPER - Phytotoxicity 0%. Herbicidal effect only on monocotyledonous and perennial weeds.

GLEAN 75 WG; STARANE 250 EC - Phytotoxicity 0%. Phytotoxicity has subsided.

Table 2 Average yield of milk thistle achenes in g/m in the years 2014, 2015 and 2016

Herbicide	2014		2015		2016	
CONTROL	108.3	cd	143.9	a	154.3	a
STOMP 400 SC	114.5	cd	177.9	c	168.5	a
BETANAL MAXX PRO	133.4	e	156.9	b	163.5	a
REFINE 50 SX	105.5	bc	169.5	c	171.0	a
BUTISAN STAR	114.3	cd	149.5	ab	161.8	a
TARGA SUPER 5 EC	118.7	cd	153.9	ab	166.0	a
GALLANT SUPER	121.7	de	151.6	ab	156.3	a
GLEAN 75 WG	68.4	a	168.9	c	164.0	a
STARANE 250 EC	93.7	b	157.8	b	161.8	a

From the table 2 is evident, that in 2014 the highest yield of the achenes was in the variant treated with herbicide BETANAL MAXX PRO, the lowest yield was in the variant treated with herbicide GLEAN 75 WG, where yield was one half lower compared to the highest yield achieved. In 2015 year the highest yield was get in the variant treated with herbicide STOMP 400 SC, the lowest yield was in the control variant. In 2016 statistically significant differences between the yields of the achenes were not found.

CONCLUSION

The monitoring of phytotoxicity of the herbicide treatment in three experimental years shows, that some included herbicides proved high selectivity against the plants of the milk thistle and in years 2014 and 2015 also the positive effect of their application on the achenes yield.

In 2014, these were TARGA SUPER 5 EC, STOMP 400, BETANAL MAXX PRO, but also GALLANT SUPER and BUTISAN STAR. In 2015, these were mainly preparations: TARGA SUPER 5 EC, GALLANT SUPER, REFINE 50 SX a STOMP 400. Similar results were achieved in 2016. The application of these herbicides had primarily effect on monocotyledonous and perennial weeds. From the point of view of phytotoxicity the application of these herbicides was the best during the experimental years: TARGA SUPER 5 EC a STOMP 400, and next already registered herbicide REFINE 50 SX. There was no phytotoxicity for products TARGA SUPER 5 EC and GALLANT SUPER in the first term. Phytotoxicity up to 5% was monitored for STOMP 400 and REFINE 50 SX.

The use of herbicides also corresponds to the yields of the achenes. In 2014, the highest yields were obtained after the application of BETANAL MAXX PRO and GALLANT SUPER. In 2015, the highest yield was obtained after the application of STOMP 400 SC, GLEAN 75 WG and REFINE 50 SX. Comparable yields were harvested also in 2016. However, as already stated in previous papers, the yields are affected by the course of weather in the given growing year, especially.

ACKNOWLEDGEMENTS

The research was financially supported by the Grant IGA TP 1/2016: New trend in the cultivation and use of milk thistle (*Silybum marianum* L.) in agriculture.

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THE ELIMINATION OF MILK THISTLE (*SILYBUM MARIANUM* L. GAERNT.) IN A SUBSEQUENT CROP

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Abstract: Milk thistle is cultivated on an area of almost 5000 hectares for the production of achenes, which are used as an important source of silymarin complex. Milk thistle is a plant sufficiently resistant to and competitive against most of the weeds, but also cultivated plants. One of the main obstacles that hinders the expansion of its area of cultivation for various usage is the fact that the subsequent crop, milk thistle becomes a difficult weed that is hard to dispose. Thanks to the use of certain preparations, like Lontrel 300 - active compound clopyralid - and Butoxone 400 - active compound MCPB (4-(4-chloro-o-tolyloxy) butanic acid), significant regulation of milk thistle plants was achieved.

Key Words: milk thistle, weeds, subsequent crop, herbicide

INTRODUCTION

Milk thistle is an annual or biennial plant of the *Asteraceae* family, which is widely used, among others things, in natural medicine (Khan et al. 2007, Alemardan et al. 2013). It is grown in Europe, Egypt, China and Argentina as a cultural and especially medicinal plant. According to different surveys it grows in the wild in some states and continents (North Africa, Australia, America) and it is a troublesome weed (Barreto 2002, Vereš and Týr 2012).

Martinelli et al. (2014) states that this plant species is widely used in both human and veterinary medicine, but also in the food industry, where - among other things - high quality milk thistle oil is used. According to this author, milk thistle is useful in animal feed, phytoenergetics and many other fields of application. In the crop rotation it can be used for example as a suitable raw material for silage maize (Habán et al. 2010). The use of milk thistle for enrichment of the feed rations, especially for highly productive animals, is a new trend. Either full achenes or oil cake are used for feeding (Sadowska et al. 2010, Wierzbowska 2013).

Milk thistle is a 20–150 cm tall, sturdy plant with an upright stalk that branches. Another characteristic feature is the massive leaf rosette of dark green colored leaves with white spots along the veins and sharp thorns at the edges. Thorns are also on the bracteae under the anthodia (AbouZid 2012, Elwekeel et al. 2013). The main stalk and the branches bear a violet-colored anthodia. Milk thistle fruit, that is the object of interest of the growers, is a grayish-downy, glossy black-brown achene. Thanks to the pappus it spreads through the wind, and not only during the harvest. The achenes germinate in a subsequent crop where they cause unwanted weed infestation (Zheljazkov et al. 2006, Abenavoli et al. 2010). According to DiTomaso et al. 2013, the achenes spread for short distances using the pappus and wind spontaneously after falling off the anthodia, but at longer distances in particular by human activity. These ways include the expansion in blends with other crops, mechanization or contaminated feed. Milk thistle plants reproduction occurs only via the seeds. The seeds can germinate either during the first rainfall or they hibernate in the seed phase and germinate early in the spring. Milk thistle achenes keep their germination ability for over 9 years.

The achenes are characterized by low or almost no dormancy (Karkanis et al. 2011). Milk thistle can be disposed of in a subsequent crop by mechanical or chemical means (DiTomaso et al. 2013).

One of the other aspects that discourage the growers due to unwanted weed infestation of subsequent crops is the uneven ripening of the anthodia, which makes the seeds fall from the anthodia before the harvest. The optimal harvest time must therefore be chosen in such a way as to avoid high harvest losses and weed infestation of subsequent crops (Habán et al. 2009, Delchev 2016).

Milk thistle is a plant very tolerant to various cultivation and mainly soil conditions. Its adaptability to soil is given mostly by the massive root system, which allows to grow milk thistle even at dry conditions (Karkanis et al. 2011, Marinelli et al. 2014). Milk thistle that has enough precipitation, especially during the summer months, produces a large amount of green matter including gradually maturing anthodia. This fact can have fatal consequences for the subsequent crop (Andrzejewska et al. 2011). According to Serima et al. (2012), effective herbicide mixtures containing trisulfuron (25%) and the Dicamba preparation (50%) are used to eliminate wide-leaf weeds, like milk thistle (*Silybum marianum*), iberis (*Bifora radians* Bieb.), rough cocklebur (*Xanthium strumarium* L.) and black nightshade (*Solanum nigrum* L.) in extensively grown wheat or maize. It can be expected that the weed infestation of the subsequent crop by milk thistle will be reduced by the means of suitable herbicide preparations.

MATERIAL AND METHODS

The experiments with the response of milk thistle to the selected herbicides that would result in its destruction in the succeeding crop were carried out in 2014–2016 on Agritec Plant Research, Ltd. plots in Šumperk. The experiments were based on Mirel seeds in the form of randomized blocks, each in four replicates. The size of the plots was 12.5 m^2, the seed rate was 8 kg/ha. The number of plants/m^2 was determined after complete emergence and during each selectivity evaluation.

Following herbicides were used for the experiments: LONTREL 300, active compound: clopyralid, amount: 0.3 l/ha in 312.5 l of water/ha, and BUTOXONE 400, active compound: MCPB (4-(4-chloro-o-tolyloxy) butanic acid), amount: 3.0 l/hain 312.5 l water. Effectiveness of the application was compared to untreated control. HEGE 32 sprayer was used for application. The evaluation of the effectiveness was carried out in all experimental years in 3 terms: 7, 14 and 28 DPA (days after application). The effectiveness of preparation was determined by the means of counting the plants in the plot.

The evaluation of results was performed by the analysis of variance (ANOVA) using the statistical program STATISTICA (data analysis software system), StatSoft, Inc. (2013), version 12. Fisher's test at a significance level of $P=0.05$ was chosen for subsequent testing.

Table 1 Overview of the applications and selectivity evaluations in the years 2014, 2015 and 2016

Year	Sow	Beginning of emergence	End of emergence	Herbicide application	BBCH	Selectivity evaluation					
						7 DPA	BBCH phase	14 DPA	BBCH phase	28 DPA	BBCH phase
2014	23.5.	29.5.	1.6.	23.6.	14–16	30.6.	16–19	7.7.	19–32	21.7.	37–61
2015	28.4.	7.5.	11.5.	4.6.	14–16	11.6.	15–18	18.6.	18–31	2.7.	37–59
2016	20.4.	30.4.	7.5.	16.6.	15–18	23.6.	16–31	30.6.	31–35	14.7.	39–67

RESULTS AND DISCUSSION

The effect of the investigated herbicides was apparent on the treated crops in all experimental years. Their effectiveness was evaluated in three terms every year.

The effectiveness evaluation in the year 2014

7 DPA (BBCH 16–19)

LONTREL 300: Significant effectiveness. Leaves and de facto whole plants were yellow to necrotic, likely with a subsequent death scenario.

BUTOXONE 400: Significant effectiveness. Leaves and de facto whole plants were yellow to necrotic, likely with a subsequent death scenario.

14 DPA (BBCH 19–32)

LONTREL 300: Significant mortality of plants. Leaves and whole plants were necrotic. Surviving plants were weaker and lower compared to untreated control.

BUTOXONE 400: Significant mortality of plants. Leaves and whole plants were necrotic. Surviving plants were weaker and lower compared to untreated control.

28 DPA (BBCH 37–61)

LONTREL 300: Significant mortality of plants. Leaves and whole plants were necrotic. Surviving plants were weaker and lower compared to untreated control.

BUTOXONE 400: Significant mortality of plants shortly after the application. Surviving plants were weaker and lower compared to untreated control.

The effectiveness evaluation in the year 2015

7 DPA (BBCH 16–19)

LONTREL 300: Significant mortality of plants. Leaves and whole plants were necrotic. Surviving plants were weaker and lower compared to untreated control.

BUTOXONE 400: Significant effectiveness. Leaves and de facto whole plants were yellow to necrotic, likely with a subsequent death scenario.

14 DPA (BBCH 19–32)

LONTREL 300: Significant mortality of plants. Leaves and whole plants were necrotic. Surviving plants were weaker and lower compared to untreated control.

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28 DPA (BBCH 37–61)

LONTREL 300: Significant mortality of plants shortly after the application. Surviving plants were weaker and lower compared to untreated control.

BUTOXONE 400: Significant mortality of plants. Leaves and whole plants were necrotic. Surviving plants were weaker and lower compared to untreated control.

The effectiveness evaluation in the year 2016

7 DPA (BBCH 16–19)

LONTREL 300: Significant effectiveness. Leaves and de facto whole plants were yellow to necrotic, likely with a subsequent death scenario.

BUTOXONE 400: Significant effectiveness. Leaves and de facto whole plants were yellow to necrotic, likely with a subsequent death scenario.

14 DPA (BBCH 19–32)

LONTREL 300: Significant effectiveness. Leaves and de facto whole plants were yellow to necrotic, likely with a subsequent death scenario.

BUTOXONE 400: Significant mortality of plants. Leaves and whole plants were necrotic. Surviving plants were weaker and lower compared to untreated control.

28 DPA (BBCH 37–61)

LONTREL 300: Significant mortality of plants shortly after the application. Surviving plants were weaker and lower compared to untreated control.

BUTOXONE 400: Significant mortality of plants shortly after the application. Surviving plants were weaker and lower compared to untreated control.

As can be seen in Table 2 and Table 3, the differences between herbicide-treated variants and the control were very high. It was also clear that the milk thistle cultivation was highly significantly affected by a growing year; this was in good accordance with the work of many other authors (Habán et al. 2009, Vaculík 2015). This confirmed, among other things, the highly conspicuous interaction between the growing year and the treatment method.

Table 2 Variance analysis for the number of plants/m^2 after the application of herbicides

Source of variance	n-1	No. of plants in pc/m^2
Year	2	31.3***
Treatment	2	2489.8***
evaluation term after 7, 14 and 28 days	2	24.8***
year*treatment	4	52.0***
year*evaluation term after 7, 14 and 28 days	4	4.7**
treatment*evaluation term after 7, 14 and 28 days	4	17.0***
year*treatment*evaluation term after 7, 14 and 28 days	8	7.6***
Deviation	81	1.0

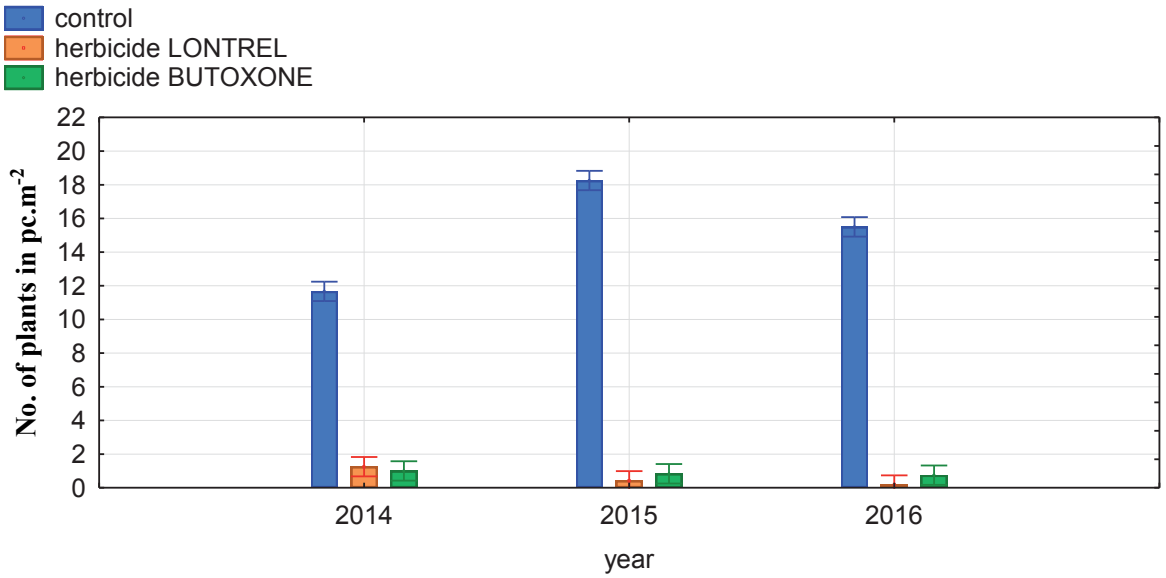
*Legend: * - $p \leq 0,05$; ** - $p \leq 0,01$; *** - $p \leq 0,001$*

Table 3 Average effectiveness of the preparations in the terms 7, 14 and 28 DPA expressed as number of plants/m^2

Herbicide	DPA	Experimental year		
		2014	2015	2016
Control	7	15.8 e	18.3 g	17.3 fg
	14	9.8 c	21.0 h	16.0 ef
	28	9.5 c	15.5 e	13.3 d
LONTREL 300	7	1.0 ab	0.8 ab	0.3 ab
	14	1.5 b	0.3 ab	0.3 ab
	28	1.3 ab	0.3 ab	0.0 a
BUTOXONE 400	7	1.0 ab	1.3 ab	1.0 ab
	14	1.0 ab	0.8 ab	0.8 ab
	28	1.0 ab	0.5 ab	0.5 ab

Legend: The average values marked with different letters in the columns differ statistically significantly at p = 0.05

Figure 1 Average number of plants/ m^2 in individual experimental years after the application of herbicides



In the case of two preparations (LONTREL 300 and BUTOXONE 400) that are intended to control the eventual occurrence of milk thistle in the subsequent crop, there has been a significant diminishing of the milk thistle plants number and it can be assumed that in combination with the competitive effect of the crop the overall herbicidal effectiveness would be excellent, i.e. 95–100%. Herbicides that are made for the disposal of broad-leaved annual and persistent weeds, such as Dicamba or herbicides containing trisulfuron, which are registered for cereals, would also be very useful. Their use for milk thistle should be a subject of a further monitoring study.

CONCLUSION

In conclusion it can be clearly stated that herbicides intended for the regulation of dicotyledonous weeds LONTREL 300 and BUTOXONE 400 are highly significantly capable to control the milk thistle infestation in a subsequent crop, most often cereal. Besides the overall herbicidal effectiveness of the above-mentioned plant protection products, the synergistic effect of the crop should be taken into the account, because the crops further strengthen the herbicidal effectiveness of given herbicides.

ACKNOWLEDGEMENTS

The research was financially supported by the Grant IGA TP 1/2016: New trend in the cultivation and use of milk thistle (*Silybum marianum* L.) in agriculture.

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THE INFLUENCE OF AGRONOMIC FACTORS ON THE GRAIN YIELD OF WINTER WHEAT

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Abstract: In the conditions of the Czech Republic, winter wheat is one of the most widely grown crops as well as cereals on arable land. The aim of the contribution was to find out the influence of different agronomic factors (pre-crop, soil tillage) as well as the year on the subsequent winter wheat grain yield in the conditions of dry land of Southern Moravia. The field experiment was conducted at the Field Trial Station in Žabčice (Czech Republic) in 2015–2017, located in the maize production area. The pre-crops for winter wheat included winter wheat, pea, alfalfa and silage maize. Two ways of soil tillage were used in the experiment, namely ploughing (to a depth of 0.24 m) and shallow loosening (to a depth of 0.15 m). From the results obtained in three years it was found that winter wheat grain yield was influenced especially by the year, the pre-crops and by combination of these two factors with soil tillage. On the other hand, statistical significance for the influence of soil tillage was not found. The yield difference between both methods of soil tillage amounted to negligible 0.11 t/ha. In terms of pre-crop, the highest winter wheat grain yields were achieved after alfalfa as a pre-crop (10.60 t/ha), the lowest yields were after winter wheat as a pre-crop (9.53 t/ha). Statistical significance was found among the pre-crops. The results from 2015–2017 also showed that the year is one of the generally most unpredictable factors which can cause different results and play an important role in generating yields. Statistical significance among individual interactions was also confirmed.

Key Words: maize production area, winter wheat, yield, pre-crop, soil tillage

INTRODUCTION

Winter wheat is one of the most widely grown crops not only in the world but also in the Czech Republic. It is our most significant as well the most grown cereal. In the Czech Republic, it takes up almost a quarter of arable land and a half of the cereals areas (Zimolka 2005). Due to the high production potential, it has gradually begun to expand significantly to higher locations, replacing the constantly reduced areas of rye and oats. Currently, it is grown virtually in all production areas (Badalíková and Bartlová 2011). Despite being grown in all production areas, locations in the maize production area can be considered the most suitable conditions for growing the winter wheat. In these areas, there is a higher probability of drier periods at the time of ripening which is necessary in order to achieve the food quality. In terms of yield generation, there is, however, the risk occurrence of drier periods in the spring period. A properly chosen growing technology consisting of different agronomic interventions can eliminate these risks to a certain extent (Smutný et al. 2007).

In order to achieve high yields when growing winter wheat, it is necessary to pay enough attention to agronomic factors whereby certain higher production ability can be achieved. The agronomic factors can include a suitable crop sequence in the crop rotation (pre-crops), in addition a suitable method of soil tillage in the particular locality conditions, the date of sowing, a suitable selection of the variety as well as sufficient fertilisation, nutrition and treatment of the stand. For instance, it has been the found that the yield can be significantly influenced under the joint action of a suitable crop and the soil tillage method (Ercoli et al. 2017). In addition to the properly used crop management practices also the soil-climate conditions of the given location as well as a particular course of the weather in the particular year are to be respected.

MATERIAL AND METHODS

The influence of agronomic factors (pre-crop, soil tillage) on winter wheat grain yields was evaluated at the Field Trial Station in Žabčice (Czech Republic) in 2015–2017, in the conditions of dry Southern Moravia. This station is located in the maize production area, which is one of the warmest and driest areas in the Czech Republic, in an altitude of 179 m and is located 25 km south from the city of Brno. The average annual precipitation for thirty years in this location amounts to 480 mm and the average yearly temperature amounts here to 9.2 °C (Table 1). Four pre-crops for winter wheat were used: winter wheat, pea, alfalfa and silage maize. Two soil tillage methods were used in the experiments: ploughing (to a depth of 0.24 m) and shallow loosening (to a depth of 0.15 m).

In 2015 and 2016, the grown variety of winter wheat was Sultan. In 2017, already the Rumor variety. The sowing rate amounted to 4 MGS/ha (millions of germinating seeds per hectare) and the sowing was done to a depth of 3 cm in the agronomic date. The total applied nitrogen dose amounted to 170 kg N/ha. In addition, P and K mineral fertilizers (90 kg P_2O_5/ha and 120 kg K_2O/ha), 1× herbicide, 1× insecticide 1× fungicide and 2× growth regulators were applied. The harvest in 2015–2017 was carried out in the first half of the month of July, using small-plot SAMPO Rosenlew SR 2010 combine harvester. The achieved yields from the harvest areas with a size of 22.5 m^2 (in four repetitions in each variant) were recalculated per hectare at the grain moisture of 14%.

Table 1 The average air temperatures and sum of precipitation in years 2014–2017, compared with temperature and sum of precipitation normal (1961–1990) at the Field Trial Station in Žabčice

Month	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	**I-XII**
2014													
Average temperature (°C)	1.1	2.7	8.5	11.8	14.5	18.8	21.5	17.9	15.6	11.5	7.5	2.4	**11.2**
Sum of precipitation (mm)	22.0	12.6	5.6	11.2	62.8	43.4	85.0	113.6	116.2	46.4	29.2	28.7	**576.7**
2015													
Average temperature (°C)	1.8	1.6	5.5	10.1	14.7	19.1	22.9	23.6	15.9	9.6	6.2	2.9	**11.2**
Sum of precipitation (mm)	20.0	7.4	28.0	9.4	33.8	22.4	22.4	106.0	23.8	48.0	24.8	17.2	**363.2**
2016													
Average temperature (°C)	-1.2	5.1	5.5	9.8	15.7	19.8	21.3	19.5	17.9	9.0	3.9	-0.5	**10.5**
Sum of precipitation (mm)	25.6	64.7	30.4	41.6	42.0	34.8	149.2	65.0	10.0	54.4	24.9	7.2	**549.8**
2017													
Average temperature (°C)	-4.9	2.0	8.0	9.2	16.0	21.0	21.4	-	-	-	-	-	-
Sum of precipitation (mm)	0.3	2.1	2.3	58.7	24.6	33.9	96.1	-	-	-	-	-	-
1961–1990													
Temperature normal (°C)	-2.0	0.2	4.3	9.6	14.6	17.7	19.3	18.6	14.7	9.5	4.1	0.0	**9.2**
Sum of precipitation normal (mm)	24.8	24.9	23.9	33.2	62.8	68.6	57.1	54.3	35.5	31.8	36.8	26.0	**479.7**

RESULTS AND DISCUSSION

The achieved results of winter wheat grain yields were statistically evaluated using ANOVA–analysis of variance (Table 2) followed by testing of mean value differences by the confidence intervals method in the statistical programme Statistica 12.0 (StatSoft software Inc., Tulsa, Oklahoma, USA).

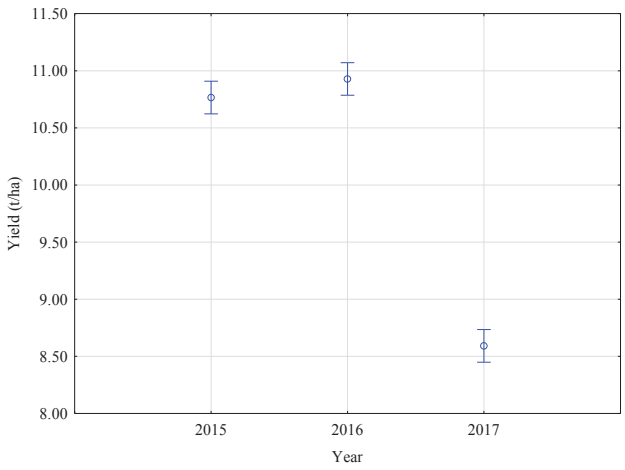
The influence of the year was statistically demonstrated on the amount of winter wheat grain yield (Figure 1). The lowest yield was achieved in 2017, namely 8.60 t/ha. On the other hand, the highest winter wheat grain yield was in 2016 (10.74 t/ha), when the difference against 2015 amounted to the negligible amount of 0.03 t/ha and 2.14 t/ha more in comparison with 2017.

Table 2 ANOVA (Analysis of variance) – grain yield of winter wheat

Source of variability	Degrees of freedom	Average square
		yield
year	2	87.14**
pre-crop	3	10.25**
soil tillage	1	0.49
year*pre-crop	6	5.68**
year*soil tillage	2	0.97*
pre-crop*soil tillage	3	1.57**
year*pre-crop*soil tillage	6	0.49
error	216	0.27

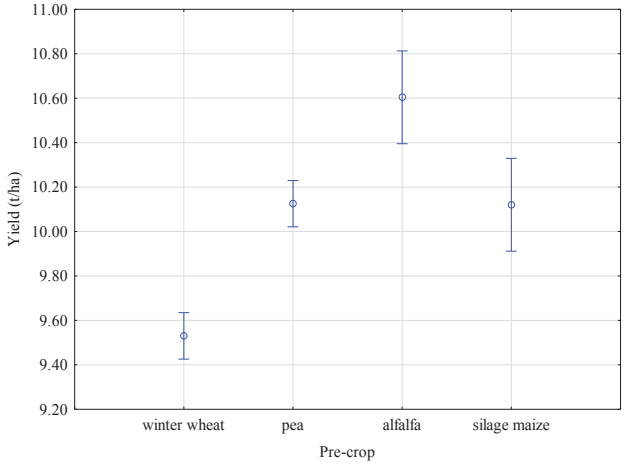
*Legend: * Statistically significant difference (P = 0.05), ** Statistically highly significant difference (P = 0.01)*

Figure 1 The influence of the year on the winter wheat grain yield



Within the influence of the pre-crops on winter wheat grain yield it was found that the highest yield was achieved after alfalfa as a pre-crop (10.60 t/ha). The lowest yield was after winter wheat as a pre-crop (9.53 t/ha). The difference between these two pre-crops amounted to 1.07 t/ha. At the same time, there was found a statistically significant difference between winter wheat and alfalfa (Figure 2) as well as between these pre-crops and silage maize and pea. Not between the pre-crops of silage maize and pea.

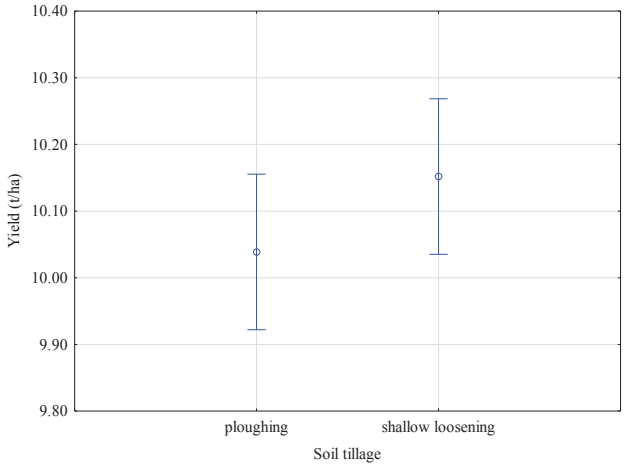
Figure 2 The influence of a pre-crop on the winter wheat grain yield



No statistical significance between ploughing and shallow loosening was found for the influence of soil tillage on the subsequent winter wheat grain yield (Figure 3). The difference between two

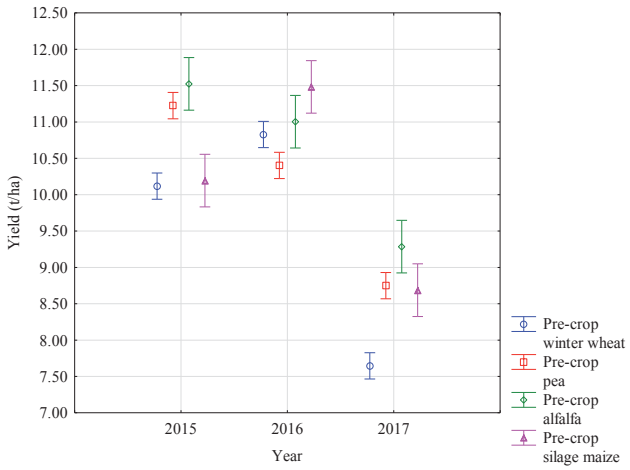
methods of soil tillage was only 0.11 t/ha, when a slightly different winter wheat grain yield was after shallow loosening (10.15 t/ha).

Figure 3 The influence of soil tillage on winter wheat grain yield



In the interaction with the year with pre-crops, the highest winter wheat yield was achieved after alfalfa in 2015 (11.53 t/ha). In the same year, the difference in comparison with silage maize was by 1.34 t/ha more, in comparison by 0.3 t/ha more and winter maize even by 1.41 t/ha more. On the other hand, the lowest yield was after winter wheat (7.65 t/ha) in 2017. It has been also found that out of all three years, the lowest winter wheat lowest yields were achieved in 2017 (Figure 4). At the same time, statistical significance was found by all four pre-crops in all three years.

Figure 4 The influence of interaction of the year with the pre-crop on winter wheat grain yield



In the interaction between the year and soil tillage it has been found that statistically significant differences after both methods of soil tillage were found between the years of 2015 and 2017 and between the years of 2017 and 2016. Whereas, between the years of 2015 and 2016, the values did not differ statistically (Figure 5). Conclusively highest grain yields were in 2016 after ploughing (10.81 t/ha), on the other hand, the lowest yields were in 2017 after ploughing (8.30 t/ha). It has been also found that in the years of 2015 and 2017, grain yield was higher after shallow loosening in comparison with ploughing, namely in 2015 by 0.10 t/ha and in 2017 by 0.11 t/ha. While in 2016, the yield after shallow loosening was lower by 0.14 t/ha in comparison with ploughing.

In the interaction of soil tillage with a pre-crop it was found that the highest yield in both soil tillage methods was achieved after alfalfa, 10.48 t/ha after ploughing and 10.73 t/ha after shallow loosening. The difference of the yield after alfalfa between these two soil tillage methods was 0.25 t/ha. Further it was found that there had been found a statistically significant difference between winter wheat as a pre-crop in both soil tillage methods and alfalfa after shallow loosening (Figure 6).

Figure 5 The influence of the year with soil tillage on winter wheat grain yield

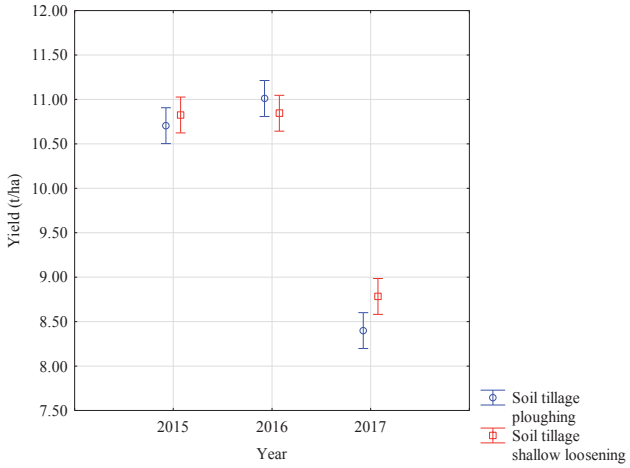
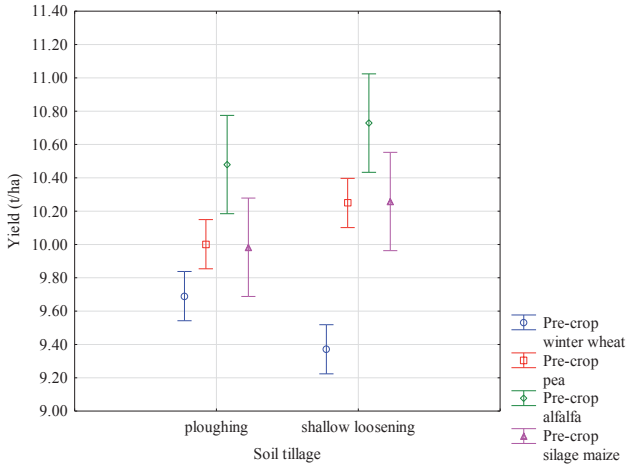


Figure 6 The influence of a pre-crop with soil tillage on winter wheat grain yield



The field experiment results from 2015–2017 showed that winter wheat grain yield is influenced not only by agronomic factors (pre-crop, soil tillage) but also by the year. Thus, high significance as well as statistical significance have been confirmed, as confirmed also by the results of Kunzová (2007) and Jug et al. (2011). It has also turned out that out of all three years, the lowest winter wheat yields were in the interaction of the year with a pre-crop. These yield differences among years could be linked with lower temperatures in winter 2016/17, when some days with frosts caused worse and slower regeneration of plants. It was negative, especially in dry condition in spring 2017. It is in opposite with warm winters in 2014/15 and 2015/16, when the plants can growth practically whole winter time. In 2016 the weather conditions were very suitable for grain formation in winter wheat. In 2015 there was lower amount of precipitation but very well distributed in time and it was effectively used for plants. But in 2017 there was typical uneven distributed precipitation whole vegetation period in combination with more frequent occurrence of hot days when evaporation increased. Less available soil moisture caused probably lower yield level in this year. As a result, this may indicate the cause of different yield results between the years of 2015 and 2017 and the years of 2016 and 2017 and the effect of the year and pre-crop factors. This is confirmed also by Neugschwandtner et al. (2015). High importance for the achieved winter wheat results can be also attributed to a suitable pre-crop which was confirmed also by Piekarczyk (2010). Our results show that the highest yield was achieved after the pre-crop of alfalfa and other two pre-crops (pea, silage maize) in comparison with winter wheat as a cereal. Similar results were found also by Hejcman and Kunzová (2010) based on their long-term experiments. The main finding was that there was found no statistical significance in the influence of soil tillage, thus between both soil tillage methods (ploughing and shallow loosening). Similarly, also Mikanová et al. (2012) indicated the same results. In addition, Rieger et al. (2008) claims that the different results of winter wheat grain yield may not always be

caused by the different soil tillage. According to Woźniak (2013), the different soil tillage method has rather a lower influence on the amount of yield, while the influence of the weather (the year) is bigger. He also points out that if there is a higher total rainfall during the vegetation period the yield is higher after ploughing than after shallow loosening. On the contrary, after a lower rainfall, the yield is higher after shallow loosening than after ploughing. Which has been also confirmed in our results, in the interaction of the year with soil tillage. Nevertheless, confirming of the influence of different soil tillage method on the winter wheat grain yield remains difficult.

CONCLUSION

The three years results show that winter wheat grain yield in the conditions of dry maize production area is influenced especially by the year (weather), the pre-crop and partly by soil tillage. This confirms not only the significance of the influence of agronomic factors, mainly the pre-crop, but also the influence of the year (the weather) not only separately, but also in the interaction with a pre-crop and in the interaction with soil tillage.

ACKNOWLEDGEMENTS

The research was financially supported by the project IGA FA MENDELU no. IP 36/2017: „Evaluation the impact of agronomic factors on yield and grain quality, selected soil properties and the economy winter wheat growing in different farming systems“.

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EVALUATION OF THE IMPACT OF DIFFERENT SOIL TILLAGE ON PHYSICAL SOIL PROPERTIES

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Abstract: The aim of the experiment was to compare the change of physical soil processes in different soil tillage towards different crops. There was conducted a field experiment at the Field Trial Station in Žabčice in 2017, within a model field experiment which is focused the management on the soil with livestock production. There were compared three different soil tillage technologies, namely ploughing (to a depth of 0.24 m), shallow loosening (to a depth of 0.15 m) and direct sowing, with winter wheat after two pre-crops (silage maize and alfalfa) and with spring barley after the pre-crop sugar beet. The evaluative physical soil properties included bulk density (g/cm^3), the total porosity (%), the minimum air capacity (%) and the maximum capillary capacity (%). The modified Kopecký-Novák method of sampling of undisturbed soil samples was used for the analyses. The depth of sampling was 0–0.10 m, 0.10–0.20 m and 0.20–0.30 m in five repetitions. Based on the achieved results it was found that the traditional soil tillage, thus ploughing has a more favourable influence on the bulk density, the total porosity and minimum air capacity than soil tillage with lower intensity (shallow loosening and direct sowing).

Key Words: soil tillage, physical soil properties, bulk density, total porosity, minimal air capacity

INTRODUCTION

Soil tillage plays an important role as well as an integral part of agro-technical intervention. Since soil tillage has an influence on the soil properties and plant production (Husnjak et al. 2002). However, it cannot be omitted that it is also a significant mean in the fight against weeds, pests and diseases.

Significant soil properties include the physical state of soil, on which the water, air, biological and thermal soil regime (Czyz 2006) is immediately depended. And they are the different soil tillage systems that considerably influence the physical properties of soils and the growth of crops (Mosaddeghi et al. 2009) and ultimately can also significantly influence the achieved crop yields. The changes induced by soil tillage most significantly relate to reduced bulk density which has an influence on the entire complex of soil physical properties, i.e. porosity, air and water capacity, thermal conductivity etc. (Czyz 2006).

It has been found that a deeper soil tillage decreases the soil strength and the soil bulk density (Laddha and Totawat 1997) but improves water storage in the soil, increases the growth of roots (Holloway and Dexter 1991) and even increases the crop production (Ghosh et al. 2006). While Mosaddeghi et al. (2009) have found that there are better physical soil properties in the system without soil tillage than in the conventional system, especially in dry and semi-dry areas. Thus we must realize that the changes of soil properties due to different soil tillage are also different depending on the soil and climate conditions of the particular areas.

MATERIAL AND METHODS

The monitoring was carried out within the rotation of crops for livestock production management in a model field experiment called AGRO 2 *(7-crop rotation – alfalfa 1st year, alfalfa 2nd year, winter wheat, silage maize, winter wheat, sugar beet, spring barley)*, based on the Field Trial Station in Žabčice. The location is found at an altitude of 179 m, in a maize production area, in a South Moravian dry area with typical inland climate. The dryness of the climate is increased by winds causing a large evaporation of soil moisture. The average annual air temperature reaches here 9.2 °C and the average rainfall amounts to 480 mm. There is a fluvisol type and heavier soil texture.

Three variants of soil tillage, namely ploughing (to a depth of 0.24 m), shallow loosening (to a depth of 0.15 m) and direct sowing, in winter wheat after the pre-crop silage maize and alfalfa and in spring barley after pre-crop sugar beet were chosen for the monitoring of physical soil properties in 2017.

Determining the physical soil properties was based on the sampling of undisturbed soil samples (in natural storage) and their following laboratory analysis. The sampling of soil samples to the so called Kopecký physical rolls (with a volume of 100 cm^3) was done in April 2017. The evaluative physical soil properties included bulk density (g/cm^3), the total porosity (%), the minimum air capacity (%). As these main physical soil properties reflect very well each mechanical intervention to the three phase soil system (solid soil matter, water and air). In addition, the maximum capillary capacity (%) was determined. These physical properties were determined at a sampling depth of 0–0.10 m, 0.10–0.20 m and 0.20–0.30 m always in five repetitions.

The Kopecký-Novak modified method used at the Department of Agrosystems and Bioclimatology of Mendel University in Brno (Kostelanský 1980) was used for the analyses. Statistic software Statistica 12.0 (StatSoft software Inc., Tulsa, Oklahoma, USA) was used for statistic evaluation.

RESULTS AND DISCUSSION

In the monitored year of 2017, there were found differences among the physical properties in the assessed experimental variants. In statistical evaluation done by analysis of variance (ANOVA) and the subsequent Tukey HSD test at a significance level $P \leq 0.05$, a statistically significant difference was found. The statistically significant difference is shown in the following tables 1 and 2. Different letters (a, b, c) designate significant difference at the significance level $P \leq 0.05$. Values of physical properties are mentioned in in these tables as averages after both pre-crops and all variants of soil tillage.

Winter wheat after silage maize

There were recorded statistical differences among individual variants when growing winter wheat after silage maize. As for bulk density and the total porosity, a statistically significant difference was found in all soil tillage variants (Table 1). As for minimal air capacity, there was a statistically significant difference between ploughing and direct sowing and between shallow loosening and direct sowing. There was no statistically significant difference between ploughing and shallow loosening. As for the maximal capillary capacity, there was found no statistically significant difference between ploughing, shallow loosening and direct sowing.

Winter wheat after alfalfa

When growing winter wheat after alfalfa, there were found statistically significant differences between ploughing and direct sowing and between shallow loosening and direct sowing both in bulk density and the total porosity, the minimum air capacity and the maximum capillary capacity (Table 1). On the contrary, there was found no statistically significant difference between ploughing and shallow loosening in all four evaluated physical soil properties.

Table 1 Average values of physical soil properties in individual soil tillage variants when growing winter wheat after the pre-crop silage maize

Pre-crop	Soil tillage variant	Bulk density (g/cm^3)	Total porosity (%)	Minimum air capacity (%)	Maximum capillary capacity (%)
Silage maize	ploughing	1.34 a	48.72 a	10.57 a	38.14 a
	shallow loosening	1.37 b	47.68 b	10.77 a	36.90 a
	direct sowing	1.42 c	45.86 c	7.87 b	37.98 a
Average		1.38	47.42	9.74	37.67
Alfalfa	ploughing	1.20 a	54.15 a	22.83 a	31.32 a
	shallow loosening	1.19 a	54.57 a	22.67 a	31.90 a
	direct sowing	1.36 b	48.18 b	11.73 b	36.45 b
Average		1.25	52.30	19.08	33.22

Legend: Different letters (a, b, c) designate a significant difference in the significance level $P \leq 0.05$.

Spring barley after sugar beet

When growing spring barley after sugar beet, there was found a statistically significant difference in all soil tillage variants in bulk density (Table 2). As for the total porosity, the minimum air capacity and the maximum capillary capacity, there was found a similar statistically significant difference as in growing winter wheat after alfalfa, thus only between ploughing and direct sowing and between shallow loosening and direct sowing. No any difference was found out between ploughing and shallow loosening.

Table 2 Average values of physical soil properties in individual soil tillage variants when growing winter wheat after the pre-crop sugar beet

Pre-crop	Soil tillage variant	Bulk density (g/cm^3)	Total porosity (%)	Minimum air capacity (%)	Maximum capillary capacity (%)
Sugar beet	ploughing	1.35 a	48.36 a	10.53 a	37.83 a
	shallow loosening	1.38 b	47.40 a	10.04 a	37.35 a
	direct sowing	1.57 c	39.96 b	6.14 b	33.82 b
Average		1.43	45.24	8.90	36.33

Legend: Different letters (a, b, c) designate a significant difference in the significance level $P \leq 0.05$.

The field experiment results have shown that as the intensity of soil tillage decreases, in our case shallow loosening and direct sowing, there is a statistically significant increase of bulk density and at the same time the reduction of the total porosity of soil. This is confirmed by most authors (Sprague and Triplett 1986, Azooz and Arshad 1996, Raus 2000, Hůla and Procházková 2008). The highest bulk density values were found after direct sowing whether in case of winter wheat after the pre-crops silage maize (1.42 g/cm^3) and after the pre-crops-alfalfa (1.36 g/cm^3) and in spring barley after the pre-crop sugar beet (1.57 g/cm^3). Horne et al. (1992) and Alegre et al. (1991) have also found, on the basis of their results, that the soil bulk density is higher in zero tillage system if compared to the traditional conventional technology (ploughing). On the contrary, the total porosity was highest in soil tillage technology by ploughing. This confirms that the total porosity is a mirror image of bulk density as claimed also by Houšť et al. (2011). The total porosity was higher after shallow loosening (54.57%) in comparison with ploughing by 0.42% only when growing winter wheat after the pre-crop alfalfa. At the same time, the direct sowing variant had a significant decrease of the minimum air capacity values in comparison with the variant with ploughing and shallow loosening of soil. The results also pointed out to a partial tendency to increase the maximal capillary soil capacity while increasing the intensity of its processing.

In spring barley, the results show that soil tillage influence values of some soil physical properties. In some cases, limits according to Lhotský methodology (1984) were obtained. Direct sowing variant had the highest values of bulk density and the lowest minimum air capacity which could be limited for root development and caused decrease of yield. It was confirmed by yield data when yield was 1.7 t/ha lower at this variant in comparison with ploughing and shallow loosening.

In winter wheat, the results show that soil tillage in interaction with pre-crop influence values of some soil physical properties, as well. In general, much more suitable values of physical parameters were obtained after alfalfa. There are significant differences among soil tillage variants, but still all values are in acceptable range. Direct sowing variant had the highest value of maximal capillary capacity. This fact was positive in dry vegetation period of year 2017 and the yield was 0.51 t/ha higher in comparison with ploughing. But this conclusion is valid only after alfalfa, when soil is in good structural staff. On the other hand, after silage maize, the grain yield of winter wheat was in this variant the lowest (difference 1.01 t/ha).

CONCLUSION

The achieved results have mostly confirmed the anticipated effects of the effects of individual soil tillage methods on the physical soil properties. When comparing the changes of physical soil properties in different soil tillage to different crops, it has turned out that the traditional soil tillage (ploughing) has a more favourable effect on bulk density, the total porosity and the minimum air capacity than low intensive soil tillage (shallow loosening and direct sowing). One-year results have shown how interact the effect of soil tillage in combination with pre-crop. When winter wheat is grown after alfalfa, soil is in good structural staff and direct sowing can be suitable variant. Especially in dry years, there is water saving effect.

ACKNOWLEDGEMENTS

The research was financially supported by the project IGA FA MENDELU no. IP 36/2017: „Evaluation the impact of agronomic factors on yield and grain quality, selected soil properties and the economy winter wheat growing in different farming systems“.

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EFFECT OF ARTIFICIALLY INDUCED DROUGHT ON GROWTH AND PRODUCTIVITY OF SELECTED CROPS WITHIN FIELD EXPERIMENT IN BOHEMIAN-MORAVIAN HIGHLANDS

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Abstract: The field experiments based on manipulating the crop environment are critical for determining the crop's response to the climatic conditions expected in the future. An experimental site in Domanínek is located by 49°31'42"N, 16°14'13"E at an altitude of 560 m (potato production area). The field experiment using rain-out shelters for soil water availability reduction was conducted in 2015/2016. The main aim of this study was to assess the impacts of different water availability (rain-out shelters vs. control) on the performance of selected field crops (spring barley, winter wheat, winter rape and silage maize). Reduction of precipitation in treatment with rain-out shelters was confirmed by measuring soil water content. The amount of precipitation during growing period was reduced by 251 mm, 277 mm, 217 mm and 240 mm for the spring barley, winter wheat, winter rape and silage maize, respectively. As a consequence, leaf area index and yields declined, however the crop responses were not consistent.

Key Words: ANOVA, drought stress, field trial, LAI, rain-out shelter, water shortage

INTRODUCTION

Long term field experiments are considered to be an irreplaceable source of information on the long-term effects of agro-technical measures on the soil environment, provide material for assessing the relationship between crop yields and weather patterns. They provide information about effects of soil management various in time and the response to changed environmental condition (Lipavský et al. 2010). According to Rühlmann and von Gagern (2009) dealing with the evaluation of the results of long-term field experiments, for example, different climatic conditions and different crops and crop rotations have an impact on the mechanisms of yield generation, nutrient balance, soil organic matter dynamics, nutrient release, nutrient intake, soil organic matter, soil biodiversity and soil biological properties.

Drought as a period of abnormal dry weather causing hydrological imbalance is the consequence of reduced precipitation and snowfall as well as increased evapotranspiration due to higher temperatures. (Konikow and Kendy 2005). It is expected that the number of drought episodes will increase (Žalud et al. 2009) and it is critical to explore how drought will affect food security in the future. The present study is focused on the impacts of the reduction in available soil moisture, which is one of the most important prerequisite for plant production. The main aim of this study was to quantify the impacts of precipitation reduction under leaf area index (LAI) and dry matter yields. Simultaneously, the applicability of experimental rain-out shelter was verified.

MATERIAL AND METHODS

Field experiment characterization

The field experiment was set up in Bystřice nad Pernštejnem at Domanínek experimental site which is situated at the altitude of 560 m. Dystric cambisol is typical soil type for this area. Other soil properties are shown in Table 1. The annual precipitation fluctuates around 610 mm and the average annual air temperature around 7 °C. Basics meteorological parameters such as air temperature and relative humidity, global radiation, total precipitation or wind speed are measured at the station.

Table 1 Soil parameters in depth 0.0–0.3 m

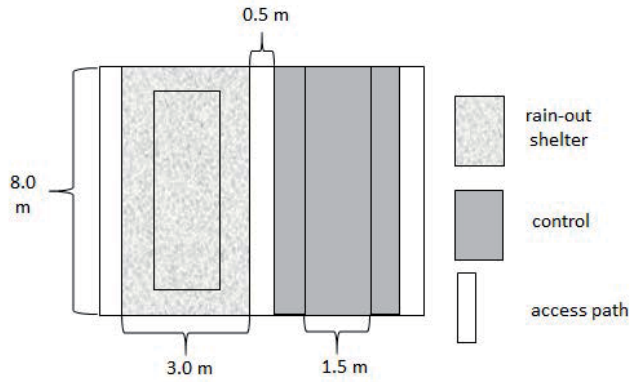
Soil texture	Field porosity (%)	Soil hydrolimits	
		wilting point (%)	field capacity (%)
Silty-loamy sand	44	8	24

The present study is focused on assessing the experiment during the 2015/2016 growing season. The selected crops have involved spring barley (variety Bojos), winter wheat (variety Bohemia), winter rape (variety Rohan) and silage maize (hybrid FAO 220 – DKC 3301). Date of sowing, harvesting and date of duration of using rain-out shelters are shown in Table 2. The crops were subsequently exposed to water deficit using mobile rain-out shelters (see Wimmerová et al. 2016) and then these variants were compared with control plots in three repetitions. Dimensions of the rain-out shelter and control plot is depicted on the Figure 1.

Table 2 Significant dates of selected crops (sowing, harvesting, sheltering) and the total and reduced amount of precipitation on individual plots and dry matter yields of selected crops

Parcel	Selected crop	Sowing	Harvesting	Rain-out shelters (2016)		Precipitation (mm)		Yield average (t/ha)	
				from	to	total	reduced	Control	shelter
1	spring barley	5. 4. 2016	15. 8. 2016	23. 5.	15. 8.	308	251	4.94	0.97
2	winter wheat	25. 9. 2015	15. 8. 2016	21. 4.	15. 8.	536	277	6.81	4.85
3	winter rape	26. 8. 2015	26. 7. 2016	25. 4.	26. 7.	507	217	2.42	1.86
4	silage maize	11. 5. 2016	29. 9. 2016	23. 5.	3. 8.	292	240	22.71	16.49

Figure 1 Dimensions of the rain-out shelter and control plot. The rectangles inside of sheltered and controlled plot depict harvest area, 2016



Methods of measurement

Weather data, especially precipitation (Table 2), was acquired from an automatic rain gauge located in the immediate vicinity of the field trial at Domanínek experimental site.

Length of the growing season, LAI (plant canopy analyser SunScan, Delta-T Device, UK), soil moisture from integrating depth of 0.0 to 0.3 m (time domain reflectometry sensor – TDR, CS 616,

Campbell Scientific Inc., UK), yields and effects of sheltered and control treatments were considered for this study. Measurements using soil moisture sensors were done only on the parcels of spring barley, winter wheat and winter rape.

Statistical evaluation

In addition to arithmetic averages, calculation of the deviations and maximum values list, the analysis of variance (ANOVA) was applied on LAI and yields by using R software and agricolae package (Felipe de Mendiburu 2017).

RESULTS AND DISCUSSION

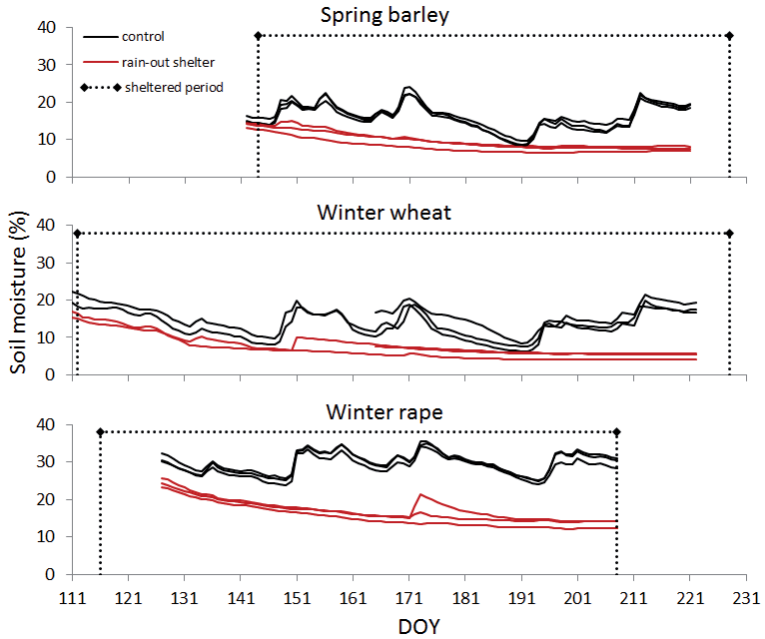
Total precipitation in the crop rotation

The amount of precipitation varied depending on the variant (sheltered vs. control) and field crop. The largest reduction of precipitation was in the case of silage maize and spring barley – 240 mm and 251 mm in 72 days and 84 days, respectively. The smallest reduction of precipitation was in the case of winter rape and winter wheat – 217 mm and 279 mm of precipitation in 92 days and 116 days, respectively. Mentioned data can be found in Table 2.

Soil moisture

According to Figure 2, differences between control and rain-out shelter plots are evident in all crops under investigation (red line below black line). In addition, the red curve is basically a horizontal flat line and proved the waterproofness of the rain-out shelters. An average deviation in soil moisture is 6.9%, 6.7% and 13.1%, respectively. Soil moisture was observed in the study by Wimmerová et al. (2016) with similar results, when control and sheltered treatment of winter wheat were compared in the year before (i.e. 2015).

Figure 2 Soil moisture content (%) in the controls (3×) and under rain-out shelters (3×) from 0.0–0.3 m. A dotted box depict sheltered period. DOY represents the day of the year 2016



Leaf area index and yields

LAI values were compared using ANOVA (Figure 3 and Table 3). According to P values (Table 3), the crop, treatment (rain-out shelter and control) and their interaction were all statistically significant ($P<0.05$) factors. Figure 3 shows differences among individual crops and rain-out shelter and control variant in more detail. Despite the fact that spring barley showed statistically significant differences between control and rain-out shelter treatments – 51% (1.76 m^2/m^2), sheltered spring barley plants were cut by hares. The effect of treatment was also statistically significant in the case of winter wheat showing

22% (1.54 m^2/m^2) decline due to rain-out shelter. The standard deviation (0.21 m^2/m^2) was consistent for both treatments.

Figure 3 Maximum mean values of the control LAI compared with maximum mean values of the sheltered LAI under selected field crops. Error bars depicted standard deviation

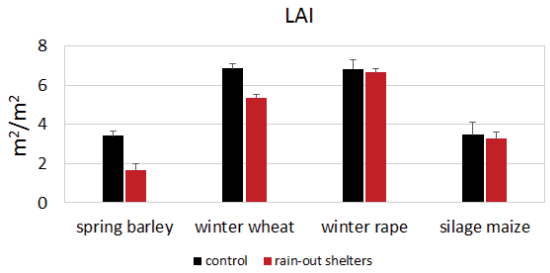


Table 3 Statistics relevance of monitored parameters under maximum mean LAI measurements. Treatment includes rain-out shelter and control variants

LAI	Df	F value	P value
Crop	3	199.631	<0.0001
Treatment	1	39.471	<0.0001
Crop:Treatment	3	8.880	0.00107

Legend: Df – degree of freedom

Regarding mean yields as well as mentioned above, the P value for all three monitored groups (crop, treatment and crop:treatment) was less than 0.05 for yields (Table 4). Silage maize with standard deviations of 1.65 t/ha (control) and 2.51 t/ha (rain-out shelter) had showed the most statistically significant differences of all crops and treatments. On average, silage maize yields decreased by 27% (6.22 t/ha) due to precipitation reduction of 240 mm/72 days (Figure 4, Table 2). Other crops had the following results – spring barley yields in rain-out shelter variant decreased on average by 80% (3.97 t/ha) in comparison with mean control variants, precipitation reduction was 251 mm/84 days; winter wheat yields in rain-out shelter treatments decreased on average by 64% (1.70 t/ha) in comparison with mean control variants, precipitation reduction was 277 mm/116 days; and winter rape yields was reduced on average by 23% (0.56 t/ha) for sheltered variants, precipitation reduction was 217 mm/92 days. The number of mean yields are mentioned in Table 2.

Figure 4 Mean yields of the control variants compared with mean yields of rain-out shelter variants. Error bars depicted standard deviation.

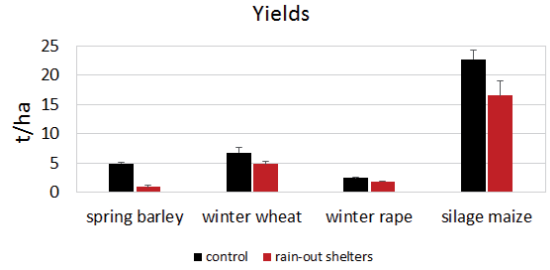


Table 4 Statistics relevance of monitored parameters under mean yields. Treatment includes rain-out shelter and control variants

Yields	Df	F value	P value
Crop	3	313.851	<0.0001
Treatment	1	47.802	<0.0001
Crop:Treatment	3	7.202	0.002825

Legend: Df – degree of freedom

Although soil moisture has been decreased in rain-out shelter treatment, UV radiation, type of crop and variety, horizontal water flow in deeper soil layers could influence individual differences between rain-out shelter and control treatments.

CONCLUSION

In general, effective differences between rain-out shelter and control treatments can only be achieved after rainy periods. This condition was realized and demonstrated with using TDR sensors therefore the soil moisture in all crops was lower than in control treatments. When the leaf area index (LAI) and yields were evaluated separately, the most significant difference was found out between rain-out shelter and control treatments for spring barley and winter wheat field crops, namely 51% (1.76 m^2/m^2) and 22% (1.54 m^2/m^2), respectively, for LAI. However, it must be taken into account that the sheltered spring barley plants were cut by hares. Within yields, the most difference was recorded among rain-out shelter and control treatments for silage maize where yield differences reached up to 27% (i.e. 6.22 t/ha).

To get the most trusted data, it is continually worked on improving the experiment. However, it is not always possible to control all biotic and abiotic factors.

ACKNOWLEDGEMENTS

The research was financially supported by the project IGA FA MENDELU no. IP 22/2017 with the support of the Specific University Research Grant, provided by the Ministry of Education, Youth and Sports of the Czech Republic.

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URL: https://mnet.mendelu.cz/mendelnet2016/mnet_2016_full.pdf. [2017-08-15].

ANIMAL PRODUCTION

COMPARISON OF LAYING INTENSITY AND EGG QUALITY THE EFFECT OF JAPANESE QUAILS (*COTURNIX JAPONICA*) WITH DIFFERENT FEATHER COLOR

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Abstract: The aim of the study was to evaluate the effect of genotype on laying intensity and egg quality in Japanese quails *Coturnix japonica L*. Four genotypes were different in plumage color, twelve quails of the same age of each genotype were included in the experiment, 48 birds in total. The observation lasted 90 days. Quails were housed in individual cages. Egg number was recorded daily, egg quality was done in two weeks intervals. Laying intensity was high in all genotypes (92.2–94.4%) without significant differences, however the highest laying intensity was in quails with aguti and mahogany types of plumage. Egg weight was significantly the highest in mahogany type (13.7 g, $P < 0.05$). Eggshell weight was significantly the lowest in quails white type plumage ($P < 0.05$), which was associated with the lowest strength of eggshell ($P > 0.05$). Albumen proportion was significantly the highest in mahogany type plumage ($P < 0.05$). On the other hand Haugh units were the highest in quails with aguti type plumage. Yolk weight was the highest in gold type and the lowest in mahogany type plumage ($P > 0.05$). Yolk colour was similar in all genotypes. The highest body weight was in mahogany type (303 g), the lowest in gold type quails (263 g).

Key Words: Japanese quail, plumage, laying intensity, egg quality

INTRODUCTION

Breeding of Japanese quails has increased due to increasing popularity of both quails meat and eggs. From former luxury delicacy has become even in Europe commonly available food. Main and traditional producers are mainly in Asia, namely China, Japan, Korea and Taiwan and in Europe they are mainly in France, Italy and Germany. There is several colour mutations, described as different plumage quail types. These quails are mainly kept as game birds in aviaries for their attractive plumage. There are very few publication reporting on performance of these plumage types. The basic feather colour in quails is aguti, which is original for wild quails and it is typical for commercial both egg and meat or dual quails. Anyway there are others plumages mainly in backyard or hobby poultry. There is recessive white plumage, dominant mahogany plumage, relatively complicated gold plumage, silver, redhead plumages or bicolor tuxedo plumage. In the world literature (Shitara et al. 1980, Tarata et al. 2016) quails are mainly divided on the basis of live body weight into egg type, dual type, meat and super meat type. Plumage mutation occurs in all these types. There is a lot of studies dealing with egg quality (Sari et al. 2016, Ghayas et al. 2017), however information about the effect of plumage mutation on egg quality are missing. Quail eggs contain 33% of yolk, 59% of albumen and 8% of eggshell. Thickness of eggshell is about 0.2 mm. Chemical composition is as follow: water 74.6%, protein 13.1%, fat 11.2% and ash 1.1%, content of energy is 632 J/egg. Concerning breeding of quails, except egg and meat production, there are research groups aiming to lower cholesterol content in the eggs by selection (Baumgartner and Hetényi 2001, Shitara et al. 1980, Hyánková and Hort 1999, Yilmaz et al. 2011).

The aim of the study was to find the effect of genotype, plumage mutations, on laying intensity and egg quality in four different plumage types of quails.

MATERIAL AND METHODS

Birds

In total forty eight females of Japanese quails were used in the experiment. There were four different plumage types of the quails, twelve of each genotype at the same age of seventy-eight days, all quails at the beginning of the experiment were already laying eggs. There were aguti type with genotype e^+/e^+ (similar plumage as in *Coturnix coturnix*), gold type with genotype Y/y^+, mahogany type *E/E* and white type *wh/wh*. The main criteria for birds' selection were as follow: balanced weight among the plumage types and colour feather purity in pedigree at least in five previous generations. Egg number was recorded daily and evaluated in week intervals as laying intensity.

Housing and feeding

Experiment took place in the experimental room of FA at MENDELU in Brno. Quails were housed in individual cages. Temperature was stable during whole experiment and ranged from 17 to 20 ºC. Day light was artificial 14 h with 10 h of dark period. The diet for parent stock (11.5 MJ ME, 16% CP) was fed ad-libitum. Cages were equipped with nipple drinkers.

Egg quality

Following characteristics of eggs quality were observed: egg weight, egg index, weight of yolk, albumen and eggshell, Haugh units, yolk colour, eggshell thickness and strength, proportion of eggshell, yolk and albumen of egg weight. Egg analysis were done in two weeks intervals when eggs were collected three consecutive days, in total about 120 eggs were analysed.

All characteristics were expressed by mean value and standard error. The effect of genotype, plumage mutation, on egg quality was analyzed using one way ANOVA and Scheffe-test, laying intensity was analyzed by Kruskal-Walliss ANOVA using the software package Unistat 5.1 (UNISTAT Ltd, ENGLAND).

RESULTS AND DISCUSSION

There was relatively high variability in live body weight of quails (Table 1). Significantly lowest weight was in gold type quails ($P < 0.05$, 263 g). The highest weight was found in mahogany type (303 g). Hyánková et al. (2002) studied heritability of live body weight in quails and its steadiness in different lines.

Laying intensity is shown in Table 2. According to Shitara et al. (1980) the top of laying intensity in quails is between 12 and 18 weeks of age, when the laying intensity reaches 90% or even more in some flocks (99%). Since 36 weeks of age laying intensity sharply decreases (Shitara et al. 1980), however Baumgartner and Hetényi (2001) reported laying intensity in 52 week of age more than 50%. Hyánkova et al. (2002) published the highest laying intensity in aguti type quails. In this experiment quails with aguti plumage also reached the highest laying intensity (94.39%). Mahogany type achieved 94.05%, gold type 92.20% and white type 92.26%. Total laying intensity in all plumage types was 93.22%.

Egg weight (Table 3) in different plumage types ranged from 12.7 to 13.7 g. The highest weight was found in mahogany type quails ($P < 0.05$), the lowest weight was found in white type quails. Egg weight is connected with live body weight of the birds. There is no negative correlation between egg and body weight as it is in chickens (Hyánková and Hort 1999). The similar situation is also in partridge (*Alectoris graeca* L.), which is close relative to quails, Kirikci et al. (2007).

Indexes of eggs were very balanced and similar in all plumage types and ranged from 1.28 to 1.34. The difference among the types were no significant. On the other hand Yilmaz et al. (2011) published significant differences in egg index among mahogany, silver and white plumage types.

Yolk weight was significantly the lowest in mahogany type ($P < 0.05$) and the highest in gold type quails. Proportion of yolk (Table 4) was significantly the lowest in mahogany type quails ($P < 0.05$, 28.7%), despite the highest egg weight in this type. It means that high egg weight was caused by higher albumen weight. The highest yolk proportion was founded in white and gold plumage types (32.0%, $P < 0.05$). Yolk colour was very similar in all types (Table 4) and it reached 5, using DSM colour fan. Yolk colour is affected mainly by pigments in feed and partly by genotype (Nelson 1968),

all plumage types fed the same diet. There was no double yolk eggs, although quails were at the beginning of laying period.

Albumen quality is shown in Table 5.

Weight of albumen was the highest in mahogany type (8.7 g) and the lowest in aguti and white types (7.7 g, $P > 0.05$). Proportion of albumen (Table 5) was the highest in mahogany type (63.9%, $P < 0.05$), which was connected with the highest egg weight in this type. The lowest albumen proportion was found in aguti type quails (60.6%). Haugh units (tab. 4) express albumen quality and there was no significant difference among the plumage types. On the other hand Yilmaz et al. (2011) published significant effect of plumage on Haugh units.

Parameters of eggshell quality are shown in Table 6. Characteristics and heritability of eggshell quality and the effect of genotype on eggshell quality deeply studied Narinc et al. (2015), Tarata et al. (2016) and Jatoi et al. (2013). These authors confirmed high heritability of eggshell quality and correlation between eggshell weight and strength. Eggshell weight was relatively balanced in this experiment. Significantly the highest weight was observed in mahogany type ($P < 0.05$) and very high weight was also recorded in aguti type quails.

The lowest eggshell weight was found in white type quails ($P < 0.05$). Proportion of eggshell was the lowest in white and gold types, however there was no significant difference among the types. Significantly the lowest eggshell thickness was found in gold type quails ($P < 0.05$).

Table 1 Live body weight of quails (g)

Genotype	Weight of quails
	Mean ± SE
Aguti	296 ± 2.14[a]
Mahogany	303 ± 2.25[a]
Gold	263 ± 1.76[c]
White	277 ± 4.30 [b]

Legend: SE – standard error; a, b – means of the same order designated by different letters are significantly different (P < 0.05)*

Table 2 Laying intensity (%)

Genotype	*Laying intensity (%)*
Aguti	94.4[a]
Mahogany	94.1[a]
Gold	92.2[a]
White	92.3[a]

Table 3 Egg weight and egg index in quails

Genotype	Egg weight (g)	Egg index
	Mean ± SE	Mean ± SE
Aguti	12.8 ± 0.06[b]	1.28 ± 0.005[a]
Mahogany	13.7 ± 0.05[a]	1.34 ± 0.005[a]
Gold	13.0 ± 0.07[b]	1.30 ± 0.006[a]
White	12.7 ± 0.05[b]	1.31 ± 0.004[a]

Legend: SE – standard error; a, b – means of the same order designated by different letters are significantly different (P < 0.05)*

Table 4 Yolk parameters of quail's eggs

Genotype	Yolk weight (g)	Yolk proportion (%)	Yolk color
	Mean ± SE	Mean ± SE	Mean ± SE
Aguti	4.1 ± 0.03[a]	31.8 ± 0.14[b]	4.9 ± 0,05[a]
Mahogany	3.9 ± 0.02[a]	28.7 ± 0.13[a]	5.1 ± 0,05[a]
Gold	4.2 ± 0.03[a]	32.0 ± 0.15[b]	4.9 ± 0,06[a]
White	4.0 ± 0.02[a]	32.0 ± 0.11[b]	5.1 ± 0,06[a]

Legend: SE – standard error; a, b – means of the same order designated by different letters are significantly different (P < 0.05)*

Table 5 Albumen parameters of quail's eggs

Genotype	Albumen weight (g)	Albumen proportion (%)	Haugh units
	Mean ± SE	Mean ± SE	Mean ± SE
Aguti	7.7 ± 0.08[a]	60.6 ± 0.15[b]	105.6 ± 1.87[a]
Mahogany	8.7 ± 0.06[a]	63.9 ± 0.14[a]	90.5 ± 1.74[a]
Gold	7.9 ± 0.08[a]	60.8 ± 0.17[b]	91.6 ± 2.17[a]
White	7.7 ± 0.15[a]	60.8 ± 0.12[b]	91.8 ± 1.56[a]

Legend: SE – standard error; a, b – means of the same order designated by different letters are significantly different (P < 0.05)*

Table 6 Eggshell parameters of quail's eggs

Genotype	Eggshell weight (g)	Eggshell proportion (%)	Eggshell thickness (mm)	Eggshell strength (N)
	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
Aguti	0.97 ± 0.067[ab]	7.6 ± 0.05[a]	0.224 ± 0.001[a]	14.5 ± 0.15[a]
Mahogany	1.01 ± 0.007[a]	7.4 ± 0.05[a]	0.227 ± 0.001[a]	14.5 ± 0.11[a]
Gold	0.94 ± 0.008[ab]	7.2 ± 0.06[a]	0.206 ± 0.001[b]	14.1 ± 0.17[a]
White	0.91 ± 0.006[b]	7.2 ± 0.04[a]	0.212 ± 0.001[ab]	13.8 ± 0.13[a]

Legend: SE – standard error; a, b – means of the same order designated by different letters are significantly different (P < 0.05)*

CONCLUSION

The main difference among the different plumage mutations were found in egg weight and proportion of egg yolk, albumen and eggshell. Egg weight was significantly the highest in mahogany type (13.7 g, P < 0.05), however yolk proportion was the lowest in this type (28.7%, P < 0.05). There was no significant difference in laying intensity (92.2–94.4%), although aguti type of quails is the most extended. The highest body weight was in mahogany type (303 g), the lowest in gold type quails (277 g).

ACKNOWLEDGEMENT

The authors would like to thank the project IGA FA IP 2017/073 for financial support. The experiment was done thanks equipment financed by project OP VaVpI CZ.1.05/4.1.00/04.0135.

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GROWTH PERFORMANCE IN LABORATORY RATS IN RELATION TO ADDITION OF MILK THISTLE PRESSED PARTS OR MYCOTOXIN CONTAMINATED FEED RATION

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Abstract: Growth performance is affected by many different factors and this impact can be positive or negative. The presence of mycotoxins in feedstuff count among negative factors, at the same time mycotoxins damage health status, especially liver. Liver have essential importance in metabolism and detoxification of organism. This article applies one´s mind to relation to feeding components with hypothetic positive (milk thistle, *Silybum marianum*) and negative (mycotoxins) impact on growth performance in laboratory rats. Experimental design was focused on comparison of three types of diet. The first type of diet was barley monodietus intended for control group, the second type of diet was addition of milk thistle pressed parts, that is known for its hepatoprotective effect (content of active substance – silymarin was 26.2 g/kg), and the third type of diet was addition of barley contaminated by mycotoxins (content of DON was 9634 µg/kg and ZEN was 2192 µg/kg). Groups with part of mouldy barley and milk thistle pressed parts on top of that were invited based on content. The experiment was tested by 25 pieces of laboratory rats divided into 5 groups. The rats in group 1 (G1) were classified like the control and were fed only with scraped barley, in group 2 (G2) and 3 (G3) fed with addition of milk thistle pressed parts (part of milk thistle in G2 was 10% and in G3 was 20%) and in group 4 (G4) and 5 (G5) fed with part of mouldy barley (part of mouldy barley in G4 was 30% and in G5 was 60%). Among these groups were discovered statistically significant differences. Higher average daily gains were occurred in groups fed with addition of milk thistle pressed parts and opposite lower average daily gains were occurred by groups fed with part of mouldy barley.

Key Words: growth performance, *Silybum marianum*, silymarin, *Fusarium*, mycotoxin, deoxynivalenol, zearalenone

INTRODUCTION

Growth performance is one of the most important indicators of dietary adequacy (National Research Council 1995). The presence of mycotoxins in animal feed can cause health disorders and can also contribute to other factors such as stress, lack of nutrition, infection by pathogenic agents etc. Negative effects of mycotoxins may particularly affect the liver, kidney, nervous system, endocrine system and immune system (Malhotra et al. 2014). Several *Fusarium* species can produce harmful mycotoxines, mainly deoxynivalenol (DON), zearalenone (ZEN), T-2 toxin, HT-2 toxin and diacetoxyscirpenol in cultivated cereals all over the world. This experiment was focused on content of DON and ZEN.

The most common mycotoxin in cereals DON is present worldwide and has relative low toxicity compared to other trichothecene mycotoxins (Kachlek et al. 2017) and a full clarification of the hepatic damage by DON has not been done yet. Some studies already demonstrated hepatic damage caused by DON and some articles presents opposite (Peng et al., 2017). ZEN is particularly known for its reproduction toxicity (Kovacs 2012) and liver toxicity (Stadnik and Borzecki 2009, Long et al. 2016).

Instead of this the feeding of milk thistle, like hepatoprotective component, could improve negative effects on performance. Complex of *Silybummarianum* agents is known like silymarin and is

able to stabile membranes of hepatocytes and improves hepatocytes resistance to toxins. Silymarin is usually used for treatment of acute or chronic disorders (Zhu et al. 2016). Milk thistle active substances appear to be safe and well tolerated (Dhiman et al. 2005). *Silybummarianum* constituents to the animal feed ration could promise for conventional methods of animal breeding (Kosina et al. 2017) and be a promising natural feed additive to improve the health condition (Cullere et al. 2016). Results of different researches indicate positive effect of feeding *Silybum marianum* on growth performance but by way of contrast this exist some researches with different ambiguous results.

Diet for laboratory animals used in this experiment formulated with scraped barley is rich in energy and may cause decreasing food consumption and can change ration of nutrients, such as competition for absorption sites among certain minerals that share common active transport systems (National Research Council 1995). Interactions between disproportionate diet and environmental factors set amplifying mechanism for liver damage (Larter 2010). This monodietus was purposed to induce health stress to reinforcement of effect of negative effect of mycotoxins and possible positive effect of milk thistle pressed parts.

The aim of this study was to determine the effect of diet with part of mouldy barley and with addition of milk thistle pressed parts in comparison with them and control group (barley monodietus). The question sounds: Will have addition of milk thistle pressed parts positive effect on weight performance? Will have diet with part of barley with high concentration of DON and ZEN negative effect on weight performance?

MATERIALS AND METHODS

Experimental design

The experiment was established by 25 pieces of laboratory rats divided into 5 groups (G1, G2, G3, G4, and G5) in experimental facilities of Department of Animal Nutrition and Forage Production in Mendel University. The rats in group G1were fed with scraped barley, the rats in G2 and G3 were fed with scraped barley with addition of milk thistle pressed parts, part of milk thistle in G2 was namely 10% and in G3 20%. The rats in groups G4 and G5 were fed with scraped barley with part of mouldy barley, part of mouldy barley in G4 was namely 30% and in G5 60%. Content of silymarin in milk thistle pressed parts was 26.2 g/kg, Content of DON in mouldy barley was 9634 µg/kg and content of ZEN was 2192 µg/kg. Served milk thistle pressed parts had flour-like structure and was mixed equally with scraped barley also the rats could not prefer any feeding component. Milk thistle variety MIREL was used. The feeding mixture and water were available *ad-libitum*. Water and feed ration were daily served and rests were removed. The length of this experiment was 28 days. Starting average rat´s weight was 90 ± 3 g. The rats were weighted in 0–7–14–21–28. The rats were sacrificed by inhalational anaesthetic Isofuran way on 28th day and liver samples were taken to histological analyse.

Determination of silymarin

The content of silymarin was performed with HPLC method in Department of Chemistry and Biochemistry at Mendel University in Brno. Analysis of silymarin was performed on a HPLC-UV / VIS instrument (Dionex Ultimate 300). Chromatography column Hypersil GOLD Dim (150 x 4.6) was used for separation by temperature 30 < °C. The sample (5 µl) was injected by autosampler. Flow rate was 1 ml/min. Content of mobile phase was: A: 0.1% formic acid, B: 100% methanol. The substances were leaved to infuse in an isocratic elution way (mobile phase A was 65% and mobile phase B was 35%). Detection of separated substances was in motion under circumstances of wavelength 288 nm.

Determination of Mycotoxins

A 2 g sample was weighed to PTFE centrifuge tubes (50 ml) followed by the addition of 10 ml of distilled water acidified (0.2% formic acid). The sample was shaken then, 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 amicrofilter with 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. 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) is used for ultra-efficient liquid chromatograph Acquity UPLC® System (Waters, Milford, MS, USA). The program Analyst® (Thermo Fisher Scientific) is 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 (weight) with standard deviation (SD). Statistical significance was determined by the examining the basic differences between control groups and G2, G3, G4, G5 by ANOVA and Scheffé's test (one-way analysis). The differences with $P < 0.05$ were considered to be significant.

RESULTS AND DISCUSSION

Growth performance (average daily gain)

Table 1 The average daily gain of rats consumed different diets (control group – fed with scraped barley. G2 – fed with scraped barley with 10% part of milk thistle pressed parts. G3 – fed with scraped barley with 20% part of milk thistle pressed parts. G4 – fed with scraped barley with 30% part of mouldy barley. G5 – fed with scraped barley with 60% part of mouldy barley.)

Day	Control group(g)	G2 (g)	G3 (g)	G4 (g)	G5 (g)
0	0	0	0	0	0
7	2.3 ± 0.5^{a}	3.4 ± 0.6^{b}	3.6 ± 0.3^{b}	3.0 ± 0.5^{a}	3.0 ± 0.3^{a}
14	2.1 ± 0.4^{a}	2.5 ± 0.3^{a}	2.4 ± 0.4^{a}	1.8 ± 0.3^{a}	1.6 ± 0.5^{a}
21	1.9 ± 0.4^{a}	1.8 ± 0.5^{a}	2.0 ± 0.7^{a}	3.0 ± 1.0^{a}	1.8 ± 0.5^{a}
28	1.7 ± 0.5^{a}	1.7 ± 0.6^{a}	3.1 ± 0.6^{b}	2.6 ± 0.7^{a}	2.4 ± 0.4^{a}

Change in index ^{a,b} shows significant difference at the level ($P < 0.05$)

The results show that the weight gain of the rats fed with mixtures of 10% the milk thistle was higher compared to rats of the control group. The highest average daily gains were found in rats fed by mixtures of 20% milk thistle. On the seventh day of the experiment, the difference between control group and G2 and G3 was found to be statistically significant, same as the difference between control group and G3. These results conclude that the addition of milk thistle pressed parts increased the growth performance. Statistically significant difference between control group and group fed with 20% part of milk thistle (G3) were observed on the 28th day too.

As far as relation between control group and groups fed with parts of mouldy barley is concerned, there were not any statistically significant differences.

Rats receiving the addition of milk thistle in their feed dose grew more intensely than the control group. Kosina (2017) describes the positive effect of the milk thistle on the growth potential of experimental animals (rabbits in this case). Therefore, the addition of the milk thistle in the feed dose could have a positive effect on the growth potential of animals. Feng (2016) states that silymarin significantly affected weight gain in mice. This trend was also observed in our experiment.

In case of mycotoxins in diet, it can be concluded that the presence of mycotoxins in the feed did not affect rats' weight gains. Thanh et al. (2016) state the DON – ZEN ingestion did not affect growth performances, average daily gain, average daily feed intake, and feed efficiency. Similar results were found by Kachlek et al. (2017).

CONCLUSION

According to our results, it is possible to draw that the growth performance was significantly higher in groups fed with addition of milk thistle pressed parts. The highest average daily gains were observed in group fed with 20% part of milk thistle (G3). Yet statistically significant differences between control group and groups fed with part of mouldy barley were not proved among the groups as individual differences in the framework of groups G4 and G5. Statistically significant differences were not proved too between groups fed with part of mouldy barley and control group. These results correspond to supposition and to other author's results.

ACKNOWLEDGEMENTS

The research was financially supported by the IGA grant, no. IP 025/2017.

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INFLUENCE OF MILKING PERIOD ON THE INTENSITY OF LYING BEHAVIOUR OF DAIRY COWS KEPT IN BOXES

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Abstract: The aim of this study was evaluating the influence of milking period on the intensity of lying behavior of dairy cows kept in boxes. This study contains also an experimental part which has been done in the farm of dairy cows, GenAgro Říčany a.s. in Říčany near Brno. In this farm, they are breeding czech fleckvieh cattle. We had mainly observed the intensity of the average time of lying in a boxing bed after dairy cow's arrival from a milking parlor, depending on milking time. During the annual observation, these effects were monitored, the average lying time in the individual lactation phases and in each row of boxes, then was observed, another effect was average lying time of the monitored cows and intensity of lying to the boxes. As supplementary information was monitored daytime pleasure during the year and milk composition. We have found out that influence on intensity and time of lying has year season and age of dairy cows. With regard to results, after return from the milking parlor was dairy cows from group 2 (youngest cows) searching for another activity and lain in the longest time. In warm months, dairy cows lain in largest amount (July, 122 pcs) These cows lain down in the longer time (May, 43.6 minutes) than cows in cold months. (November, 87 pcs).

Key words: rest, dairy cows, boxing bed, milking, lying cows

INTRODUCTION

To dairy cows must be allowed a sufficient time to lying and resting. That is important for maximal milk production. Equally important is comfort and tranquility in stable (Leonard et al. 1994). If dairy cows are not stable in optimal conditions, are they developing different activity than lying. And that has got negative influence on rumination and milk production (Doležal and Staněk 2015). There are many various activities that cows can do during a day, but the most important for them are rest and lying (Munksgaard and Simonsen 1996).

MATERIAL AND METHODS

For this study was chosen behavioral observation of production stables for dairy cows. The observation took place in facility GenAgro Říčany a.s. In this production stables were housed 400 pcs of dairy cows of czech fleckwieh cattle, which was divided in 4 groups according the performance. Group 1 was dairy cows in first phase of lactacion. Goup 2 was youngest cows on firs lactation and groups 3 and 4 on second and third phase of lactation. Each of groups went to milking parlor separately. Dairy cows from group 1 went first and from group 4 went last. Dairy cows were observed from arriving from milking parlor to laying down of first 20 cows.

Observations were made during one calendar year, from December 2015 to November 2016. In total were made 12 observations. Every observation was made at the end of the month and always during the morning milking of cows (from 4:30 AM to 11:00 AM) Every group was going for milking separately. For a start of observation was important arrival of first cow from the milking parlor, then each group was observed for one hour. For our study was most important time interval, from arrival of individual cows to lay down to one of the boxes. This time interval has been written into the sheet and statistically evaluated. Time interval and intensity of lying were main aim of our study.

RESULTS AND DISCUSSION

In Table 1 we can see number of cows that lain down to the boxing bed during the year. Most cows lain down in period from May to September, largest number of cows lain in July (122 pcs). The largest intensity of lying was in group 1 in September (37 pcs) and the least intensity of lying during the observation was in April in group 4 (16 pcs).

Table 1 Intensity of lying of dairy cows – number of cows in individual groups (pcs)

Month/group	Totally	Group 1	Group 2	Group 3	Group 4
	pcs	pcs	pcs	pcs	pcs
December	95	25	24	18	28
January	98	27	23	21	27
February	93	24	22	29	18
March	99	28	24	24	23
April	91	30	22	23	16
May	118	29	28	31	30
June	105	27	28	27	23
July	122	29	30	36	27
August	116	30	31	28	27
September	115	37	30	27	21
October	95	25	22	27	21
November	87	21	24	20	22

Menke et al. (2015) stated in their study that cows which are on the top of group hierarchy lies more than cows which are lower in hierarchy. Petříčková (2012) found out that lying is the most fluctuating behavior during a day. The most were cows lying from 11:00 PM to 12:00 PM and from 2:00 AM to 3:00 AM and other important period of lying author observed at noon in time of stable tranquility. The author also states that dairy cows lay more in summer than in Autumn and least in Spring. This statement is identical with results of our study.

From Table 2 is obvious that dairy cows in group 1 lain down fastest in December. On average 29.6 minutes from arrival from the milking parlor. On the other side longest time to lay down was measured in April (45.0 minutes).

Table 2 Average time to lying down of all monitored cows (minutes)

Month/group	Totally	Group 1	Group 2	Group 3	Group 4
	minutes	minutes	minutes	minutes	minutes
December	41.5	37.6	50.8	30.1	45.1
January	38.1	36.3	40.3	31.4	43.1
February	40.7	35.5	47.9	45.4	31.0
March	39.8	36.7	50.3	41.2	31.0
April	42.3	45.0	54.3	35.4	30.8
May	41.6	43.9	54.4	37.1	32.7
June	42.6	41.9	46.5	44.3	36.8
July	39.8	36.3	56.5	37.2	35.7
August	34.8	43.1	48.4	36.8	34.8
September	43.2	41.4	55.0	39.1	34.6
October	34.0	34.8	45.5	32.8	22.7
November	32.9	29.6	36.5	31.5	33.5
Totally $\bar{x}$	39.3	38.5	48.9	36.8	34.3

In group 2 fastest of dairy cows lain down to the boxes again in November in 36.5 minutes after first cow arrive from the milking parlor. In this group dairy cows lain slowest in July in 56.5 minutes.

Dairy cows from group 3 lain to the boxes slowest in February (45.4 minutes) and fastest in December (30.1 minutes). In group 4 lain cows in October in 22.7 after first cow arrive from the milking parlor and slowest in December (45.1 minutes). On average from groups lain cows fastest in November (32.9 minutes) and slowest in September (43.2 minutes). Dairy cows went to the milking parlor before feed intake and therefor their priority was different than lying down. In group 2 was housing cows on first lactation and therefor their time to lay down was longest even if they were fed, because they were very active. Shortest time from arrive of cows from the milking parlor was in group 4. In our opinion, it was caused by cows leaving group fed. After they arrive from the milking parlor these cows found box for rest. They didn´t want to intake food.

Table 3 Average time of lying of first 20 monitored cows (minutes)

Month/group	Totally	Group 1	Group 2	Group 3	Group 4
	minutes	minutes	minutes	minutes	minutes
December	41.7	40.6	50.2	29.7	46.2
January	38.1	36.5	40.8	30.1	44.4
February	40.8	36.6	49.0	45.9	31.0
March	39.8	37.3	52.6	41.6	31.4
April	42.5	45.5	54.4	37.2	30.8
May	43.6	45.7	57.5	39.4	31.8
June	41.4	39.5	47.4	41.7	36.9
July	39.0	35.2	55.0	31.3	34.7
August	40.0	44.3	44.3	37.8	34.0
September	41.3	39.7	53.0	38.7	33.9
October	33.3	33.8	44.7	32.2	22.6
November	33.1	31.4	35.3	31.5	34.1
Totally $\bar{x}$	39.6	38.8	48.7	36.4	34.3

In Table 3 was included first 20 cows which in every section lain fastest. Out of these 80 cows lain fastest cows from group 4 in October (22.55 minutes). The slowest cows was from group 2 in May (57.50 minutes). On average cows lain slowest in May (43.60 minutes) and fastest in December (33.30 minutes). How can we see in „Totally" column in Table 3, cows from group 2 lain down in the longest time. In our opinion, this fact could be caused by that in section 2 was housed primary dairy cows on first lactation (youngest cows in stable). Against older cows, young cows walked in a section for longer before lying down to the box. Shortest time to lay down was observed in the group 4. In our opinion lain this group fastest because they went for milking already fed and after returning from the milking parlor they were directly looking for the boxing bed.

Resting time of dairy cows is influenced by many factors, for example nutrition, type and size of boxing bed, quality of used bedding in boxes, number of cows in group and season (Doležal and Staněk 2015). Out of research Brzozowska et al. (2014) has season got significant influence on dairy cows lying. In spring has been observed decline of lying down and very sharp decline of lying in summer months. In winter cows spend more time by lying than in different seasons. This statement matches with our results. Same results published Uzal Seyfi (2013) in their study.

CONCLUSION

In summary, more dairy cows lain down in summer months and less in winter. This result manifested itself in each of groups. Most cows from group 1 lain down in April and August and least in November. In group 2 most in August and least in October. In group 3 most in July and least in December. And in group 4 most in May and least in April.

Average time to lay down of all cows was longest in warmest months of the year. Dairy cows were lying in larger amount, but in longer time interval. Fastest time to lay down was observed in November and slowest in June. This trend was similar in all observed groups. In group 1 lain dairy cows fastest in November and slowest in April. In group 2 slowest in July and fastest in November. In

group 3 fastest in December and slowest in February. And in group 4 fastest in October and slowest in December. If we consider only first twenty cows from each group, slowest cows were from group 4 and fastest from group 2 (youngest cows). Studies in this area are very different, results of some of them are similar to ours. To confirm our results is required to develop more studies in this area.

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ANALYSIS OF BREEDING AND PERFORMANCE OF HORSES IN THE CZECH REPUBLIC BASED ON EVENTING COMPETITIONS

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Abstract: This work deals with riding competitions – eventing and includes statistical evaluation of the effect of the breed, age, year of start and sex on the performance of the horses. Data were taken from the Survey of Sport Horses of 2005 and 2015. These data are issued by the Czech Equestrian Federation. Data were further processed and statistically analysed using the program STATISTICA 2012. Evaluation of the sport performance was based on the auxiliary points (AAP). The breed, age and year of start have a statistically significant effect on performance. The breed with the highest performance was the Irish sport horse with 17.4 AAP. The best results were achieved by horses of 13 to 16 years of age (AAP 11.81). The most frequently used breeds in the Czech Republic are the Czech warm-blood and the English Thoroughbred. The performance of imported breeds was better than of horses born in the Czech Republic. The performance of horses in 2015 was better than in 2005. We can see an increasing tendency in the performance. In conclusion we compared the situation in the Czech Republic over the past ten years by comparing the gradual change in their representation of the sex, breeding and country of birth.

Key words: eventing, sports breeds of horses, performance

INTRODUCTION

The popularity of riding – eventing is steadily increasing. It is an attractive discipline which has its roots in the military. Although it is not so widespread as show jumping, it is still the most attractive and most popular riding discipline and its popularity is continuously increasing.

Eventing is made up of three completely different parts, i.e. dressage, cross-country and show jumping (Pellarová et al. 2015). These three phases take place on separate consecutive days during which a competitor rides the same horse throughout (FEI 2017).

In this three-day event the jumping test takes place on the last day after a veterinary inspection (FEI 2017). The dressage test is executed on the first day of eventing. The starting pair executes the prescribed movements on the dressage rectangle (usually measuring either 20 × 60m or 20 × 40m). For deviations in the respective movements the judges allot penalty points (Grodl 2009). They evaluate the correct sitting position and precise execution of the respective movements (Motyginová 2001). Cross-country is the most demanding phase of the entire three-day event. It is a test of endurance and speed (Micklem 2004). The cross country test constitutes the most exciting and challenging all-round test of riding ability and horsemanship. This part of the competition focuses on the ability of athletes and horses to adapt to different and variable conditions of the competition (weather, terrain, obstacles, etc.) showing jumping skills, harmony and mutual confidence (FEI 2017). After the rider and horse successfully master the cross-country test they are faced with the final test – show jumping (Petrmannová 2013). The show jumping test is executed on the last day of the classical three-day event. It is a test of willingness and concentration of the horse, psychic resistance of the rider and his art of strategy. It puts to test the ability of the horse to regenerate. The winner is the rider with the fewest penalty points for all the three tests (Grodl 2009).

When choosing the horse preference is given to a young, healthy and well-bred horse. Important is also the type of the horse and the body conformation (Dušek 1999). According to Misař and

Jiskrová (2001) when choosing a horse for eventing it is important that the horse has an extended and smooth gait, deftness and courage to master difficult natural fences. The speed of the horse must be sufficient, the constitution hardy and the horse tenacious. The horse of choice should be able to make decisions for himself. Although this is not a prerequisite it helps the horse cope with pitfalls of the course much better (Dillon 2012). Lerche (1956) pointed out the importance of conformity of the body conformation of the horse to prevent unbalanced straining. The greatest emphasis is on the limbs of the horse (Paalman 1998).

Horses of any kind of breed intended for sporting activities can be used in the discipline. In the Czech Republic it is mostly the Czech warm-blood, the Slovakian warm-blood bred in the Czech Republic and the English Thoroughbred (ČJF 2015). In general a very good horse for this discipline is a horse with a greater proportion of blood of the English Thoroughbred.

MATERIAL AND METHODS

Determination of the comparative base

Here we used the Survey of Sport Horses of 2005 and 2015. From the database of the ČJF we selected the horses that started in the respective year at least three times, of which minimally one start was in eventing; 529 horses in total. We also used data from charts of the best horses in eventing competitions in the respective years.

Methods of determination of average auxiliary points (AAP)

The horses in the charts are arranged according to the AAPs per one start. Auxiliary points are obtained by conversion of the actual result of the horse in eventing competitions converted by means of the following matrices, see Table 1.

Table 1 Matrices of calculations of horses in eventing (ČJF 2015)

Difficulty code	Degree of competition		Disability Is subtracted	Penalty points							
				<45	45.01 –55	55.01 –65	65.01 –75	75.01 –85	85.01 –110	110.01 –150	>150
37	CCI4*	TT	0	34	33	32	31	30	29	27	25
36	CNC/CCN/CIC/CCI 3*	TT	0	30	29	28	27	26	25	23	21
35	CNC/CCN/CIC/CCI 2*	ST	-1	26	25	24	23	22	21	19	17
34	CNC/CCN/CIC/CCI 1*	ST	-2	22	21	20	19	18	17	15	13
33	L	L	-3	18	17	16	15	14	13	11	9
32	ZL	ZL	-2	14	13	12	11	10	9	7	5
31	Z	Z	-1	11	10	9	8	7	6	4	2

Statistical analysis

The data were evaluated statistically using the programme STATISTICA 2012.

Descriptive statistics of the AAPs in 2005 and 2015

The following were evaluated: *arithmetical mean, modus, median, maximum, minimum and standard deviation.*

Statistical analysis using the ANOVA method (Analysis of Variance) and following tests

We used multi-factorial analysis. When the effect was statistically significant we assessed the differences among the age groups, sexes, breeds and years of start using the method of Scheffe's multiple comparisons.

Factors followed:

Age

- Group 4–6 years – 140 horses
- Group 7–12 years – 329 horses
- Group 13–16 years – 54 horses
- Group 17 and more years – 6 horses

Breed

- Czech warm-blood – 284 horses
- Slovakian warm-blood + Kinský horse – 33 horses
- English Thoroughbred – 107 horses
- Irish sports horse – 11 horses
- German warm-blood breeds + Swedish warm-blood + Danish warm-blood – 23 horses
- Dutch warm-blood + French riding horse – 13 horses
- Horse of the warm-blood type – 14 horses
- Horses with no pedigree – 23 horses
- Arabian thoroughbred + Shagya Arab – 3 horses
- Hafling – 3 horses
- Others (**) – 15 horses

(**) Wielkopolska and Malopolska horse, Polish noble half-bred horse, Ukrainian riding horse

Sex

- Stallions – 53 horses
- Mares – 203 horses
- Geldings – 273 horses

Year of start

- 2005 – 233 horses
- 2015 – 296 horses

RESULTS AND DISCUSSION

The average AAP points of horses starting in eventing competitions were 10.75 in 2015 and 7.80 in 2005. In 2015 the average AAP points were by 2.95 higher than in 2005. Also the mean values, the minimum and maximum were higher than in 2005. The minimum value was -0.3 in 2005 and 0 in 2015. The value of the maximum value was 22.20 in 2005 and 25.33 in 2015, see Table 2. The higher value of AAP points in 2015 demonstrates that the present-day performance of horses is higher. The same results were achieved by Horká (2016) who evaluated the jumping performance of horses. She stated that from 2005 to 2014 the performance increased by 3.14 points. The results of Ricard and Chanu (2000) suggest that selection on jumping performance will lead to some positive correlated response for eventing performance.

The results of the effect of the respective factors for AAP are presented in Table 3, where the factors were: age, sex, breed and year of start.

Table 2 Descriptive statistics of evaluations of the performance of horses in eventing competitions in 2005 and 2015

AAP variable	Mean	Median	Modus	Modus frequency	Minimum	Maximum	Standard deviation
2005	7.80	7.83	8.00	11.00	-0.30	22.20	4.27
2015	10.75	10.39	9.00	13.00	0	25.33	5.07

Table 3 Effect of the respective factors on the performance of horses in eventing competitions

Performance	Age	Sex	Breed	Year of start
AAP	**	-	**	**

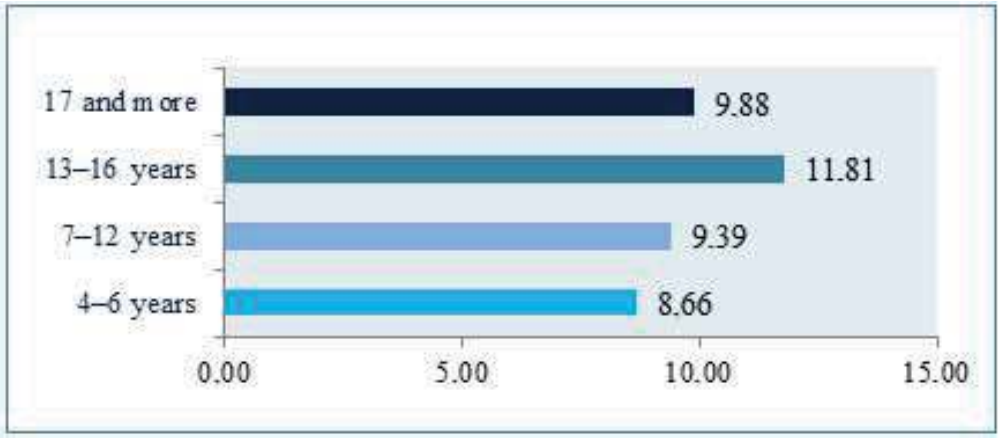
Significance: P ≤ 0.01 (**) statistically highly significant, P ≤ 0.05 (*) statistically significant

The effect of the breed, age and year of start had a statistically highly significant effect on the performance of the horses. The statistically significant effect of the sex was not proved.

Following tests using Scheffe's test

In following tests of the age by means of Scheffe's test it was discovered that performance of the category of horses of the age of 13–16 years was statistically highly significant compared to the youngest age category of 4–6 years and age category of 7–12 years; see Figure 1.

Figure 1 Average values of AAP in the respective age categories



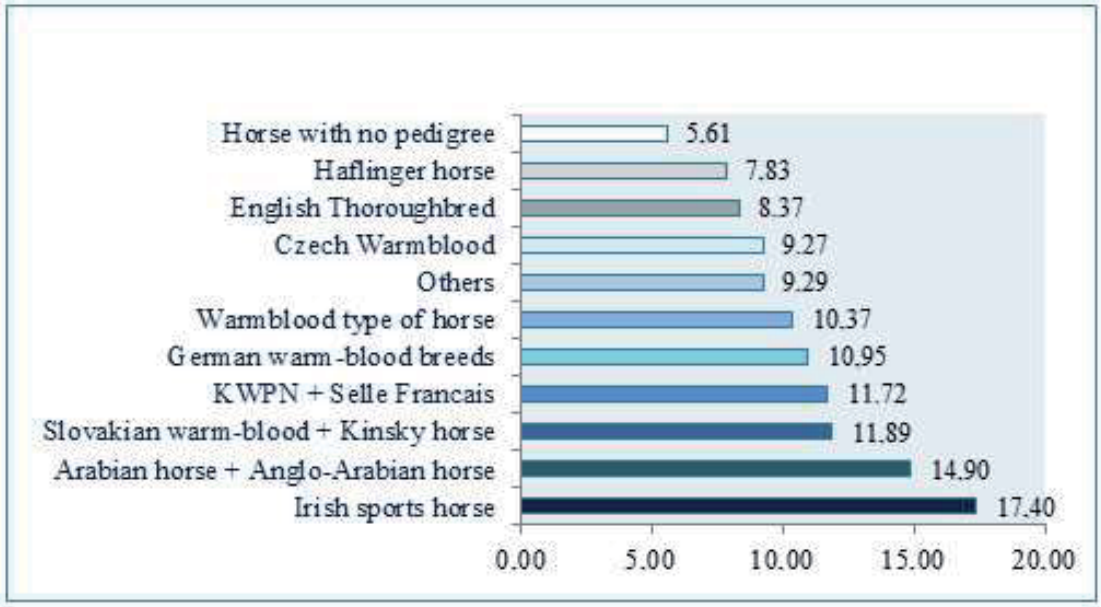
In subsequent tests of the year of start we discovered that performance had increased. In 2005 AAP was 7.8. In 2015 performance increased (by 3 AAP) to AAP 10.8.

Also Dibalová (2009) confirmed that the performance of horses in eventing competitions increased; she reported that performance increased gradually from 2000 to 2007, i.e. by 5.99 points, corresponding with our results and proving the gradual increase in performance.

It was discovered on the base of factor breed that the performance of the Irish sport horse was statistically highly significantly higher than that of the Czech warm-blood (by 8.13 AAP), the English Thoroughbred (by 9.03 AAP) and of horses that had no pedigree (by 11.79 AAP) and was statistically significantly higher than of horses of other breeds (by 8.11 AAP). The performance of the Czech warm-blood was statistically highly significantly higher than of horses that had no pedigree.

The performance of the Irish sport horse was unbeatably the best (AAP 17.4). Dibalová (2009) concluded that worldwide this breed is the most successful breed in eventing competitions due to its high performance and in the Czech Republic its popularity is constantly increasing.

Figure 2 Average values of auxiliary points of the respective breeds



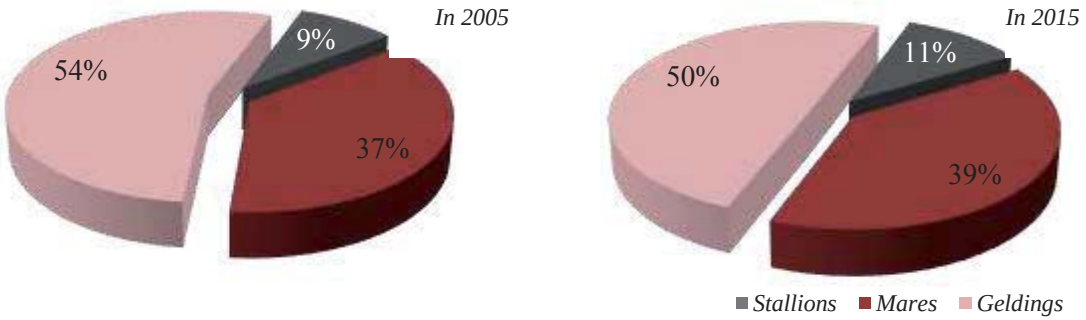
Overall evaluation of eventing competitions in the Czech Republic and across the world

The graphs below show the proportional representation of sex of horses starting in eventing competitions in the Czech Republic in 2005 and 2015. In this competition preference is given to geldings over mares and stallions. The reason for the popularity of geldings is that their temperament is calmer, they are even-tempered and better manageable.

Dibalová (2009) added that geldings are used more frequently for eventing competitions because they are mentally more balanced thanks to which their performance is more stable.

Performance of stallions is considerably better than both that of mares and geldings (Whitaker et al. 2008). However, stallions are used least of all, possibly because they are not manageable easily. According to Krčová (2013) stallions require specific handling and for inexperienced riders they might be dangerous.

Figures 3 and 4 Sex representation of horses starting in eventing competitions



CONCLUSION

The objective of the present study was to evaluate the effects of the individual factors on the performance of horses in eventing competitions. We monitored the results of the individual factors for AAP, they were the following: age, sex, breed and year of start. Other factors in the competitions were country of origin of the horses, the breed and the effect of stallions imported from abroad. We compared a period of ten years (2005–2015) and we found that performance was influenced by the breed, age of horse and year of start.

It was also discovered that at present the reigning horses in eventing competitions are the Irish sport horses. Although these horses do appear in the Czech Republic they are in a minority. The most

easily available horses for Czech breeders are Czech breeds, such as the Czech warm-blood and the Slovakian warm-blood bred in the Czech Republic, followed by the English Thoroughbred.

In terms of the age of the horses the highest performing horses were horses from 13 to 16 years of age (11.81 AAP). Their performance gradually increased up to the age of 15 years (15.01 AAP) and then began to decline. The highest performing breed was the Irish sport horse (17.4 AAP). The performance of the horses increased in 2015 as compared to 2005. Germany has progressed considerably in breeding. Surprising results were achieved with the Czech warm-blood; its performance over the studied period increased ranking it immediately after the German warm-blood breeds and confirming the great progress in breeding of the Czech warm-blood for sports performance.

Another finding (based on our tests) was that sex had no effect on performance. In both of the above mentioned years geldings were the most frequently used horses in eventing competitions, followed by mares and least of all stallions. The other factor was the country of origin of the horse. The overwhelming majority of starting horses was born in the Czech Republic. The number of horses imported to the Czech Republic is increasing. During the monitored period the number of imported horses increased by 8%.

In conclusion I would like to add that if the Czech Republic wants to compete in eventing competitions with riders from abroad, I recommend that they either acquire an imported horse (primarily an Irish sport horse or a German warm-blood breed) or one of our Czech sport breeds (Czech warm-blood). However, a horse competing in an eventing competition must also show speed and stamina. That is the reason why breeders frequently tend to the English Thoroughbred.

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SERUM GLUCOSE AND ALT CONCENTRATIONS DURING DIFFERENT LEVELS OF TRAINING IN HORSES

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Abstract: This work is targeted on influence of various factors on biochemical blood parameters of horses, especially physical activity, or stress. The main goal is to evaluate knowledge about blood content and use of biochemical indicators in horse diagnostics. Sixteen Czech Warmblood horses were used in experiment. All of this horses were stallions in age 3 years. They were divided on the basis of their stabling and training status. Group A, free range stabled, trained only easily before the blood sampling, and group B, in a box stabled, with intensive training, jumping and dressage six times in a week. Lower glucose and Alanine Aminotransferase (ALT) levels were determined in blood of easily trained horses (group A). Differences between the observed groups were significant only for glucose. The moderate negative correlation between ALT and glucose was found in group B (r = -0.405). This correlation was very low in group A.

Key Words: horse, glucose, ALT, stress, biochemical parameters

INTRODUCTION

The information about biochemical response of horses submitted to physical exercise such as dressage and jumping is limited and controversial. The training of horse involves periodic exercises that cause structural, functional and behavioral changes (Lindner 2000). These exercises prepare horses for the competitions. High performance of the athlete horse is determined by many complicated interdependent physiological and hematochemical processes (Warwick 2004). Analyzes and study the modifications of blood parameters is very important for understanding of horse physiology, function and types of energy utilized (De Miranda et al. 2009). Biochemical and hematological parameters have been studied during different kinds of physical effort, such as trot races (Piccione et al. 2009), or 130 km long endurance ride (Noleto 2016). After the warm up the glucose values showed a significant decrease and a significant increase after the show jumping test (Fazio et al. 2014). Glucose significantly decreased also during endurance race for 120 km in Scandinavian studies (Larsson et al. 2013).

Alanine Aminotransferase (ALT), formerly known as glutamic pyruvic transminase is not specific to liver. Increases in ALT may by caused by acute liver failor, but also by myositis; therefore ALT is not useful for predicting liver diseases of horses. (Reeder et al. 2009). Normal range of ALT is between 0.08–0.25 μkat/l (Inlab Medical 2001)

The aim of the study was to evaluate glucose and ALT concentration in blood of horses with different training levels. And to determine whether there is a corelation between concentration of ALT and glucoses of low and high intensity training horses.

MATERIALS AND METHODS

Animals and breeding conditions

Sixteen Czech Warmblood horses (stallions) in age 3 years were used in this experiment. Two groups were formed, eight horses each. This division was based on stabling and training status: group

A, free range stabled, trained only easily before the blood sampling, and group B, in a box stabled, with intensive training, jumping and dressage six times in a week.

All horses were stabled in Provincial Stud Farm Tlumačov. The area was located in altitude 200 meters above sea level. During the experiment was average air temperature 7.6 °C.

Collection of blood samples

Blood was sampled from *vena jugularis externa.* During sampling there waseffort of avoiding horse excessive excitation. ALT and glucose was analysed from blood serum spectrophotometrically using XT20i automatic analyser (Thermo Fisher Scientific, Finland). Currently available commercial kits by Biovendor-laboratory medicine were used for analysis.

Statistical evaluation

The data were expressed as means ± SEM. For comparisons Student's t-test was used. Statistica 8.0 statistical software (StatSoft Inc., Tulsa, USA) was used to analyse all data from this experiment. The level of statistical significance was defined as P=0.05. Correlations among the glucose and ALT concentration of the animal´s blood were evaluated by means of the correlation coefficient at the level of probability (P=0.05).

RESULT AND DISCUSSION

Lower glucose and ALT levels were determined in blood of horses easily trained (5.74 ± 0.23 mmol/l and 0.19 ± 0.02 µkat/l) compared with intensive trained horses (7.51 ± 0.22 mmol/l and 0.21 ± 0.02 µkat/l). Based on the statistical analysis, differences between the observed groups were significant with glucose (p = 0.00002), but not significant with ALT (p = 0.50851).

Table 1 glucose level in blood of both groups (mmol/l)

	Average	Min	Max	s_x	V_x
Group A	5.74	5.26	6.22	0.23	4.01
Group B	7.51	7.06	7.98	0.22	2.93

Legend: Group A – free range, easy training; Group B – box stable, intensive training; S_x – standard deviation; Vx – coefficient of variation; Min – minimum value (mmol/l); Max – maximum value (mmol/l)

Table 2 ALT level in blood of both groups (µkat/l)

	Average	Min	Max	s_x	V_x
Group A	0.19	0.17	0.25	0.02	10.53
Group B	0.21	0.17	0.25	0.02	9.52

Legend: Group A – free range, easy training; Group B – box stable, intensive training; ALT – Alanine Aminotransferase; S_x – standard deviation; Vx – coefficient of variation; Min – minimum value (µkat/l); Max – maximum value (µkat/l)

The correlation between Glucose and ALT in the group of easily trained horses was not existent (r = 0.067) as can be seen on Figue 1. Very low correlation was determined in the group of intensive trained horses (r = -0.405) as can be seen on Figure 2.

Hanák and Olehla (2010) claimed, that training of young horses caused glucose increase in blood. This statement agrees with result of this work. Ferraz (2010) also proved, that concentration of glucose was raised with intensive training and higher glucose concentration could be an indicator of performance and readiness for the competition. Malinowski (2004) reported, that higher glucose level may also be an indicator of horse stress. Cortisol, glucocorticoid of the adrenal gland, acts to assist the animal in relieving stress by increasing glucose to provide energy which enables the horse to escape from the stressor.

Results of ALT agree with normal reference ranges for horses as same as concentration of glucose (Cit VFU 2007, Cal Vet 2013, Laboklin 2016, Inlab Medical 2001). Higher ALT levels in blood of intensive trained horses compared with easily trained horses corresponds to the results of the study made by Larson (2013) The possible explanation of this increase would be the greater muscle growth which is related to more intensive cell changes.

In the Gudasheva (2016) study, it was found that jumping exercise induce changes not only in ALT but also increases of biochemical parameters: creatin kinase (CK), aspartate aminotransferase (AST), triacylglycerols, total cholesterol and creatinine within the reference ranges. Gudasheva (2016)

claimed, that changes in these parameters reflects the changes in skeletal muscle and kidney functions as well as alterations in the type of used energy.

Figure 1 The correlation between the concentration of blood glucose and ALT in the group of easily trained horses

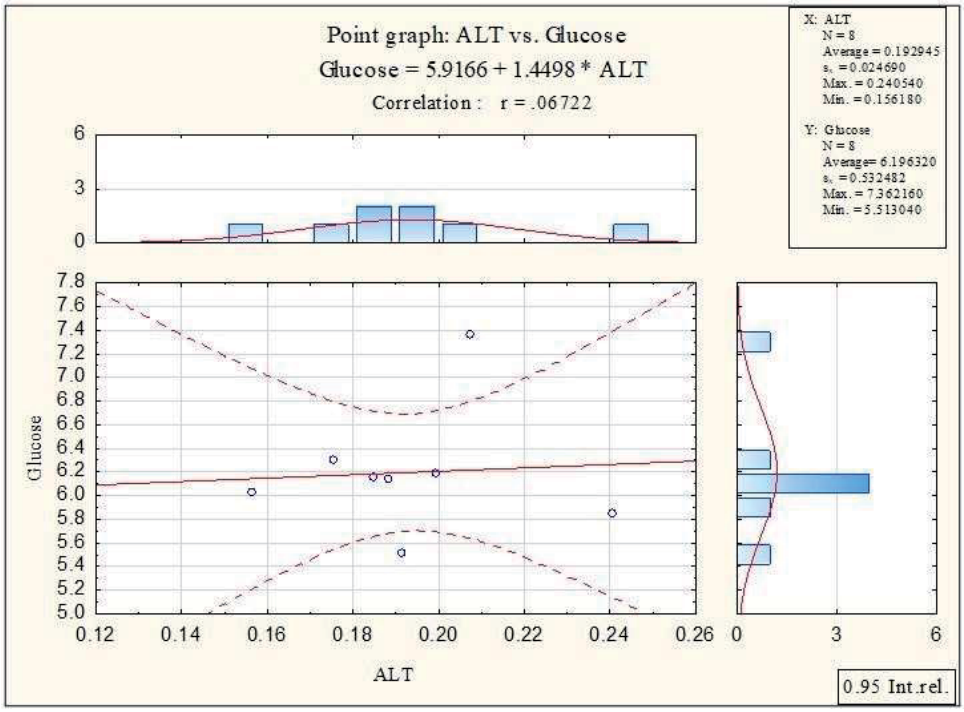
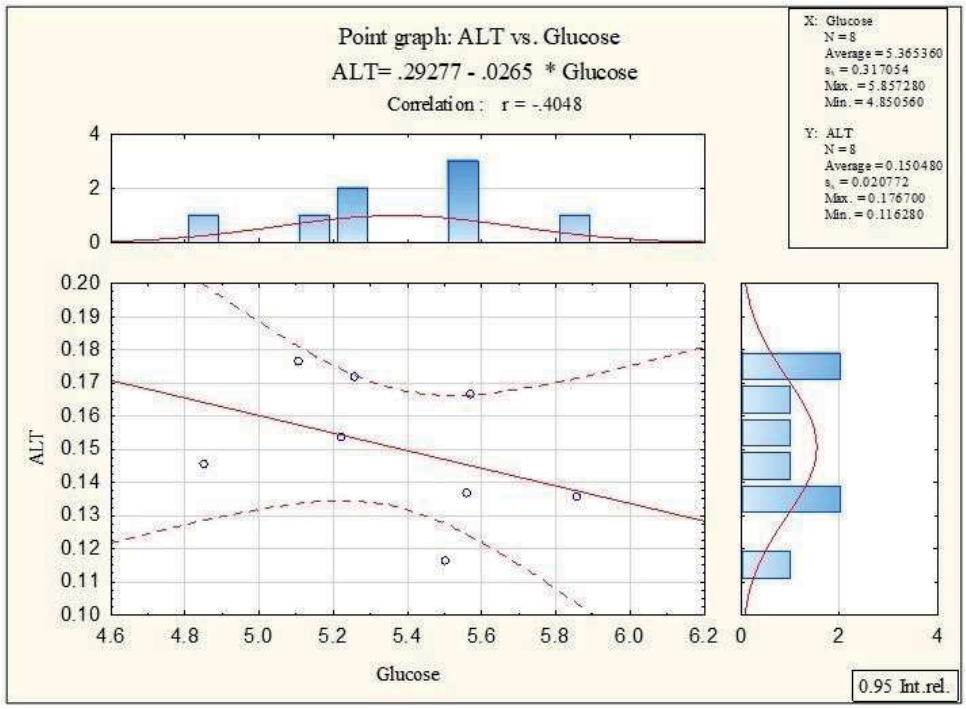


Figure 2 The correlation between the concentration of blood glucose and ALT in the group of intensive trained horses



CONCLUSION

Lower glucose and ALT levels were determined in blood of easily trained horses, than in blood of intensive trained horses. Differences between the observed groups was significant for glucose but

was not significant for ALT. In of intensive trained horses, there was found very low correlation between ALT and glucose (r = -0.405). This correlation was not found in of easily trained horses.

ACKNOWLEDGEMENT
The study was supported by the grant project IGA IP 7/2017.

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THE INFLUENCE OF BREED, SEX AND LITTER SIZE ON THE GROWTH INTENSITY OF LAMBS

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Abstract The purpose of this study was to evaluate the growth intensity on the basis of weight gains and live weight of the Suffolk and Charollais breeds. The experiment also included the assessment of the fat and muscle depth of meat breeds. All the monitored indicators were evaluated depending on the breed, sex and litter frequency. Selected meat breeds were reared in ŠZP Žabčice. The lambs were weighed 4 times and the weight was recalculated to 30, 70, 100 and 200 days of age. Measured parameters of muscle and fat depth were statistically significant ($P < 0.01$) in lambs divided by breed and sex. While control weighing, no statistical differences were found ($P > 0.05$) during evaluation of the intensity of growth using average daily increments and weights. The biggest depth of the muscle had ewe lamb of the Suffolk breed (2.94 cm), followed by the rams of the same breed (2.74 cm). The muscle and fat depth of the studied breeds was significantly ($P < 0.5$) higher for the Suffolk breed than for the Charollais breed.

Key Words: lamb, Suffolk, Charollais, weight, growth

INTRODUCTION

According to Czech Statistical office, 232 000 sheep were bred in 2015. Nevertheless, only 24 118 ewes were in yield control. Of this number, 5716 Suffolk ewes and 436 Charollais ewes were in yield control. Favorable situation has arisen in the sale of animals to abroad. On the other hand, the consumption of lamb meat has been very low in a long-term, 0.4 kg per person a year. The world average consumption of lamb meat has reached 39 kg per year (Horák et al. 2012).

There is a number of factors which influence animal growth. External factors (nutrition, veterinary care, environmental conditions, etc.), as well as internal factors (breed, sex, litter frequency, genotype, etc.). Growth can be assessed by daily gain or by measuring individual body parts. In this study, growth will be evaluated through daily gains. The same issue has been addressed by Gutierrez et al. (2005) and Analla et al. (1998).

MATERIAL AND METHODS

Breeding conditions

Evaluation of growth ability was performed in school farm (SF) of Mendel University in Žabčice. The study included 79 animals of breed Suffolk and Charollais. In the winter, sheep are stabled on deep litter. During summer time, sheep with their lambs are stabled on the pasture, where water and shelter from bad weather are available. The sheep breeds had the same breeding conditions including matter rations.

The yield control (YK) at school farm was conducted by a breeder of the Association of breeders of sheep and goats. Weighing was performed with a precision of 0.1 kg. In framework yield control of meat sheep breeds was done using 100 daily weighing and ultrasonographic measurement of depth of *musculus longissimus lumborum et thoracis* (m.l.l.t.) and depth of fat.

Methodology of experiment

Average weight gains were calculated from the weights measured at following intervals:

- 30–70 days
- 30–100 days
- 30–200 days
- 70–100 days
- 70–200 days
- 100–200 days of age

The model equation for evaluating the weights and average weight gains of lambs:

$Y_{ijkl} = \mu + PV_i + PO_j + PL_k + PL_k \times PO_j + e_{ijk}$

Y_{ijkl} – measured value

μ – overall average

PV_i – effect i–th litter size (singles, twins, triplets)

PO_j – effect j–th sex (ram, ewe lamb)

PL_k – effect k–th breed (Suffolk, Charollais)

e_{ijk} – residue

RESULTS AND DISCUSSION

The growth of lambs was evaluated according to litter size, sex and breed. Very notable statistical differences ($P < 0.01$) between sexes and breeds were recorded while measuring m.l.l.t and fat on 100 days of age. Table 1 and Table 2 shows that the influence of breed, sex and litter frequency on daily gains and weights of lambs was not statistically conclusive ($P > 0.05$). During weighing on 30 days of age the weight of singles was 14.66 kg, individuals amongst twins were 13.9 kg and individuals amongst triplets were 10.76 kg. These results are identical to results of studies conducted by Dobeš (2007). This trend was similar in all other weightings.

The singles had the most notable depth of the m.l.l.t. (2.34 cm) then the twins (2.25 cm) and the lowest was the depth of the muscle of triplets (1.02 cm). Daily gains of rams at intervals 30–70 days, 30–100 days and from 100 to 200 days were higher than daily gains of ewe lambs. Ewe lambs had higher daily gains from 30 to 200 days and from 70 to 100 days. While comparing sexes, there were no significant differences ($P > 0.05$) amongst all recorded weights in concrete intervals. Charollais breed reached the highest daily gains on all control periods. The only exception was period of 70–100 days, where bigger daily gains were achieved by lambs of Suffolk breed. A similar result was also measured by Hošek et al. (2008). These increases were not statistically significant ($P > 0.05$).

The biggest weights of animals on 30, 70 and 100 days had Suffolk breed. The individuals of Charollais breed had bigger weight than individuals of Suffolk breed while weighing on 200 days. The significantly bigger depth of muscle (2.36 cm) and fat (0.37 cm) on 100 days of age was measured for Suffolk. Lambs of Charollais had the depth of muscle 1.43 cm and depth of fat 0.19 cm. The most notable daily gains on 30–100 days had rams of Charollais breed (345.33 g/day) the second one was ewe lambs of Suffolk breed (331.48 g/day) and the third highest daily gains had rams of Suffolk breed (317.13 g/day).

The significant differences ($P < 0.01$) in depth of muscle (m.l.l.t.) and fat were measured between breeds and sexes. The biggest depth of muscle had females of Suffolk breed (2.94 cm) followed by significantly ($P < 0.01$) lagged rams of Suffolk breed (2.74 cm). The rams and ewe lambs of Charollais breed had depth of muscle of 2.67 cm and 2.6 cm. Notable difference ($P < 0.5$) occurred while comparing the depth of fat between breeds. The lambs of Suffolk breed had the depth of fat 0.37 ± 0.18 cm and the lambs of Charollais breed 0.19 ± 0.19 cm. Milerski (2001) reported similar results in his study.

Table 1 The weight of animals and depth of muscle, fat and their statistical significance

Character	Factor	n	Weight in 30 days (kg) $\bar{x} \pm S_x$	Weight in 70 days (kg) $\bar{x} \pm S_x$	Weight in 100 days (kg) $\bar{x} \pm S_x$	Weight in 200 days (kg) $\bar{x} \pm S_x$	Depth of muscle in 100 days (cm) $\bar{x} \pm S_x$	Depth of fat in 100 days (cm) $\bar{x} \pm S_x$
Litter size	Singles	6	14.66 ± 1.68	27.98 ± 3.76	38.30 ± 3.80	57.50 ± 4.90	2.34 ± 1.16	0.33 ± 0.17
	Twins	25	13.90 ± 3.32	26.11 ± 5.25	36.72 ± 6.61	55.46 ± 3.88	2.25 ± 1.19	0.35 ± 0.20
	Triplets	7	10.76 ± 2.15	22.32 ± 4.19	32.49 ± 5.34	46.46	1.02 ± 1.28	0.14 ± 0.17
Gender	Ram	22	13.34 ± 3.62	26.29 ± 5.72	36.26 ± 6.74	54.91 ± 5.75	2.35 ± 1.01	0.35 ± 0.16
	Ewe lambs	16	13.58 ± 2.54	24.90 ± 4.05	36.10 ± 5.58	56.56 ± 4.60	1.61 ± 1.49	0.25 ± 0.24
Breed	Suffolk	25	13.87 ± 3.28	26.00 ± 4.99	36.51 ± 5.80	54.66 ± 4.47	2.36^{a} ± 1.11	0.37^{a} ± 0.18
	Charollais	13	12.62 ± 2.90	25.13 ± 5.38	35.58 ± 7.12	59.03 ± 9.72	1.43^{B} ± 1.39	0.19^{b} ± 0.19
Breed *Sex	SF* Ram	14	13.84 ± 3.89	26.09 ± 5.80	36.04 ± 6.09	53.87 ± 4.37	2.74^{A} ± 0.39	0.41^{A} ± 0.86
	SF* Ewe l.	11	13.91 ± 2.48	25.90 ± 4.00	37.11 ± 5.65	56.56 ± 4.60	2.94^{B} ± 0.4	0.48^{A} ± 1.03
	CH* Ram	8	12.46 ± 3.13	26.65 ± 5.96	36.64 ± 8.21	59.03 ± 9.72	2.67^{A} ± 0.31	0.37^{B} ± 0.97
	CH* Ewe l.	5	12.88 ± 2.81	22.69 ± 3.55	33.88 ± 5.31	–	2.60^{A} ± 0.16	0.32^{B} ± 0.42

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 daily gains and their statistical significant

Character	Factor	n	Daily gains 30–70 (g/day) $\bar{x} \pm s_x$	Daily gains 30–100 (g/day) $\bar{x} \pm s_x$	Daily gains 30–200 (g/day) $\bar{x} \pm s_x$	Daily gains 70–100 (g/day) $\bar{x} \pm s_x$	Daily gains 70–200 (g/day) $\bar{x} \pm s_x$	Daily gains 100–200 (g/day) $\bar{x} \pm s_x$
Litter size	Singles	6	332.99 ± 81.73	337.70 ± 60.38	252.47 ± 37.90	343.98 ± 81.78	218.70 ± 38.49	189.38 ± 15.48
	Twins	25	305.07 ± 79.00	326.00 ± 78.24	239.17 ± 29.27	353.91 ± 138.84	211.39 ± 29.84	161.81 ± 37.73
	Triplets	7	288.95 ± 57.77	310.39 ± 50.02	213.15	338.99 ± 51.15	187.67	145.56
Gender	Ram	22	323.71 ± 76.98	327.38 ± 73.53	237.95 ± 30.57	332.28 ± 132.37	210.87 ± 32,01	168.72 ± 26.15
	Ewe lambs	16	282.86 ± 68.18	321.66 ± 67.72	245.60 ± 30.10	373.40 ± 92,66	212.59 ± 25.92	154.36 ± 58.76
Breed	Suffolk	25	303.35 ± 63.47	323.45 ± 60.28	234.99 ± 21.24	350.24 ± 83.84	207.81 ± 23.73	163.81 ± 38.47
	Charollais	13	312.59 ± 96.71	327.91 ± 89.10	267.49 ± 59.21	348.34 ± 169.43	231.05 ± 57.68	172..64 ± 11.7
Breed *Sex	SF* Ram	14	306.08 ± 58.88	317.13 ± 46.50	230.57 ± 15.95	331.86 ± 58.69	205.82 ± 23.67	167.74 ± 28.98
	SF* Ewe l.	11	299.87 ± 71.68	331.48 ± 76.03	245.60 ± 30.10	373.63 ± 106.39	212.59 ± 25.92	154.36 ± 58.76
	CH* Ram	8	354.57 ± 98.13	345.33 ± 107.82	267.49 ± 59.21	333.01 ± 214.86	231.05 ± 57.68	172.64 ± 11.70
	CH* Ewe l.	5	245.42 ± 45.17	300.05 ± 43.53	–	3702.89 ± 64.08	–	–

CONCLUSION

The purpose of this thesis was to evaluate the influence of breed, gender and litter size on the intensity of growth. The depth of *musculus longissimus lumborum et thoracis* and fat was measured as well. The observation did not provide proof of significant influence ($P > 0.05$) of the monitored breeds on the intensity of growth but notable differences ($P < 0.05$) were detected when measuring depth of muscle (m.l.l.t.) and fat between the breeds. The Suffolk had bigger depth of muscle and fat than Charollais.

The influence of gender on the growth ability was not significantly demonstrated. The depth of muscle was notably ($P < 0.01$) the most significant amongst rams of Suffolk breed. Notable difference was found between sexes within the breeds, measuring the depth of fat. The difference in weights and weight gains amongst the lambs from different litter of size was not demonstrated ($P > 0.05$).

ACKNOWLEDGEMENTS

This study was supported by the project No. TP7/2017 of MENDELU, Faculty of AgriSciences Internal Grant Agency.

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THE EFFECT OF GENOTYPE AND PASTURE ON CHICKENS PERFORMANCE AND DIGESTIVE TRACT DEVELOPMENT

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Abstract: The aim of the study was to estimate the pasture intake in different genotypes and to evaluate the effect of genotype on carcass quality and digestive tract development. Three different genotypes were involved in the experiment; fast growing Ross 308 both sexes, slower growing JA 757 both sexes and layer-type cockerels ISA Dual, fifty chickens of each genotype. From ten days of age all hybrids were fed on fresh pasture in separate feeders to accustom this feed. Since day eighteen in Ross 308 and JA 757 and since day twenty-seven in ISA Dual daily pasture intake was observed. The experiment was finished when pasture intake plateau was achieved in each hybrid. Consequently, chickens were slaughtered at the age 49 in Ross 308, 64 in JA 757 and 90 in ISA Dual. The maximal daily pasture intake was significantly the highest in ISA Dual cockerels 25.8g in comparison with JA 757 and Ross 308 (16.1, 13.1 g/d respectively, $P < 0.05$). Both length of intestine and caeca were shorter in ISA Dual in comparison with JA 757 and Ross 308 ($P < 0.05$). The lowest weight of gizzard was found in Ross 308. Carcass quality was the best in Ross 308 including the lowest proportion and weight of abdominal fat ($P < 0.05$). Intake of pasture improve feed conversion ratio (Ross 308 1.50, JA 757 1.86, ISA Dual 2.49).

Key Words: ISA Dual, Ross 308, JA 757, carcass quality, length of gut

INTRODUCTION

In the European Union the number of organic and free range chicken production has been increased, between years 2012 and 2016 it was about 43.3% (Eurostat 2017). According to the legislation (Commission Regulation (EC) No 889/2008) access to free range with grassland is one of the basic requirements for organic or free range production. In USA the use of mobile processing units for pasture poultry is growing rapidly too (Angioloni et al. 2016).

According to some authors intake of plant particles can be regarded as a valuable feed supplement (Ponte et al. 2008a). Lorenz et al. (2013) estimated that green fodder could cover about 10–15% of total daily dry matter intake in broilers. Similarly, Fanatico (1998) stated keeping broilers at pasture, and then, allowing the birds to forage on plants, seeds, insects and worms, can reduce the costs of feed by 30%.

The intake of pasture can be affected by many factors: genotype, age, technology, pasture quality, season or soil quality (Hörning et al. 2002, Horsted et al. 2007, Dal Bosco et al. 2014). According to Skřivan (2015) fast growing chickens are also suitable for grazing mainly because of meat enrichment by unsaturated fatty acids and vitamin E. On the other hand due to high growth intensity and breeding goals broilers have lower movement activity (Skřivan 2015). Ponte et al. (2008b) recommend on the basis of detected etiological observations, movement activity and adaptability for free range chicken meat production mainly slow growing genotypes or even layer-type cockerels.

The aim of the study was to estimate the pasture intake in different genotypes and evaluated the effect of genotype on carcass quality and digestive tract development. The hypothesis was that depending on the birds age the pasture intake has increased, therefore prolonged fattening period was used.

MATERIAL AND METHODS

The experiment took place in the building M of FA MENDELU in Brno. Three different hybrids were used; fast growing Ross 308 both sexes, slower growing JA 757 both sexes and layer-type cockerels ISA Dual, fifty chickens of each genotype. All chickens were in the same room, in twelve boxes with litter, nipple drinkers and pan feeders, four replications for each hybrid. Access to feed and water was at libitum. Light day was 18h. Three feed mixtures were used during the experiment BR1 (crude protein 19.3%, crude fat 5.3%, crude fibre 3.3%), BR2 (crude protein 19.0%, crude fat 5.3%, crude fibre 3.5%) and BR3 (crude protein 17.7%, crude fat 6.0%, crude fibre 4.0%).

From ten days of age all hybrids were fed on fresh pasture in separate feeders to accustom this feed. Since day eighteen in Ross 308 and JA 757 daily pasture intake was observed. Due to very low intake of pasture in ISA Dual the beginning of measurement started at age twenty-seven days. The average pasture intake was evaluated on weekly basis. When there was no significant increase in amount of pasture intake between consecutive weeks, recognized as maximum at libitum intake, the experiment in the hybrid was ended. Consequently, chickens were slaughtered at the age 49 in Ross 308, 64 in JA 757 and 90 in ISA Dual. Ross 308 fed BR1 till 9d, BR2 till 42d and BR3 till 49d of age. JA 757 fed BR1 till 22d, BR2 till 56d and BR3 till 64d of age. ISA Dual fed BR1 till 35d, BR2 till 83d and BR3 till 90d of age.

The composition of pasture was as follow: 50% of genus *Trifolium* with majority of *Trifolium repens* L., 40% of genus *Poaceae* with majority of *Lolium perenne* L. and 10% of herbs namely *Bellis perennis* L. and *Ajuga reptans* L. Fresh pasture was weighted and supplied to the chickens in all boxes each morning in sufficient quantity and after 12h the residues were weighted. Daily feed pasture intake was calculated. At the end of experiment ten males and ten females in Ross 308 and JA 757 and ten cockerels of ISA Dual were slaughtered. Yields of carcass, breast meat without skin, legs with bones and skin and abdominal fat were evaluated. Digestive tract development was assessed based on weight of empty gizzard including cuticle and length of intestine and caeca.

The effect of hybrids on observed characteristics was analysed using one way ANOVA and LSD-test using the software package Unistat 5.1 (Unistat Ltd, England).

RESULTS AND DISCUSSION

The pasture intake and characteristics of genotypes performance including carcass quality are shown in Table 1. Both the average and maximal pasture intakes were significantly the highest in ISA Dual ($P < 0.05$). Regardless prolonged feeding of BR1 and prolonged fattening period in ISA Dual till 90 days of age and in JA 757 till 64 days of age the live body weight was significantly the highest in Ross 308 ($P < 0.05$). There was significant difference in carcass weight in ISA Dual in comparison with JA 757 and Ross 308 ($P < 0.05$). Significant difference in carcass weight between Ross 308 and Lohmann Dual also observed Habig et al. (2016).

FCR was the worst in ISA Dual ($P < 0.05$), however the FCR are interestingly low, which confirm hypothesis of Fanatico (1998) that keeping broilers at pasture can reduce the costs of feed by 30%. Although there was no significant difference in carcass weight between JA 757 and Ross 308, the carcass yield was the highest in Ross 308 ($P < 0.05$). Both weight and yield of abdominal fat was the highest in ISA Dual ($P < 0.05$). High yield of breast meat is one of the breeding aim in fast growing chickens, consequently breast weight as well as and yield were the highest in Ross 308 ($P < 0.05$). On the other hand leg yield was the highest in ISA Dual ($P < 0.05$), however the weight of legs was the lowest in this genotype ($P < 0.05$).

Table 2 shows the digestive tract development. Despite the lower growth intensity in ISA Dual, there was no significant difference in gizzard weight between ISA Dual and Ross 308 and the proportion of gizzard from carcass weight was even significantly higher in ISA Dual ($P < 0.05$). On the other hand Ross 308 and JA 757 had significantly longer both intestine and caeca in comparison with ISA Dual ($P < 0.05$). Kokoszynski et al. (2017) also reported the highest length of intestine in Ross 308 in comparison with hybrids Hubbard Flex or Hubbard F15.

Table 1 Characteristics of hybrids performance and carcass quality

Hybrid	ISA Dual	JA 757	ROSS 308
Age at slaughter (days)	90	64	49
	Mean ± SE*	Mean ± SE*	Mean ± SE*
Daily pasture intake [g/day]	16.60 ± 0.39^{b}	10.20 ± 0.28^{a}	10.00 ± 0.29^{a}
Maximal pasture intake [g/day]	25.84 ± 1.96^{e}	16.11 ± 1.26^{d}	13.13 ± 0.99^{c}
Live body weight [kg]	2.62 ± 0.03^{a}	3.35 ± 0.06^{b}	3.18 ± 0.06^{c}
Feed conversion ratio	2.49 ± 0.14^{c}	1.86 ± 0.05^{b}	1.50 ± 0.04^{a}
Carcass weight [kg]	1.76 ± 0.01^{a}	2.41 ± 0.06^{b}	2.38 ± 0.07^{b}
Carcass yield [%]	66.30 ± 1.18^{a}	72.00 ± 0.99^{b}	74.30 ± 0.42^{c}
Breast weight [g]	296.20 ± 5.30^{a}	601.80 ± 15.77^{b}	758.80 ± 18.86^{c}
Leg weight [g]	604.20 ± 8.44^{a}	743.40 ± 22.55^{b}	689.00 ± 23.90^{b}
Abdominal fat weight [g]	92.40 ± 8.29^{b}	89.90 ± 6.74^{b}	40.50 ± 3.82^{a}
Breast yield [%]	16.82 ± 0.30^{a}	25.03 ± 0.50^{b}	31.92 ± 0.35^{c}
Leg yield [%]	34.30 ± 0.42^{c}	30.79 ± 0.40^{b}	28.82 ± 0.32^{a}
Abdominal fat yield [%]	5.23 ± 0.45^{c}	3.75 ± 0.28^{b}	1.70 ± 0.16^{a}

Legend: SE – standard error; a, b – means of the same order designated by different letters are significantly different (P < 0.05)*

Table 2 Characteristics of digestive tract development

Hybrid	ISA Dual	JA 757	ROSS 308
Age at slaughter (days)	90	64	49
	Mean ± SE*	Mean ± SE*	Mean ± SE*
Length of intestine [cm]	160.00 ± 2.24^{a}	181.40 ± 4.63^{b}	184.80 ± 3.76^{b}
Length of intestine [cm/100g carcass]	9.08 ± 0.11^{b}	7.59 ± 0.22^{a}	7.81 ± 0.14^{a}
Length of caeca [cm]	16.90 ± 0.26^{a}	19.40 ± 0.27^{b}	19.70 ± 0.23^{b}
Gizzard weight [g]	34.00 ± 1.55^{ab}	34.70 ± 1.39^{b}	30.00 ± 1.09^{a}
Gizzard yield [%]	1.93 ± 0.09^{c}	1.45 ± 0.06^{b}	1.28 ± 0.05^{a}

Legend: SE – standard error; a, b – means of the same order designated by different letters are significantly different (P < 0.05)*

CONCLUSION

The maximal daily mean pasture intake was significantly the highest in ISA Dual cockerels 25.8 g in comparison with JA 757 and Ross 308 (16.1, 13.1 g/d respectively, $P < 0.05$). Both length of intestine and caeca were shorter in ISA Dual in comparison with JA 757 and Ross 308 ($P < 0.05$). The lowest weight of gizzard was found in Ross 308. Carcass quality was the best in Ross 308 including the lowest proportion and weight of abdominal fat ($P < 0.05$). Intake of pasture improve feed conversion ratio (Ross 308 1.50, JA 757 1.86, ISA Dual 2.49).

ACKNOWLEDGEMENT

The authors would like to thank the project IGA FA IP 2017/073 for financial support and Botanical garden and Arboretum of MENDELU for pasture supply.

The experiment was done thanks equipment financed by project OP VaVpI CZ.1.05/4.1.00/04.0135.

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INCIDENCE OF PATHOLOGICAL CHANGES OF SPERMATOZOA DEPENDING ON THE AGE OF BOARS

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Abstract: The aim of this study was to assess the incidence of pathological changes of spermatozoa in breeding boars with special focus on character of changes during the activity of boars in insemination centre. A total of 1200 semen samples from 150 boars aged 9–36 months were analysed. The experiment was carried out from January to April 2017. Abnormalities of spermatozoa were assessed microscopically on stained native semen smears on glass slides. The percentage of spermatozoa with pathological defects differed among age categories. Total proportion of spermatozoa with morphological defects decreased with age. The highest proportion of spermatozoa with abnormal size and shape of head, proximal droplet and folded tail was recorded in younger boars (bellow 18 months of age). More frequent defects of acrosome were observed in the semen of boars older than 25 months. Also positive and negative correlations were found among individual groups of morphological abnormalities.

Key words: boar, ejaculate, age of boar, spermatozoa, defects of spermatozoa

INTRODUCTION

One of the basic factors, that determine profitability of animal production, is fertility of sows and boars. Numerous and balanced litters, as same as numbers of weaned piglets significantly influence efficiency of pig production (Gadea 2005). Exploitation of potential fertility of sows depends to a large extent on boars (Tsakmakidis et al. 2010). Fertilization is a result of many mutually determined events occurring in strict order and a normal course of each phase depends on full maturity of gametes. Besides high level of sexual activity, breeding boars should also reach production of large amounts of high quality semen (Frydrychova et al. 2011). Morphological evaluation of semen in terms of changes in spermatozoa which occur in boars with age, can be useful for prediction of fertility in boars (Lipensky et al. 2014). Morphologically abnormal spermatozoa can decrease fertilization rate and embryonic development.

Regarding the fact that spermatogenesis in boars lasts nearly 45 days, which are followed by approximately 10–12 days of maturation, most of the abnormalities in spermatozoa arise during this period (Vogler et al. 1991). In this respect, the morphology of spermatozoa reflects the health of seminal tubules and to a lesser extent also of epididymis. Abnormalities of sperm morphology are traditionally classified according to localization of defect (head, midpiece, tail) or the place where the defect originated (primary: testes, secondary: epididymis, tertiary: accessory glands/after ejaculation).

The aim of this study was to assess the extent of changes in incidence of pathological spermatozoa in breeding boars of different age categories.

MATERIAL AND METHODS

The experimental population consisted of Duroc boars from insemination centre Velké Meziříčí (Czech Republic). The boars were subjected to analysis of sperm morphological changes from January to April 2017. Morphological evaluation of spermatozoa was performed in 150 randomly selected boars, 30 individuals of each age category. All the selected boars were in good health, without visible malformations and showed a normal level of libido. Semen collection was realized manually in a sampling room once per week in all the categories (King and Macpherson 1973). Twice per month,

semen smears from native ejaculates were made for sperm morphology evaluation. The dried smears were stained according to Cerovsky (1976), in saturated solution of Congo red and 0.5% aqueous solution of crystal violet. Morphological examination was performed on light microscope with an objective for oil immersion at 1500 × magnification. The results of morphological evaluation were recorded using DeSMA software. For each spermiogram, a total of 500 sperm cells were evaluated, of which percentage share with pathological defects was calculated subsequently. The defects of spermatozoa were divided as follows: defects in head size (macrocephaly, microcephaly), defects in head shape (tapered, pyriform, amorphous, round, pin, crest, malformed basis), free heads, acrosomal defects (condensation, vacuolization, released acrosome, no acrosome), abnormal tail insertion (abaxial, paraxial, retroaxial), proximal cytoplasmic droplet, bent tail, folded tail, primary coiled tail (dag effect) and other abnormalities (double head, diadem defect, persistent acroblast, broken tail, duplicate tail, agenesis).

The data were divided into 5 categories according to the age of boars (8–12, 13–18, 19–24, 25–30 and 31–36 months). Overall number of the analysed ejaculates was 1200; 240 ejaculates from each age category.

The recorded data were processed and statistically evaluated using STATISTICA CZ software, version 12.0. Statistical differences among categories were determined by t-test and correlations by Spearman's coefficient.

RESULTS

The incidence of pathological defects in spermatozoa depended on the age of boars. Increasing and decreasing trends were observed for individual morphological changes with increasing age and in some cases the proportions of abnormalities stayed without statistically significant changes for the whole time of observation.

Table 1 and Figure 1 document that overall percentage of pathological spermatozoa decreased significantly with age of boars, from 11.15% in boars < 12 months of age to 8.58% in boars between 31–36 months of age ($p < 0.01$). Proportion of spermatozoa with defects in head size ranged from 0.72% to 0.65% till the age of 18 months, in boars between 19 and 24 months it decreased to 0.27% ($p < 0.01$). From the 25th month of age the number of these abnormalities grew again up to the level of 0.51%, nevertheless this increase was not statistically significant. The highest proportion of spermatozoa with head shape defects was also found in the youngest boars, it was 1.14% in the age category bellow 12 months and 1.08% in the age category from 13 to 18 months. The proportion decreased to 0.64% between 19 and 24 months ($p < 0.01$) and there were no significant changes in this proportion in later age categories. The percentage of free heads in semen of boars before the 30th month of age ranged from 0.17% to 0.31%, in boars from 31 to 36 months it increased significantly to 0.51% ($p < 0.01$). The acrosomal defects showed an increasing trend. Before the age of 24 months the proportion of spermatozoa with problematic acrosome was 0.48–0.63% and then it decreased from 0.78% in the category 25–30 months ($p < 0.05$) to 1.01% in the category 31–36 months ($p < 0.01$). There was a highly statistically significant decrease of proportions of spermatozoa with proximal cytoplasmic droplet with increasing age. The semen of boars younger than 12 months contained 5.47% of sperm cells with proximal droplet. In boars from 13 to 18 months the proportion decreased by 1%, to 4.47% ($p < 0.01$). The value decreased again to 3.76% in boars from 19 to 24 months ($p < 0.01$) and in the next age category (from 25 to 30 months) the value decreased only insignificantly, to 3.41%. The lowest percentage of spermatozoa with proximal droplet ($p < 0.01$) was recorded in boars between 31 and 36 months of age (2.84%). The incidence of spermatozoa with bent tail in boars younger than 12 months reached 1.31%, then it decreased to 1.93% in the age category 13–18 months, however this increase was not statistically significant ($p > 0.05$). At the age of 19–24 months it increased significantly again to the level of 3.76% ($p < 0.01$). The proportion of sperm cells with bent tail decreased gradually again to the level of 1.88% in the age category 31–36 months ($p < 0.01$). The highest percentage of spermatozoa with folded tail was found in younger boars, in the category bellow 12 months it was 0.97% and in the category between 13–18 months it was 0.73%. It decreased to 0.31% later, in boars between 19–24 months ($p < 0.01$). There were no statistically significant changes in boars older than 24 months, their semen contained 0.29–0.31% of sperm cells with folded tail. On

the contrary, the youngest age category showed the lowest proportion of spermatozoa with coiled tail, the value was 0.27% and it increased gradually up to the level of 0.78% in boars between 19–24 months of age ($p < 0.01$). Subsequently the percentage of sperm cells with this defect decreased from 0.54% in the age category between 25–30 months to 0.40% in the age category between 31–36 months ($p < 0.01$). There were no statistically significant differences observed in the case of spermatozoa with abnormal insertion of tail. This defect occurred in 0.12% (age 19–24 months) to 0.37% (age 25–30 months) of sperm cells. Similarly, no increasing or decreasing trend was found for other defects, their proportions varied from 0.20% (age 31–36 months) to 0.32% (age < 12 months).

Table 1 Morphological changes in spermatozoa of boars from observed age categories

Defects (average ± SE*)	**Age of boars (months)**				
	< 12	**13–18**	**19–24**	**25–30**	**31–36**
n	**30**	**30**	**30**	**30**	**30**
Total proportion of pathological sperm cells	11.15 ± 0.35^{C}	10.88 ± 0.24^{BC}	10.79 ± 0.28^{B}	9.89 ± 0.31^{AB}	8.58 ± 0.30^{A}
Defects in head size	0.72 ± 0.09^{B}	0.65 ± 0.08^{B}	0.27 ± 0.03^{A}	0.45 ± 0.08^{AB}	0.51 ± 0.06^{AB}
Defects in head shape	1.14 ± 0.18^{B}	1.08 ± 0.11^{B}	0.64 ± 0.09^{A}	0.82 ± 0.10^{A}	0.69 ± 0.07^{A}
Free heads	0.31 ± 0.05^{A}	0.27 ± 0.04^{A}	0.17 ± 0.04^{A}	0.25 ± 0.01^{A}	0.51 ± 0.05^{B}
Acrosome defects	0.48 ± 0.09^{A}	0.59 ± 0.06^{A}	0.63 ± 0.11^{Aa}	0.78 ± 0.09^{bC}	1.01 ± 0.12^{D}
Abnormal insertion of tail	0.16 ± 0.04	0.21 ± 0.03	0.12 ± 0.03	0.37 ± 0.05	0.22 ± 0.04
Proximal droplet	5.47 ± 0.32^{D}	4.47 ± 0.28^{C}	3.76 ± 0.25^{B}	3.41 ± 0.33^{B}	2.84 ± 0.19^{A}
Bent tail	1.31 ± 0.18^{A}	1.93 ± 0.21^{A}	3.85 ± 0.31^{C}	2.74 ± 0.20^{B}	1.88 ± 0.19^{A}
Folded tail	0.97 ± 0.09^{B}	0.73 ± 0.05^{B}	0.31 ± 0.07^{A}	0.29 ± 0.07^{A}	0.32 ± 0.06^{A}
Coiled tail	0.27 ± 0.03^{A}	0.47 ± 0.08^{AB}	0.78 ± 0.12^{B}	0.54 ± 0.14^{AB}	0.40 ± 0.08^{A}
Other abnormalities	0.32 ± 0.03	0.21 ± 0.04	0.26 ± 0.06	0.24 ± 0.05	0.20 ± 0.07

Legend: SE– standard error; A,B,C,D – values with different superscripts within a row are highly significantly different ($p < 0.01$); a,b, – values with different superscripts within a row are significantly different ($p < 0.05$)*

Table 2 presents correlation coefficients among the observed morphological abnormalities. High, moderate, weak and very weak correlations, both positive and negative, were found between the morphological changes. A high positive correlation was found between the defects of head size and head shape (0.89) and between the defects of head size and folded tail (0.84), between the defects of head shape and folded tail (0.73), between the folded and coiled tail (0.89), between the proximal cytoplasmic droplet and other defects (0.73), between the proximal droplet and total proportion of pathological spermatozoa (0.85) and between other defects and the total proportion of pathological spermatozoa (0.72) ($p < 0.01$). A high negative correlation was recorded between the defects of head size and the folded (-0.86) and coiled (-0.88) tail ($p < 0.01$). A negative correlation was observed also between the proportion of free heads and the folded (-0.84) and coiled tail (-0.81) ($p < 0.01$). The defects of acrosome were in a negative correlation with the defects of head shape (-0.71), the proximal

droplet (-0.82) and the total proportion of pathological spermatozoa (-0.85) ($p < 0.01$). The folded tail was in a high negative correlation with the bent (-0.74) and coiled tail (-0.76) ($p < 0.01$).

Figure 1 Morphological changes in spermatozoa of boars from observed age categories

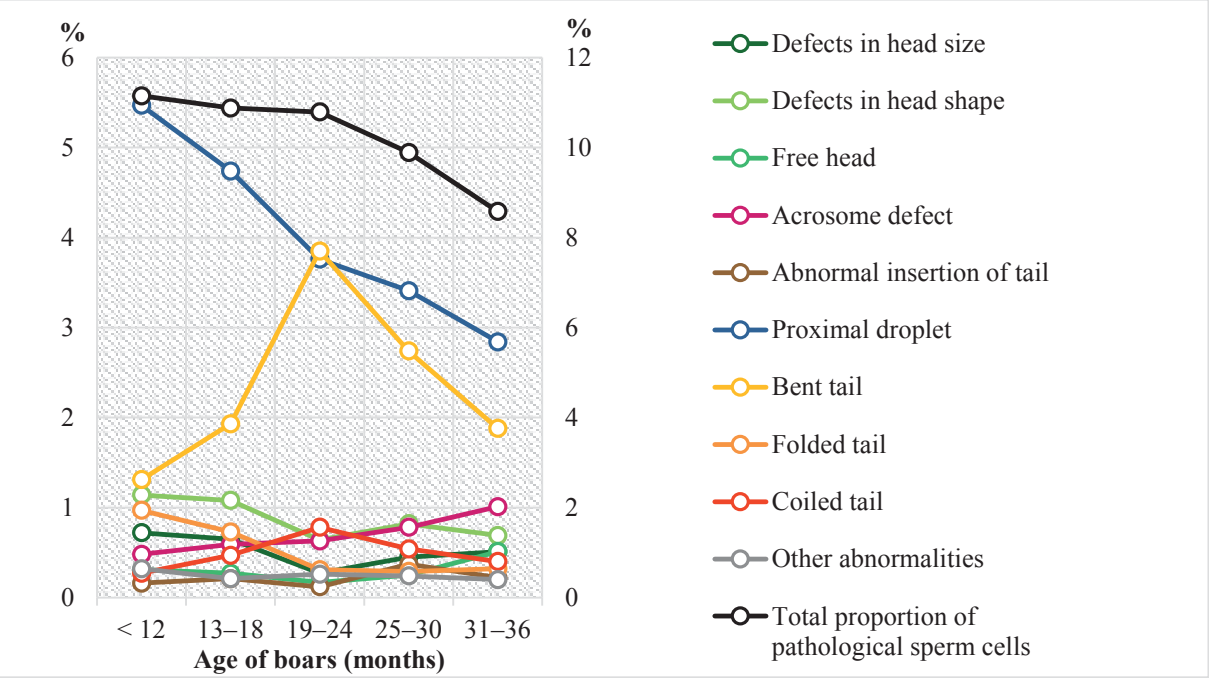

Table 2 Correlation coefficients between observed morphological abnormalities of spermatozoa

	Defects									
	DHSh	FH	AD	AIT	PD	BT	FT	CT	OA	A
DHSi	0.89**	0.68**	-0.60**	0.03	0.61**	-0.86**	0.84**	-0.88**	0.09*	0.55**
DHSh		0.37*	-0.71**	0.08	0.65**	-0.63*	0.73**	-0.65**	0.29*	0.66**
FH			0.11	0.29	0.10	-0.84**	0.54**	-0.81**	0.36*	-0.09
AD				0.57**	-0.82**	0.27*	-0.64**	0.31*	-0.68**	-0.85**
AIT					-0.55**	-0.08**	-0.37*	-0.12	-0.57**	-0.56**
PD						-0.31*	0.68**	-0.28*	0.73**	0.85**
BT							-0.74**	0.89**	0.13	-0.25*
FT								-0.76**	0.20*	0.66**
CT									0.08	-0.29**
OA										0.72**

*Legend: DHS – defects in head size, DHSh – defects in head shape; FH – free heads, AD – acrosome defects, AIT – abnormal insertion of tail, PD – proximal droplet, BT – bent tail, FT – folded tail, CT – coiled tail, OA – other abnormalities; ** – correlations are statistically highly significant, with $p < 0.01$; * – correlations are statistically significant, with $p < 0.05$.*

DISCUSSION

The results of the present study indicate that the incidence of morphological abnormalities in spermatozoa depends on the age of boars. In insemination, most boars produce semen with values in normal range, but in some cases the fertility of boars is decreased without an evident reason. A total proportion of spermatozoa with morphological defects in boars at the age of 8 to 36 month was within the standard range accepted for boars utilized in breeding. The same findings were described by Wierzbowski (1999) and Strzezek (2002). Based on the results of this study it can be stated that the semen obtained from younger boars can contain higher proportion of sperm cells with morphological

defects than the semen of older boars. These conclusions correspond to the findings of Bazaszewska et al. (2014) and Stostak and Burys (2011). The question arises whether this is related to sperm concentration, because younger boars reach higher values of sperm concentration than older boars (Kamanova et al. 2016) and according to Bazaszewska et al. (2014) the sperm cells have less favourable conditions for development after the release from seminal tubules in semen with high sperm concentrations. Semen of young boars also contained high proportions of sperm cells with proximal droplet. During the passage of sperm cells to epididymis and their maturation, the droplet normally shakes and flows away. High frequency of sperm cells with proximal droplet in semen can indicate a spermatogenesis disorder (Johnson 1997). The sperm cells with droplets are essentially not matured (Lewis 2014) and a high proportion of spermatozoa with this defect leads to fertility disorders. Some authors suggest that impaired maturity of spermatozoa is associated to anomalies of head shape (Huszar et al. 1994). Our research revealed a moderate correlation between the incidence of proximal droplets and the defects of head shape. Stostak and Burys (2011) state that semen of young boars is characterized by lower concentration of free heads when compared to older boars (25–36 month of age). The present study documented that this defects is most problematic in the boars between 31–36 months of age. According to Blom (1981), abnormalities of head size and shape and midpiece defects, proximal cytoplasmic droplet and individual pathological changes present in high proportion affect fertility more than defects of tail.

CONCLUSION

Thorough detection of morphological defects and elimination of their causes are necessary prerequisites for obtaining semen of a high quality with high fertilizing ability. The results of morphological evaluation of semen in this study suggest that the incidence of pathological spermatozoa changes with the age of boars. Also the character of defects changes. The semen obtained from younger boars contains higher proportion of pathological spermatozoa than the semen of older boars (older than 19 months). The sperm cells of younger boars are characterized by higher incidence of the defects in head size and shape and the proximal cytoplasmic droplet. On the contrary, the boars older than 25 months are more prone to the acrosomal defects. In conclusion, the percentage of pathological spermatozoa in ejaculate decreases with increasing age of boars.

ACKNOWLEDGEMENTS

This study was supported by the project of MENDELU Internal Grant Agency, Faculty of AgriSciences No. TP 7/2017.

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EFFECTS OF MONENSIN ON THE COPPER, ZINC AND IODINE CONTENTS IN MILK OF DAIRY COWS

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Abstract: The effect of monensin (intraruminal bolus, 32.4 g) on the copper (Cu), zinc (Zn) and iodine (I) contents in milk of Holstein dairy cows (10,200 liters per lactation) was observed during three experiments (E group: monensin, n = 8, C group: n = 8). Milk was examined twice between the 2nd and 8th week of lactation. The positive effect of monensin resulted in a lower level of beta-hydroxybutyric acid in milk and the higher Cu contents (15.8 % to 28.2 % higher) and Zn (1.8% to 14.4 % higher) in milk. No difference was observed in the iodine (I) content in milk.

Key Words: copper, zinc, iodine, milk, ketosis

INTRODUCTION

Ketosis ranks among the frequent production diseases in the ascending phase of lactation. This disease is associated with the deposit fat reduction in relation to energy deficiency and increased ketogenesis (Duffield 2000 and Litherland et al. 2011). A subclinical form of the disease is manifested by an increase in ketone bodies in body fluids, by a decline in milk production, an increase in a milk fat content, and by a decline in the non-fat dry matter (Suthar et al. 2013). The data on changes in minerals in milk of cows with ketosis is not very common. The statistically insignificant decline was stated, for example, by King (1979). Changes in the content of trace elements in connection with ketosis are measured in blood serum. As an example, the lower copper (Cu), cobalt (Co) and manganum (Mn) contents in blood serum of cows with clinical ketosis were discovered by Kaya et al. (2016). The statistically significant lower zinc (Zn) content in blood serum in dairy cows with subclinical ketosis was established by Zhang et al. (2010). Trace elements are characterized by unequal ability of transition from feed to milk. For example, the Cu content in milk does not correlate with the content in a feed ration (Kinal et al. 2007). On the other hand, the iodine (I) content correlates positively with its intake (Trávníček et al. 2010).

As for the efficiency aspect of anti-ketogenetic profylaxation, monensin ionophore in the form of intraruminal boluses is the most commonly used (Šlosárková et al. 2015). Monensin positively affects fermentation in the rumen to produce propionic acid (Duffield et al. 2008).

The aim of the study was to evaluate the submitted Zn, Cu and I contents in milk of dairy cows with subclinical ketosis and cows treated with ionophore monensis.

MATERIAL AND METHODS

The intraruminal bolus containing monensin (32.4 g) was validated during 3 experiments using dairy cows (Holstein-Friesian breed) with an average production of 10,200 litres of milk during lactation. Both the experimental group (E) (n = 8, monensin was applied 3 weeks before parturition)

and control group (C) (n = 8) were used during each experiment. Each group composed of 4 dairy cows for the 1st lactation and 4 dairy cows for the 2nd to 5th lactation was examined twice between the 2nd and 8th week after parturition. The milk was examined twice during the experiments: between the 2nd and 4th week of lactation and between the 5th and 8th week of lactation. The feed ration was identical in the control and experimental groups. The contents of trace elements per 1 kg of dry matter of fodders were: copper (Cu) 12.2 mg, zinc (Zn) 63.0 mg and iodine (I) 1.3 mg. The presence of beta-hydroxybutyric acid in milk (BHB) was determined by the infra-red spectroscopy (FT-MIR) method. The atomic absorption spectrophotometry (AAS) method was applied to determine the content of Zn and Cu in milk while using a UNICAM 969 AA spectrometer. Iodine in milk was determined on the basis of alkaline ashing by a spectrophotometric method according to Sandell-Kolthoff. The analysis was compared to a reference sample. All data was analyzed through the Statistica Program.

RESULTS AND DISCUSSION

The average concentration of beta-hydroxybutyric acid (BHB) and tracked elements in milk are included in Table 1. The intraruminal administration of monenzin 3 weeks before parturition influenced positively a reduction of the BHB concentration in milk during all three experiments. The average BHB content in milk of dairy cows within the experimental groups ranged from 0.078 to 0.144 mmol/l and from 0.222 to 0.328 mmol/l ($P < 0.01$) in milk of dairy cows within the control groups. The BHB concentration over 0.200 mmol/l in milk of dairy cows in the control groups already corresponds to the subclinical ketosis (Geishauser et al. 2000).

Milk of dairy cows in the experimental groups contained the higher average Cu and Zn content (Cu: higher by 15.8 % to 28.2%, Zn: higher by 1.8 % to 14.4%) compared to the control groups. The iodine content in milk was higher in dairy cows treated with monensin only during the second experiment. The average content of elements in milk of dairy cows in the first lactation is shown in Table 2. The biggest differences between the experimental and control groups were also identified in the Cu content (24.8 %). The difference in the Zn content was only 6.3% and 4.2% for the I content.

Figures 1 to 3 show the dynamics of elements during the first 8 weeks of lactation. The Cu and Zn contents in milk were higher in all three attempts between the 2nd and 4th week of lactation as compared to the 5th and 8th week of lactation. The most significant difference in the Cu and Zn contents (Cu: higher by 19% to 41%, Zn: higher by 3% to 19 %) was observed between the 2nd and 4th week of lactation. The iodine content in milk did not show similar trends (Figure 3).

Table 1 Contents of beta-hydroxybutyric acid and copper, zink and iodine in milk of dairy cows

Experiment	Group	n analysis	BHB (mmol/1)	Copper (mg/l)	Zinc (mg/1)	Iodine (mg/1)	Fat[1] (kg)	Protein[1] (kg)
1.	E	16	0.078 ± 0.039[a]	0.445 ± 0.025[d]	3.405 ± 0.250	0.18 ± 0.04	157.8 ± 40.1	119.6 ± 22.9
	C	16	0.222 ± 0.099[a]	0.360 ± 0.040 [d]	3.345 ± 0.275	0.20 ± 0.05	140.8 ± 31.6	117.5 ±24.
2.	E	16	0.144 ± 0.069[b]	0.455 ± 0.125[g]	3.385 ± 0.425[f]	0.32 ± 0.05	147.5 ± 16.9	116.4 ± 15.4
	C	16	0.328 ± 0.138[b]	0.355 ± 0.055[g]	2.960 ± 0.400[f]	0.29 ± 0.06	137.8 ± 25.6	113.8 ± 22.7
3.	E	16	0.122 ± 0.069[c]	0.330 ± 0.040[e]	2.913 ± 0.275	0.20 ± 0.05	142.6 ± 21.6	116.4 ± 15.7
	C	16	0.303 ± 0.071[c]	0.285 ± 0.025[e]	2.750 ± 0.265	0.22 ± 0.04	130.8. ± 18.9	110.0 ± 18.1

a:a, b:b, c:c, d:d,e:e, f:f $P < 0.01$

Legend: E – experimental group; C – control group; BHB – beta-hydroxybutyric acid; 1 – 100 days lactation

Subclinical ketosis is associated with significant changes in the composition and production of milk (Suthar et al. 2013). Ketosis was accompanied by a decline in the Cu and Zn contents in milk

(Tables 1, 2; Figures 1–3) during the given experiments. This statistically significant lower Cu content in milk of dairy cows in the control groups can be related to the metabolic liver congestion in connection with ketogenesis. The liver is an important depot and metabolic organ for Cu contrary to Zn (Lech and Sadlik 2007, 2011). The iodine content in milk of dairy cows in the control groups was not different from the experimental groups. It is concentrated in the body outside the liver, especially in the thyroid gland (Peksa et al. 2013). As opposed to Cu and Zn, the iodine content in milk also corresponds with its intake (Trávníček et al. 2010). Due to the fact that Cu is part of casein (O´Neill et al., 1989), its decline in milk of the control groups may be combined with a decline in the milk protein content in connection with ketosis (Suthar et al. 2013). Lower milk protein production during the first 100 days of lactation was also found in dairy cows with subclinical ketosis (control groups) in our experiments (protein reduction of 1.81% to 5.4%). There was also a decline in milk and fat production. The results have already been published (Hladký et al. 2016).

Table 2 Contents of beta-hydroxybutyric acid and copper, zink and iodine in milk dairy cows (1st lactation)

Group	n analysis	BHB (mmol/1)	Copper (mg/1)	Zinc (mg/1)	Iodine (mg/1)
E	24	0.123 ± 0.066[a]	0.392 ± 0.083[b]	3.290 ± 0.480	0.224 ± 0.083
C	24	0.323 ± 0.125[a]	0.314 ± 0.071[b]	3.093 ± 0.422	0.215 ± 0.060

[a:a] $P < 0.01$, [b:b] $P < 0.05$

Legend: E – experimental group; C – control group; BHB – beta-hydroxybutyric acid

Figure 1 Dynamic of copper content in milk (mg/l)

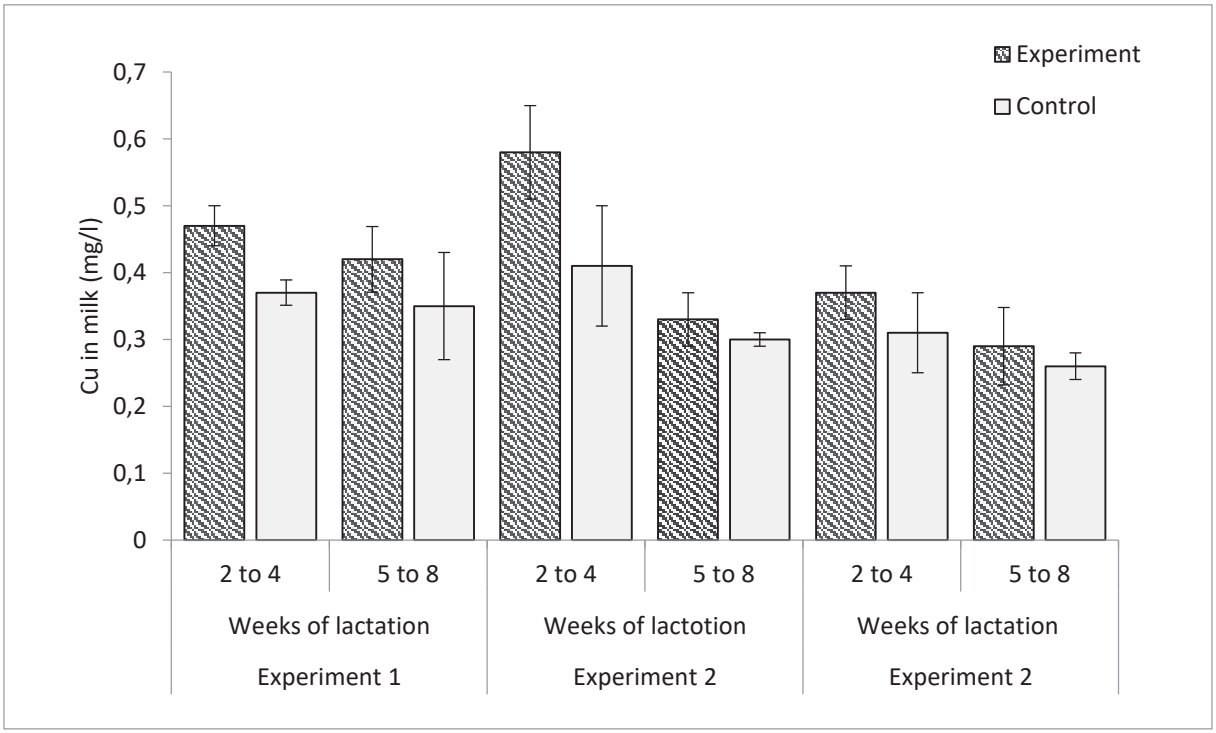


Figure 2 Dynamic of zinc content in milk (mg/l)

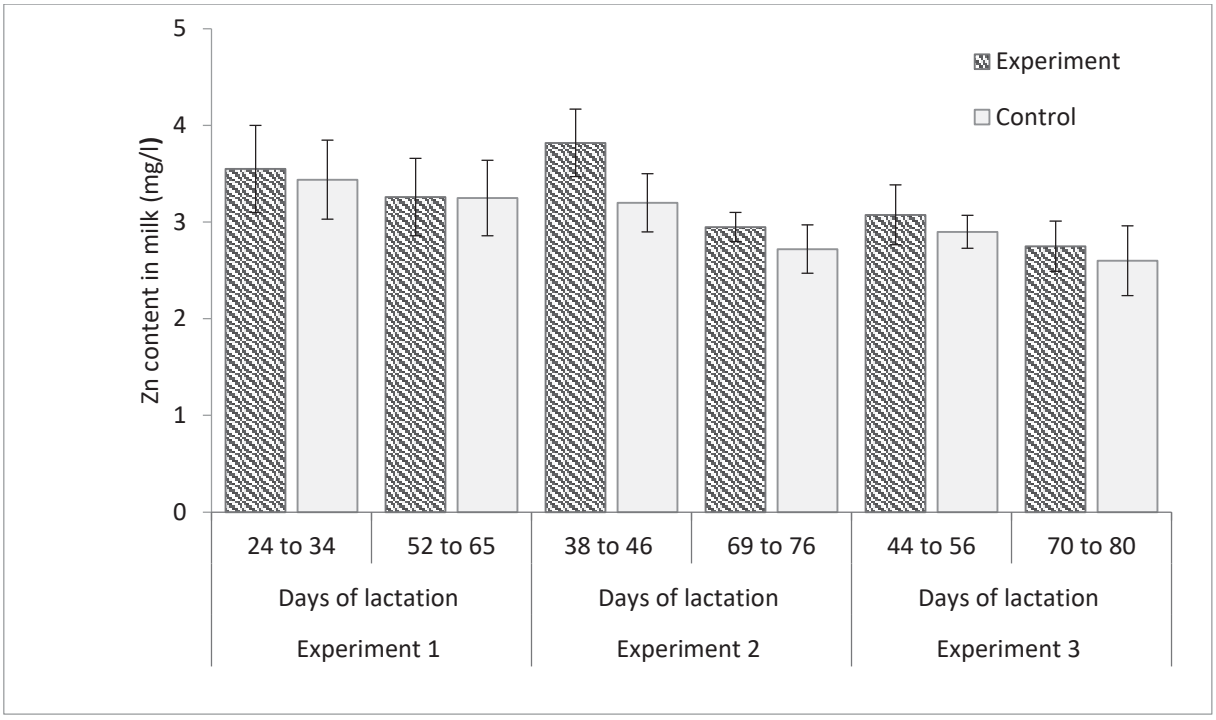
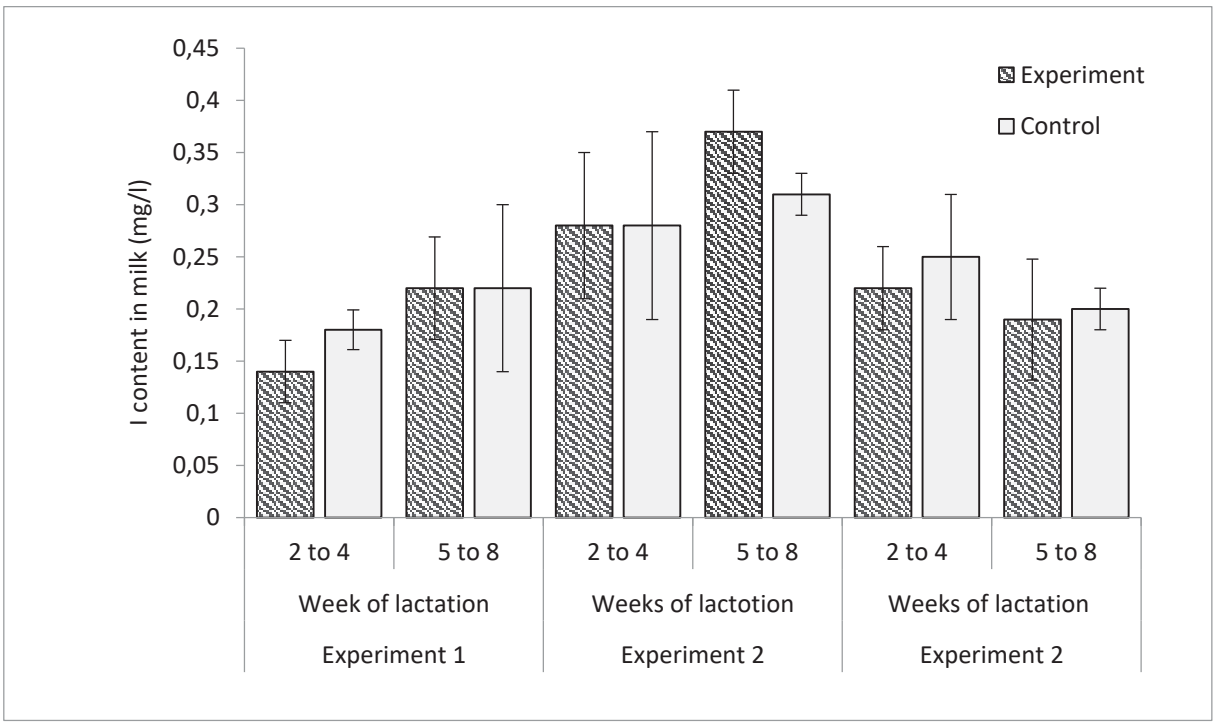


Figure 2 Dynamic of iodine content in milk (mg/l)



CONCLUSION

The intraruminal administration of monensin (32.4 g) 3 weeks before parturition had a positive effect on a reduction of subclinical ketosis and on a higher Cu content in milk.

ACKNOWLEDGEMENTS

The research was financially supported by the University of South Bohemia in České Budějovice (GAJU–002/2016/Z) and by the Ministry of Agriculture of the Czech Republic (NAZV KUS QJ 1510339).

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INFLUENCE OF THE STABLE ENVIRONMENT TEMPERATURE ON THE REPRODUCTION OF THE HIGH-PRODUCING DAIRY COWS

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Abstract: The goal of this study is to evaluate the influence of the stable environment temperature on the successful conception of Holstein cattle cows. Cows (n = 292) were divided into two groups, cows inseminated during the natural heat period and cows included into synchronization programme Ovsynch. The experiment was performed at a school farm in Žabčice. Data were collected in the period from June 2016 to February 2017. The results of the study proved the influence of the temperature of the environment on the conception of the cows. The analysis showed that cows in the synchronization programme had the best ($p < 0.01$) conception rates in February (45.46%). Very similar conception rates were also detected in cows with natural heat (50.00%). The further important finding was the different sensitivity of the animals to the temperature of the environment. The difference between the decrease or increase of conception rates in the group of cows with natural heat and the cows with synchronized heat was 2 months.

Key Words: Holstein cattle, reproduction, Ovsynch, air temperature

INTRODUCTION

Higher milk production negatively influences reproduction in the herd. Therefore problems with a conception of cows appear more often. These problems express themselves by an increase in silent, indistinctive heats and cessation in reproduction cycles of the cows. The problems with conception lead to elongation of service intervals, increase in usage of insemination doses and higher consumption of hormones necessary for regular reproduction cycle.

In the herds, the cows are monitored and the first heat is being detected. The cows in which the heats are not distinctive are included in the synchronization programmes. The presence of "phantom cows" (cows, which are not in the gestation period and their oestrous cycle has ceased) represents a challenge for synchronization programmes to restore their oestrous cycle (Lucy et al. 2004). When the ovarium acyclia appears it is necessary to use GnRH and $PGF_{2\alpha}$ for the correct function of the oestrous cycle. Currently, synchronization protocols are used for that. These protocols are used for heat synchronization and timing of insemination of cows (Pursley et al. 1997). Synchronization protocol Ovsynch can be used both for the first insemination of the cows and for repeated insemination of the cows in which pregnancy was not proved (Sterry et al. 2006). Incorrect timing of the beginning of the heat synchronization can lead to ovulation of premature follicles, which are a result of improper synchronization of the follicular wave after the first GnRH injection (Lucy et al. 2004).

Synchronization protocol Ovsynch consists of an application of GnRH (day 0), which eliminates current follicular wave, application (day 7), which causes luteolysis and the following insemination (8 to 18 hours after application of $PGF_{2\alpha}$).

As the reproduction has a low heritability ($h^2 = 0.05 - 0.2$), it is significantly influenced by external factors such as stable, climate, environment etc. (Louda et al. 2008). Climate conditions play an important role in the conception of cows. High temperatures can influence the reproductive cycle (De Rensis and Scaramuzzi 2002). Thermal stress also influences the ability of the cows to conceive (Lucy 2002). Thermal stress is the main factor which contributes to low conception rates of the cows, which are inseminated in the summer months (Al-Katanani et al. 1999). The decrease in conception rates can be 20–30% in the summer months compared to the winter months. (De Rensis et al. 2002).

The aim of this paper is to evaluate the influence of stable environment temperatures on conception rates of high-producing dairy cows.

MATERIAL AND METHODS

The experiment was carried out at a farm in the South Moravian Region (school farm Žabčice). In the experiment cows inseminated in so-called natural heats and cows included in the Ovsynch programme were observed. The average length of a service period (SP) in the monitored population was about 150 days. The experiment took place from June 2016 to the end of February 2017. The study included 292 cows of Holstein cattle. Natural heats were detected based on the visual evaluation of the heat signs of the cows and consequently confirmed by ultrasonographic examination of the ovaries. The breeding cows included in the Ovsynch programme were inseminated based on a time schedule of the given synchronization programme. Before the insemination itself, ovaries of those cows were also ultrasonographically examined for the confirmation of follicle size. Insemination was performed using a rectal technique. The pregnancy of the cows was checked ultrasonographically 30 days after the insemination. The cows were kept in a free stall pens, fed by unified rations. Data about the outside temperature were collected from the weather station in Žabčice, in the stable, the temperature was measured using HOBO sensors located in the central part of the stable in the height of a cow's head. Data were processed using STATISTICA 12.0 program.

RESULTS AND DISCUSSION

Influence of a stable environment temperature and success rates of the conception in the period between June 2016 and February 2017 are provided in Table 1. Conception rates for the cows without the synchronization and for those using the synchronization protocol Ovsynch were evaluated. It is evident, that the cows included into the synchronization programme Ovsynch had the best ($p < 0.01$) conception rates in February (45.46%), at the average stable temperature 8.23 ± 0.85 °C. Similar results were also observed in the cows which were not included in the synchronization programme. They also had the best ($p < 0.01$) conception rates in February (50.00%). From the collected data it is possible to observe a difference in the values of the worst conception rates, where the cows from the synchronization programme had the worst ($p < 0.01$) conception rates in November (7.41%), at the average temperature of 7.32 ± 1.61 °C. Compared to that, the breeding cows without synchronization protocol had the worst ($p < 0.01$) conception rates in September (6.25%) at the average temperature 20.14 ± 0.94 °C. Sambraus (1997) and Vokřálová et al. (2007) state that the optimal temperature span for the cows is between 0 and 20 °C, when this temperature is exceeded, the cows suffer from the thermal stress. The temperature has to be measured inside the stable because there is a rather significant difference between the inside and outside temperature (Table 1). Dirandeh et al. (2014) carried out an experiment where synchronization programme Ovsynch was tested in two groups of cows in the period of thermal stress. The first group was given GnRH on the 6th day after ovulation (O6), the second group was given GnRH randomly, without detecting the ovulation. Better conception rates appeared in the group O6 (31%) compared to the second group (25%). Vasconcelos et al. (1999) state, that the correct time of GnRH application influences the conception rates of the cows. In their study, they found out that the cows in the middle part of the heat cycle (5th - 9th day of the cycle) have a higher probability of ovulation than the cows in another part of the heat cycle. That means that there is a higher possibility of conception. Lucy et al. (2004) came to the same results and they state that the incorrect time of the GnRH application can lead to ovulation of premature follicles. Premature follicles release a low amount of estradiol, which means a higher probability of embryonic mortality (Perry et al. 2005). Stevenson and Phatak (2005) did not identify any differences in the conception rates between the PreSynch and Ovsynch.

According to Lucy (2002), the thermal stress leads to the increase of body temperature of an individual. Increased body temperature influences function of the ovaries, embryos quality and the embryo development in general. The organism is able to regulate the necessary physiological processes with minimum energy consumption only in the zone of thermal neutrality, which is individual for each cow (Dolejš et al. 2000). These temperature differences can negatively influence the heat of the breeding cows. De Rensis and Scaramuzzi (2002) state that the thermal stress can influence the course of oestrous cycle and conception. This finding corresponds with the results of our

experiment. The thermal stress does not affect the follicles development and the heat cycle on the very day, but with a delay as long as several months. In the cows with natural heat, the decrease is visible from September (6.25%), compared to the cows with synchronized heat, where the decrease in conception is visible from November (7.41%). The increase can be detected in the first group (with natural heat) from December (39.40%) and in the second group (with synchronized heat) from January (22.65%). In February the results of the conception are similar. What is interesting is, that the decrease an increase in conception rates in cows with heat synchronization is two months delayed. The decrease in the conception rates in cows with natural heat is visible in September (6.25%). In the cows with synchronized heat, the decrease showed up in November (7.41%). The same trend could be also identified also in an increase of conception rates. In the cows with natural heat, the increase in conception rates appears in November (15.82%) and in the cows with synchronized heat in January (22.65%).

Table 1 Influence of the temperature on the successful conception in individual months

Period	Number of cows (pcs)	Average outside temperature on days of insemination (°C)	Average stable temperature on days of insemination (°C)	Number of pregnancy cows (pcs)	Number of pregnancy cows after ovsynch (pcs)
June	42	20.30 ± 3.55	25.24 ± 1.55	23.81^{AB}	23.81^{AE}
July	34	14.80 ± 3.31	25.90 ± 1.43	29.42^{B}	38.24^{BC}
August	16	19.46 ± 1.90	22.26 ± 1.13	43.75^{C}	31.25^{CE}
September	32	16.94 ± 3.61	20.14 ± 0.94	6.25^{D}	15.63^{A}
October	33	7.73 ± 3.55	10.40 ± 1.10	11.12^{DE}	18.19^{A}
November	27	3.76 ± 3.09	7.32 ± 1.61	15.82^{ADE}	7.41^{D}
December	33	0.15 ± 3.36	3.61 ± 1.15	39.40^{C}	10.7^{D}
January	53	-5.41 ± 2.99	-0.21 ± 1.16	20.76^{ABE}	22.65^{AC}
February	22	1.42 ± 3.44	8.23 ± 0.85	50.00^{C}	45.46^{B}
∑	292				

** A, B, C, D, E = significant differences $p < 0.01$*

CONCLUSIONS

Based on the results of our experiment, it can be stated that the temperature of the stable environment significantly influences the conception of the dairy cows of the Holstein cattle. The analysis showed that both cows with the natural heat and the cows in synchronization programme Ovsynch had best conception rates in February. The decrease in the conception rates in the cows with natural heat is visible in September (6.25%). The group of cows with synchronized heat showed a reaction to the thermal stress in November when the conception rates fell to 7.41%. The increase in conception rates again appeared with two months delay. In the group of cows with natural heat the conception rates increased in November (15.82%) and in the second group of cows, with synchronized heat, the conception rates increased in January (22.65%). These results support the theory that cows better cope with the lower temperatures and that the high temperatures negatively influence heat cycle and the correct development of follicles in general.

ACKNOWLEDGEMENTS

The research was financially supported by the project TP IGA MENDELU 7/2017.

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FACTORS INFLUENCING THE PERFORMANCE OF THE ENGLISH THOROUGHBRED HORSES

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Abstract: The target of the work was to make a database of the Best 3yo english thoroughbred horses done by the IFHA and analysed their performance by rating with the choosen effects. We´ve been analysing 1284 3yo horses with the minimum rating of 115 racing in the years 2000 to 2016. We chose the seven main effects which were Year of racing, country of origin, country of training, sex, distance, sireline and line of the maternal sire. We´ve been analysing whole database by the analysis of variance by the UNISTAT, 6.5 and then we checked the proven differencies by the multiple analysis by Scheffe method. We found as highly influential to the performance of the horses effects country of training, sex and distance. We found out the high differences between horses trained in Ireland and horses trained in non European countries in case of effect country of training. Then we found out the differencies between colts (with the best average rating) and fillies. Another differencies we found out between the groups of intermediares, millers and sprinters as well as between millers and long distance runners. We cannot prove the differencies in other chosen effects.

Key words: English thoroughbred, rating, IFHA

INTRODUCTION

English thoroughbred horses are the fastest horses around the world. As Dušek (1999) published, the most important stallions of the breed was Byerley Turk, born in 1680, Darley Arabian, bought in Syria in 1712 and finally Godolphin Barb, born in 1724. These three stallions are considered to be the founders of the breed. Although it had been a sporting activity for hundreds of years, British horseracing really began to get organised in the second half of the 18th century. An essential part of that was the establishment of formal rules of racing. Detailed recording of the horses competing, the results, and their pedigrees was started at this time (Weatherbys.co.uk 2015). As Varola (1981) published, and the Schulmann (2011) after thirty years too, the main role in the thoroughbred breed are not the genetics but almost the social and economical factors. Vlček presented that the breeder´s ideal horse had to be the horse with good performance as 2yo and with increasing performance at 3yo and hold the performance at 4yo and older in the 30 to 50th years of the 20th century. The best stallion could cover about 40-50 mares per season and even the best one couldn´t separate from the others and cover most of mares so this maintained the full range of the sirelines (Schulmann 2011). The internationalisation and mostly commercial thoughts in the breeding of the thoroughbreds have shown the new wave of breeding for faster runners and early runners. The british most wanted sire Soldier of Fortune covered 304 mares in 2016 as well as french sire Rajsaman covered 219 mares in 2016 (Blacktypepedigree.com 2016). And other most desired sires covered similar number of mares (Vlček 2016). That means that the most mares were covered by only a few most wanted and best sires and the others, maybe same quality sires, can´t get more chance to have a good offspring at all because of the great marketing pressure from the biggest studfarms. These are the goals that have a role in the construction of the blood lines in the thoroughbred breed (Schulmann 2011).

The main goal of this article was to analyse the influence of the chosen effects (sireline, line of the maternal sire, year of racing, country of origin, country of training, sex and distance) on the performance of the best 3yo thoroughbred horses by the rating from the IFHA (International Federation of Horseracing Authorities). The ratings are compiled under the auspices of The International Federation of Horseracing Authorities (IFHA) by racing officials & handicappers

representing the five continents who compile the ranking order by agreeing on the rating for each horse. The ratings are based on the performance of horses in elite races held during the designated period which takes in account the quality of opposition and achievements of each horse. Throughout the year the Longines Rankings are published at regular intervals and the consolidated annual rankings are released in January. The annual rankings denote the champions in the various distance categories for example sprint or mile, surface either turf or dirt/artificial and also the fillies & mares category (IFHA 2017)

MATERIAL AND METHODS

We made the database of the best 3yo thoroughbred horses from the annual „Blue Books" from IFHA in the 2000 to 2016. We record the rating, name, sex, sire, dam, dam sire, sireline and line of the maternal sire, country of origin, country of training, trainer and distance for each horse from the database made on www.pedigrequery.com. We had already 1284 thoroughbred horses. As the next step we divided the database by the chosen effects:

- Year of Racing – 2000 (90), 2001 (85), 2002 (67), 2003 (69), 2004 (53), 2005 (51), 2006 (64), 2007 (76), 2008 (68), 2009 (92), 2010 (88), 2011 (99), 2012 (80), 2013 (72), 2014 (89), 2015 (74), 2016 (77)
- Country of origin – ARG (15), AUS (46), FR (89), GB (185), GER (40), IRE (297), SA (BRZ + CHI + PER + URU, 13), JPN (86), NZ (11), OTH (CAN + ITY + SAF + SPA, 8) and USA (494)
- Country of training – AUS (49), FR (212), GB (267), GER (46), IRE (153), ITY (15), JPN (91), SA (ARG + BRZ + CHI + PER + URU, 29), OTH (CZ + HUN + SPA + CAN + NZ + SAF + SIN + UAE, 15) and USA (402)
- Sex – F (296), C (943), G (45)
- Distance – S (109), M (440), I (326), L (360), E (49)
- Sireline – Blandford (12), Djebel (12), Man O´War (11), Pharos (914), Sickle (297), Other DA (38)
- Line of the maternal sire – Blandford (18), Djebel (19), Fairway (13), Gainsborough (27), Herold (12), Man O´War (24), Pharos (828), Ribot (28), Sickle (271), Sun Teddy (17), Other DA (27)

We registered the influence of each of the effect on the rating by the analysis of variance by the UNISTAT, 6.5 and then we checked the proven differencies by the multiple analysis by Scheffe method.

RESULTS AND DISCUSSION

We had 1284 horses which run in years 2000 to 2016 in database. The horse performance, in this case racing performance, is the multifactorial characteristics. We´ve been analysing the horse performance with seven chosen effects.

You can find the main numbers of chosen and tested effects in Table 1. Every horse got the rating by their performance in the best races around the world and in the international database are written horses with rating not less than 115. We found out as highly influential to the performance of the horses effects country of training, sex and distance. We cannot prove the differencies in other chosen effects. Then we checked the proven differencies by the multiple analysis of each effect by the Scheffe method.

Evaluation of the effect Country of training

As the highly provable diferency we evaluated the effect country of training. We found out the high differences between horses trained in Ireland (average rating 119.18) and horses trained in non European countries (such was JPN, AUS, USA and the countries of South America) as you can find in Table 2. The Great Britain had the second highest average rating (118.29), followed by french trained horses (117.86) and after them the most numerous group of horses trained in USA (117.84). These results show the most common known facts that the best horses are bred and trained in Ireland, GB and USA. This is caused by the long history of breeding and training of the thoroughbred horses, their

perfection in managing the stud and training yards and as the last not least the quality of the trainers. The lowest average rating numbers have horses trained in Italy, that´s because of the lost of the best trainers there.

Table 1 Analysis of variance

Variability	Sum of the squares	Deg. freed.	Average square	Stat F	Prob
Main efects	1710.906	56	30.552	2.895	0.0000
Year of racing	244.621	16	15.289	1.449	0.1113
Country of origin	22.207	10	2.221	0.210	0.9954
Country of training	339.998	9	37.778	3.579	0.0002
Sex	287.146	2	143.573	13.603	0.0000
Distance	428.965	4	107.241	10.161	0.0000
Sireline	78.061	5	15.612	1.479	0.1937
Line of the maternal sire	66.056	10	6.606	0.626	0.7927
Explained	1710.906	56	30.552	2.895	0.0000
Mistake	12950.502	1227	10.555		
Total	14661.408	1283	11.427		

Table 2 Scheffe´s multiple analysis for effect Country of training

Groups	No.	Average	ITY	SA	AUS	OTH	JPN	GER	USA	FR	GB	IRE
ITY	15	115.9333										
SA	29	115.9655										**
AUS	49	116.7755										**
OTH	20	116.8000										
JPN	91	117.3626										**
GER	46	117.3696										
USA	402	117.8433										**
FR	212	117.8632										
GB	267	118.2996										
IRE	153	119.1830		**	**		**		**			

Shorts: ITY (Italy), SA (South America), AUS (Australia), OTH (Others), JPN (Japan), GER (Germany), USA (United States of America), FR (France), GB (Great Britain), IRE (Ireland)

You can find the range of rating points of the horses trained in Ireland and GB was wider (21 points) than for the most numerous group of horses trained in USA (19 points) as well as wider range of rating points of the horses trained in Germany, where the only 49 horses in database had rating range of 13 points.

Table 3 Main characteristics for effect Country of training

	No.	Average	Median	Varriation coefficient	Minimum	Maximum	Range
AUS	49	116.7755	116.0000	0.0179	115.0000	123.0000	8.0000
FR	212	117.8632	117.0000	0.0295	115.0000	132.0000	17.0000
GB	267	118.2996	117.0000	0.0295	115.0000	136.0000	21.0000
GER	46	117.3696	116.0000	0.0256	115.0000	128.0000	13.0000
IRE	153	119.1830	118.0000	0.0352	115.0000	136.0000	21.0000
ITY	15	115.9333	115.0000	0.0124	115.0000	120.0000	5.0000
SA	29	115.9655	115.0000	0.0116	115.0000	120.0000	5.0000
JPN	91	117.3626	116.0000	0.0223	115.0000	125.0000	10.0000
OTH	20	116.8000	116.5000	0.0146	115.0000	121.0000	6.0000
USA	402	117.8433	117.0000	0.0276	115.0000	134.0000	19.0000

Shorts: ITY (Italy), SA (South America), AUS (Australia), OTH (Others), JPN (Japan), GER (Germany), USA (United States of America), FR (France), GB (Great Britain), IRE (Ireland)

Evaluation of the effect Sex

As the second effect we found as highly conclusive on the performance was the effect Sex. We evaluated 943 colts, which were with the average rating of 119.19 highly different than 296 evaluated fillies with the average rating 117.20 written to Table 4. The last 45 horses were geldings with average rating of 117.17. It´s common known fact that the performance of colts are higher than of fillies which we proven in our research over the database of 3yo thoroughbred horses. We should think about the number of horses in the each sex category which in fact has the influence to the performance too. We had about 75% less fillies than colts. This is caused by the big presure to the colts to became sires at studs and need to have highest performance to be chosen into stud and be wanted by other breeders and owners of breeding mares. That´s why colts have much more running in the classical year of 3yo and have highest performance. Instead of this mares have to be managed to get into stud as early as possible. So their performance is lower and they have less races run than colts.

Table 4 Scheffe´s multiple analysis for effect Sex

Groups	No.	Average	G	F	C
G	45	117.1778			
F	296	117.1959			**
C	943	118.1941		**	

Shorts: G – Gelding, F – Filly, C – Colt

We can see the range of the rating for group of colts are wide as well as the range of rating for group of fillies even they have only 296 horses and the range is 15 points written in Table 5.

Table 5 Main characteristics for effect Sex

	No.	Average	Median	Varriation coefficient	Minimum	Maximum	Range
C	943	118.1941	117.0000	0.0305	115.0000	136.0000	21.0000
F	296	117.1959	116.0000	0.0217	115.0000	130.0000	15.0000
G	45	117.1778	116.0000	0.0194	115.0000	123.0000	8.0000

Shorts: G – Gelding, F – Filly, C – Colt

Evaluation of the effect Distance

The last effect we evaluated as highly influenced on the performance was the effect distance of the races. We found out the highly differences between the group of Intermediaters (with average rating 118.57), sprinters (with average rating 117.18) and group of millers (with average rating 117.45) see in Table 6. Then we proven the differencies between group of millers (117.45) and group of long distance runners (118.26) written in Table 6. The highest average rating we found in the group of intermediaters 118.57 and group of long distance runners 118.26, which are the distances of the main classical races for 3yo horses. These are the most important races for the breeding career of the English thoroughbreds a that´s the reason they have the highest rating over the other races. The lowest average rating we found in the group of extra long distance runners (117.12). Extra long distance runner are horses performed in races with the distance more than 2700 m so not every horse could reach such a long distance as they are 3yo. We had lower number of participants in this group as well. Mostly the older horses reach the extra long distance races and they run less often that´s why there aren´t so much horses in the group as 3yo.

Table 6 Scheffe´s multiple analysis for effect Distance

Groups	No.	Average	E	S	M	L	I
E	49	117.1224					
S	109	117.1835					**
M	440	117.4523				**	**
L	360	118.2611			**		
I	326	118.5736		**	**		

Shorts: S – sprinters, M – millers, I – intermediares, L – long distance runners, E – extra long distance runners

We can see the wide range of rating in the group od millers (21 points) as well as in intermediares and long distance runners (17 points). These are the most numerous groups in database so the range could be wider than in other once. In these groups we can find the best representatives of the breed as well as the medium quality horses. We can see smaller range of rating points in groups of millers and extra long distance runners (Table 7).

Table 7 Main characteristics for effect Distance

	No.	Average	Median	Varriation coefficient	Minimum	Maximum	Range
S	109	117.1835	116.0000	0.0210	115.0000	126.0000	11.0000
M	440	117.4523	116.0000	0.0255	115.0000	136.0000	21.0000
I	326	118.5736	117.0000	0.0325	115.0000	136.0000	21.0000
L	360	118.2611	117.0000	0.0302	115.0000	132.0000	17.0000
E	49	117.1224	117.0000	0.0215	115.0000	125.0000	10.0000

Shorts: S – sprinters, M – millers, I – intermediares, L – long distance runners, E – extra long distance runners

We cannot prove the statistical diferrencies between groups of the effects Country of origin, Sireline, Line of the maternal sire and Year of racing. There are significant representatives of the decendant sire Darley Arabian, resp. Pharos and Sickle, in the groups of Sirelines and Lines of maternal sire. Other lines are in minority. So we think about the way of breeding of English thoroughbreds, their shorter breeding base and the usage of the sires and breeding mares form only one descendant sireline.

CONCLUSION

We´ve been analysing 1284 3yo horses with the minimum rating of 115 during the years 2000 to 2016. We divided the whole database into groups of seven choose effects which are Year of racing, country of origin, country of training, sex, distance, sireline and line of the maternal sire. We´ve been analysing whole database by the analysis of variance and then we check the proven differencies by the multiple analysis by Scheffe method. We found as highly influential to the performance of the horses effects country of traning, sex and distance. We found out the high differences between horses trained in Ireland (average rating 119.18) and horses trained in non European countries in case of effect Country of training. Then we found out the differencies between stallions (118.19) beside mares (117.20). Another differencies we found between the groups of intermediares (118.57), millers (117.45) and sprinters (117.18) as well as between millers (117.18) and long distance runners (118.26). We cannot prove the differencies in other chosen effects.

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EVALUATING THE IMPORTANCE OF THE STALLION SCYRIS IN THE BREEDING OF THE CZECH WARMBLOOD

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Abstract: The aim of this thesis was to evaluate the importance of the stud stallion Scyris in the breeding of the Czech Warmblood. To evaluate the stud horse we used his offspring born in the years 2011−2016. Two underlying databases were then created in the programme Microsoft Office Excel 2007. The first was used to assess linear regression and the second to assess correlation using the Pearson correlation coefficient. On the basis of aggregate statistics from these measurements, it can be stated that the dams are unbalanced in exterior terms, and stallion 606 Scyris (POL) did not completely succeed in balancing out this variability in his daughters. In the next step, the correlation dependence of individual body measurements was assessed in daughters and dams. It was determined that there is medium correlation dependence between the attributes TWH (tape withers height), SWH (stick withers height) and CBC (cannon bone circumference), so it can be said that the influence of dams on the bone mass and withers height of daughters is probable but is not conclusive. With the attribute ChC (chest circumference) low correlation dependence between dams and daughters was recorded. This suggests that the dams have a lesser influence on the bulkiness of daughters. When assessing the linear regression, we recorded a decreasing tendency of the regression coefficient with regard to offspring class which exhibits 16% reliability and according to our test emerges as statistically conclusive. So the assessed class of daughters of the sire 6062 Scyris (POL) does not improve – on the contrary, it decreases slightly. A high value for the downward trend was recorded with the jump index of the dams and is documented by 21.66% reliability. Our test emerged as highly statistically conclusive and we can conclude that the mares mated with stallion 6062 Scyris (POL) stallions are, in the course of years, breeders selected for lesser quality mares.

Key words: Czech Warmblood, Thoroughbred, stallion, offspring

INTRODUCTION

The main aim of this thesis was to evaluate the importance of the stud horse 6062 Scyris (POL), which belongs to the English Thoroughbred breed, in the breeding of the Czech Warmblood.

His importance in breeding was evaluated by performing a comparison of his 48 descendants, where we assessed and compared their exterior variability and the variability of the mated dams. The next step in evaluating the offspring was to carry out linear regression, correlation between the dams and daughters, and comparison of offspring colour with the aim of determining whether stallion 6062 Scyris (POL) carries a certain gene that influences colouring.

At present, the main point of breeding horses is to create a high-quality partner for sporting and recreational purposes. Sporting performance has become one of the main assessment criteria in the breeding of horses. The aim of improving individual breeds is to create the best possible athlete for the type of competition for which the breed is destined. This makes the correct choice of horses for breeding very important.

Horse breeding very often involves crossing different breeds, which serves to mate individuals with a different genotype with the aim of creating new breeds (known as “an infusion of blood”) and is used mainly with warmbloods to improve or refine their build.

Characteristics of the Czech Warmblood breed

Dušek (2011) states that the rather variable conformation and the existence of quite a diverse range of colours are due to the relatively short period of improvement and the number of breeds used.

The Association of Czech Warmblood Breeders, which is in charge of the breed registry in the Czech Republic, defines the breeding objective in its breeding programme and rules thus: The aim of improving the Czech Warmblood is to produce a noble, sound and easy-to-ride horse whose temperament, character, spacious and elastic mechanics of movement, and sound health make it suitable for all types of performance-related equestrian sport within the FEI disciplines as well as for leisure activities.

The adult horse has a medium body frame with good lines, a solid foundation and no obvious or genetically determined faults or diseases (SCHČT 2017).

Table 1 Body measurements according to the standard for the Czech Warmblood

Body measurements	Mares	Stallions
Stick withers height (cm)	161–167	162–170
Cannon bone circumference (cm)	19.5–22	21–22.5

(SCHČT 2017)

Characteristics of the English Thoroughbred breed

As Koubek et al. (1957) state, the forms of the English Thoroughbred are sometimes quite divergent.

According to Hermsen (2002), the Thoroughbred is a generally noble and elegantly built horse.

Schmiedová (2012) then states that the English Thoroughbred is now very different in outward appearance from the horses from which it originated. It has a medium to large, rectangular frame. This was achieved through selecting the fastest individuals for breeding purposes. The average height of the English Thoroughbred is now around 160 cm measured by stick.

Edwards (1992) writes that the head of the English Thoroughbred is noble, dry and very fine and the profile is straight. The cheeks must not be fleshy or the jaw coarse. The ears are fairly long and very mobile.

According to Hermsen (2002), the neck is long and slightly arched and passes into pronounced withers.

Schmiedová (2012) states that the chest must be capacious in order to allow for sufficient development of the heart and lungs.

According to Dušek (2011), the top line is relatively long, straight and well linked. The hindquarters are long, sloping and muscular; according to Edwards (1998), they must be strong, because they provide the strength required for speed.

Edwards (1998) describes the shoulder blade of the English Thoroughbred as long and angled. In combination with the pronounced withers, it determines the long, low and economical movement.

The cannon bone of the English Thoroughbred is relatively short and strong enough to support the body at high speeds. The strength of the cannon bone is genetically determined, but its firmness is influenced by the conditions of rearing and training. The pastern is relatively long and should be sufficiently firm and flexible with the correct bend. The hooves should, of course, be regular (Schmiedová 2012).

The colouring of the English Thoroughbred is also very variable. The most frequent colours are bay, brown, chestnut, black and grey. It can very often be found with markings, both on the head and on the limbs (Stead 2015).

Berns (2001) writes that the English Thoroughbred is a horse born for speed.

Zuda (1969) cited in Byrtusová (2007), states that a constant property acquired through the environment is early maturity. It is the earliest maturing breed. The most characteristic property is its hard constitution, expressed by its respiratory system, disproportionately spirited temperament and

generally high responsiveness of the nervous system, which mainly comes into play during performance.

Importance of the English Thoroughbred in breeding warmblood sporting breeds

Kopecký et al. (1977) state that because of its outstanding qualities the English Thoroughbred was used to improve other breeds of horses or directly contributed to their creation. Due to the spacious mechanics of movement of the hard constitution and exterior, the English Thoroughbred became the corrector of the properties of a large proportion of other warmblood utility types and breeds.

Hanušová (2007) cited in Müllerová (2010), claims that even today warmblood breeding still needs to be improved; otherwise, as a result of the stronger influence of the dam's genetic potential, it will gradually revert to the original heavier working type. The negative aspects which the Thoroughbred often brings to breeding are: poor foundation; insufficient mechanics of movement in walk and trot; small, undistinguished joints; excessive sensitivity, nervousness, etc.

Specific aspects of selecting an English Thoroughbred suitable for improving the Czech Warmblood

Zelník et al. (1958) write that breeding stallions are correctors of breeding material in local breeding, which is why they must always have more perfect conformation than mares. The chosen stallion must above all be suitable for the relevant area in which he is to be used for breeding. His origins should be such as to improve the quality of breeding in this area. A newly classified breeding stallion intended for improvement should not have exterior or character flaws which are already present in the breed.

An important aspect which needs to be taken into account is that the English Thoroughbred's influence on offspring in warmblood breeding is very individual. This is particularly true with regard to individual heredity from the stallion. With a certain type of mare or even just with a certain breed, only some stud horses will assert their influence.

MATERIAL AND METHODOLOGY

Selection of offspring by the stud horse in question

The underlying database was created using data published on the internet site of the Association of Czech Warmblood Breeders. On this website we determined that in the years 2011–2016 a total of 48 foals born by the breeding stallion 6062 Scyris (POL) were recorded in the breed registry for the Czech Warmblood. All the data available there were then used to create the database and statistics. For ease of reference we created tables and graphs expressing the number and percentage of foals by sex in the given years and the distribution of offspring by sex.

Database creation and selection of a suitable statistical processing method

The database of offspring was created in the programme Microsoft Office Excel 2007. The data acquired were then processed using the Pearson correlation coefficient and the linear regression function in the programme UNISTAT 6.5.

Selection of characteristics for evaluating the offspring of stallion 6062 Scyris (POL)

The underlying database for evaluating linear regression includes the following data for each horse: name, sex, year of birth, colour, class, jump index, dam's name, dam's colour, dam's class, dam's breed, year of birth, jump index, damsire's name, damsire's colour, damsire's class, damsire's breed, damsire's year of birth, damsire's jump index.

We observed how the class of all 48 descendants changes dependent on their year of birth. We also observed a change in the dependence of the class of the 14 assessed daughters on their year of birth. We then evaluated how the dependence of the jump index of the 48 descendants' dams on their year of birth changes. And finally we evaluated the change in the dependence of the class of the 48 descendants' dams on their year of birth.

We evaluated the Scyris stallion on the basis of 48 offspring who are enrolled in the breed book of the Czech warmblood. In a linear regression, we evaluated Scyris's offspring according to their year

of birth in order to take into account the external environmental influences that did not affect the genotype acquired by the father, however, it would affect the phenotypic expression of the sign.

The underlying database for evaluating the Pearson correlation coefficient includes the following data for each horse: descendant's name, descendant's SWH, descendant's TWH, descendant's ChC, descendant's CBC, dam's name, dam's SWH, dam's TWH, dam's ChC, dam's CBC.

RESULTS AND DISCUSSION

Characteristics of the comparative base

The database contains 48 descendants of the stallion 6062 Scyris (POL) which were born in the years 2011–2016, although no foals were born in a Czech Warmblood stud farm in 2015. The reason for this is that in 2014 the stallion was acting as a stud horse in Germany. The stallion 6062 Scyris (POL) was most favoured by Czech Warmblood breeders in the year 2013. There was then a decline in the rate of his offspring in the years that followed.

Monitoring variability in mated dams and their offspring

The class and basic measurements were determined for 14 daughters of 6062 Scyris (POL) and their dams. These measurements were then used to evaluate the minimum, maximum and mean values. The mean values obtained were then rounded off in accordance with the standard recording of body measurements in horses, where the cannon bone circumference (CBC) is rounded off to one decimal place and the remaining values (chest circumference – ChC, stick height at withers – SWH, tape height at withers – TWH) were rounded off to whole numbers. The mean values of the dams and daughters were then compared, and according to the results it can be stated that:

- average SWH value of daughters (164 cm) is the same as in dams (164 cm)
- average TWH value of daughters (173 cm) is 2 cm lower than in dams (175 cm)
- average ChC value of daughters (190 cm) is 6 cm lower than in dams (196 cm)
- average CBC value of daughters (20.4 cm) is 0.6 cm lower than in dams (21.2 cm)

From this we concluded that stallion 6062 Scyris (POL) reduces the shin circumference of his daughters, which is demonstrated by the lower CBC values for daughters than is the case with the dams. Which is undesirable for the breeding of the Czech warmblood, according to a member of the studbook.

Table 2 Of variability basic body measurements from aggregate statistics

	SWH of daughter	TWH of daughter	ChC of daughter	CBC of daughter	SWH of dam	TWH of dam	ChC of dam	CBC of dam
Diameter	164,071	173,357	190,214	20,414	164,142	175,214	195,714	21,200
Dispersion	18,071	17,478	43,565	0,349	18,285	17,873	71,912	0,650
Standard deviation	4,251	4,180	6,600	0,590	4,276	4,227	8,480	0,806
Variation coefficient	0,025	0,024	0,034	0,028	0,026	0,024	0,043	0,038

After establishing the variability from aggregate statistics, we determined that dams and their daughters are highly variable, which is confirmed both by the values of the variation coefficient and by the values of variance and standard deviation. This variability can be seen most clearly with the values ChC of daughter and ChC of dam. The values obtained confirm that the stud horse 6062 Scyris (POL) did not level out the variability in the properties of the mated mares, as the breed's advice wanted to.

Correlation

According to the correlation dependence of individual body measurements in daughters and dams, it was determined that there is medium correlation dependence between the attributes TWH, SWH and CBC, and according to this finding it can be stated that the influence of dams on the bone mass and withers height of daughters is probable but not conclusive. With the attribute ChC, low

correlation dependence between dams and daughters was recorded. This suggests that the dams have a lesser influence on the bulkiness of daughters.

Table 3 Correlation dependence of individual body measurements between daughters and dams

	CBC of daughter	ChC of daughter	SWH of daughter	TWH of daughter
CBC of dam	0.531	0.408	0.457	0.567
ChC of dam	-0.003	0.170	-0.242	-0.083
SWH of dam	0.364	0.154	0.494	0.539
TWH of dam	0.301	0.195	0.480	0.543

Linear regression

Table 4 Results of linear regression of mother class, class of daughters, offspring classes and mother's jump index, depending on the year of birth of the offspring

Dependent variable	Regression coefficient	Reliability value (%)	Probability F
Class of dams	0.003	0.28	0.718
Class of daughters	-0.094	15.48	0.163
Class of offspring	-1.243	16.00	*
Jump index of dams	-4.986	21.66	**

When evaluating the linear regression of the dams' class, we record an increasing tendency of the regression coefficient, although this exhibits a very low reliability value. This dependent variable also emerged as inconclusive in the test.

In the case of the daughters' class, the regression coefficient decreases, with explanatory power of 15.48%, and here the test is again inconclusive.

With the offspring's class, we again recorded a decreasing tendency of the regression coefficient, which exhibits 16% reliability and according to our test emerges as statistically conclusive. Based on this, it can be stated that the assessed class of offspring's of the sire 6062 Scyris (POL) does not improve – on the contrary, it decreases slightly.

The jump index of dams exhibits a high value for the downward trend (-4.9860) which is documented by 21.66% reliability. In this case, the test emerged as highly statistically conclusive. Based on this, it can be concluded that the mares mated with stallion 6062 Scyris (POL) are of relatively low quality in performance terms.

Analysis of the colouring of the offspring of stallion 6062 Scyris (POL)

It was determined that a total of 10 out of the 48 descendants had a grey dam and the remaining 38 were of other colouring. It was also established that 28 out of the 48 descendants are of grey colouring and the remaining 20 are of other colouring. Expressed in percentages, 21% were from grey dams and 79% from dams of another colour, and with the offspring 58% are grey and 42% of the offspring are of a different colour. From what has been established here, it can be stated that the breeding stallion 6062 Scyris (POL) carries a heterozygous allelic pair of G genes (Gg). Although in the heterozygous assembly this gene does not have a 100% influence on grey colouring, if it encounters a heterozygous set of the G gene in the dam, their offspring will be grey in colour.

CONCLUSION

The main aim of this thesis was to evaluate the importance of the stud horse 6062 Scyris (POL), which is a member of the English Thoroughbred breed, in the breeding of the Czech Warmblood. Two databases were compiled from all the background information on the descendants. The first was used to evaluate linear regression, where we observed changes in the class of all 48 descendants and a change in the dependence of the class of the 14 assessed daughters, and we evaluated a change in the dependence of the jump index and class of the dams of all 48 descendants –all of this depending on their year of birth. The second database was used to evaluate the Pearson correlation coefficient, where

we observed and assessed the correlation dependence of the individual body measurements in the 14 daughters that had been assigned a class and their dams. In this thesis we also recorded the minimum, maximum and mean values of the individual body measurements of the 14 daughters and their dams, and the numbers belonging to either sex, and we also performed an analysis of colouring. From all the information ascertained, we came to the conclusion that stallion 6062 Scyris (POL) did not balance out his offspring in exterior terms as expected and carries a heterozygous set of alleles in the G chromosome, which influences grey colouring. In the coming years it would be interesting to re-examine the results and conclusions that we have reached in this thesis.

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THE INFLUENCE OF ENVIRONMENTAL CONDITIONS AND THE MONTH OF BIRTH ON THE PROSPERITY OF FOALS OF THE CZECH WARMBLOOD BREED ON THE STUD FARM ŠCHK – KUBIŠTA

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Abstract: The experiment was conducted in the stud farm of our choice. In the first phase we assessed the prosperity of the foals based on the effect of the month of birth. For this purpose, we selected 14 high-pregnant mares which were monitored from 1 March 2017 until 26 August 2017. After foaling the born foals were monitored individually. The foundation database was made out using the Microsoft Excel programme. The General Linear Model GLM, Unistat 6.5. programme was used to process the results. If the effect was statistically significant, the differences among the effects were determined using multiple comparisons by means of Scheffe's test. The test was conducted at levels of significance of $P < 0.05$ and $P < 0.01$. The dependent variables were the following: body temperature, incidence of defects and weight gains of the individual foals. The sources of variability were the following: effect of the month of birth, effect of sex, effect of the outdoor temperature. We found that the outdoor temperature had a statistically significant effect on their body temperature and incidence of defects (health problems). The other effects were statistically insignificant.

KeyWords: foals, temperature, horse, prosperity, environment

INTRODUCTION

The present situation in horse breeding in the Czech Republic is relatively good. The numbers of horses are continuously increasing, interest in horse breeding as well as working in this area is also on the rise. It is important for the breeders and owners of horses to be well informed about the optimal conditions they must provide for the horses. Only a few studies deal with the effect of temperature on the ethology of horses and the prosperity of foals.

Newborn foals are growing very fast initially, although direct selection for this property has never been done. In medium sized breeds, the daily flock is 1.5 kg during the first month of life. The birth weight of the foal will double within 30–35 days and will increase to two months. Its further increase is slower. (Meyer and Coenen 2003). In the newborn foals, the percentage of suction time is 12.3% within 24 hours. The average suction frequency in the monitored period is 115 times. This means that the foals are sampled 4.8 times in each hour of the observed period (Duruttya 2005). Regular control of the health of the foals is very important during the intake period. Especially in large breeds there are often respiratory diseases in this period. Therefore, it is important to measure the temperature regularly, preferably daily and follow the temperature curve (Jiskrová and Misař 2001).

Weaning is stressful both for the foal and the mare. The mare usually gets over this period quickly, but for the young foal weaning is more often than not a great strain. Stress may cause the foal to temporarily lose its appetite, it impairs its condition and increases its susceptibility to parasitic and virus infections (Hošák 2009). Dušek (2007) pointed out that stress as a marked change in the optimal environment, particularly if it is sudden, greatly strains the organism of the foal. Stress may also affect the behaviour and body temperature of the horse. The changes may be sudden or may be the result of a long term effect of stressors. These stressors differ and may be the following: effects of the environment – e.g. thermal effects; transport; micro or macro climate; nutritional effects; effects of infectious diseases; psychological effects.

In order to provide satisfactory and sufficiently conclusive information on this issue and to monitor the thermal indicators in the stable we chose a closed herd turnover stud farm counting 140 horses of the Czech warmblood breed of all age categories.

MATERIAL AND METHODS

The experiment was conducted at the stud farm of our choice. ŠCHK – Kubišta near Hradec Králové. This stud farm has approximately thirty-one horses of breed Czech warm-blooded. The horses are hunted here in a combined way (in the stable and pasture). There are 4 categories of horses in the stud. Mares with foals, mares that are empty, young stallions in training and young mares in training, mares in rearing and stallions in rearing. In the first phase we evaluated the **prosperity of the foals based on the effect of month of birth**. For this purpose, we selected 14 high pregnant mares before foaling which were monitored from 1 March 2017 until 26 August 2017. After the mares gave birth the foals were monitored individually from their birth to weaning, i.e. from March to October. The body temperature was measured at ambient temperature fluctuations above 5 °C. The temperature and relative humidity of the environment in stable were measured with a thermohydrograph in two hour intervals over the entire experiment; for statistical evaluations we used the highest and lowest temperatures during the respective days.

The dependent variables were as follows:

- **The body temperature,** – the body temperature of the foals was measured when the outdoor temperature fluctuated above 5 °C at 18:00 in the stable.
- **Incidence of defects** – health problems. After consultation with a veterinarian health problems were categorized as follows:
  - No problems = 0
  - Enlarged lymphat degree 1 = 1
  - Enlarged lymphat degrees 2 = 2
  - Enlarged lymphat degrees 3 = 3
  - Brittle hoof horn = 4
  - Contracted hooves on pastures = 5
- **Growth** were monitored on the basis of beneficial growth by means of the weight index = heartgirth² x length / 11877.4¨ = weight of horse. The weight of the foal was monitored at the end of every month. Since the horse is characterized by its sporting performance and not by meat production, it has been determined to measure the increment only once a month.

The sources of variability were the following:

- **Effect of the month of birth** – Selected foals were born between the months of March and April. These foals were compared to each individual increment.
- **Effect of the temperature environment** – the temperature environment was measured by a thermohydrograph from COMET SYSTEM. The thermohydrograph was placed in a herd of mares with foals. Temperature measurement was set every two hours during the mares in the stable. From each measured day, the lowest and highest temperatures were selected. These data were subjected to static analysis of the General Linear Model GLM and subsequently evaluated by Schefe's test.
- **Effect of sex** – Total were observed 14, 9 of which were colts and 5 were fillies

The database was made out using the Microsoft Excel programme. The General Linear Model GLM, Unistat 6.5. programme was used to process the results. If the effect was statistically significant, the differences in the values were determined using multiple comparisons by means of Scheffe's test. The test was conducted at the levels of significance of $P < 0.05$ and $P < 0.01$.

RESULTS AND DISCUSSION

Monitoring the prosperity of foals based on the month of birth and the temperature of the environment.

*Table 1 The effect of outdoor temperature on the dependent variable – **Body temperatures of the individual foals** based on the general linear model*

Source of variability	Probability
Month of birth	0.2307
Sex	0.0989
Outdoor temperature	0.0128

Legend: Red figures = P < 0.05

Table 1 shows that the outdoor temperature had a statistically significant effect on the body temperature of the foals. These results confirm our practical breeding experiences that the body temperature of foals increases when the changes in temperatures are high. Therefore, we recommend regular health examinations to be carried out in the case that a higher body temperature causes changes in the health condition of the foal.

*Table 2 The effect of outdoor temperature on the dependent variable – **Incidence of defects = health problems** based on the general linear model*

Sources of variability	Probability
Month of birth	0.1521
Sex	0.9065
Outdoor temperature	0.0310

Legend: Red figures = P < 0.05

Table 2 shows that the outdoor temperature had a statistically significant effect on the incidence of defects. The results of this table confirm our previous assertions and recommend the breeder to check the health condition on a regular basis. It is important to note that the incidence of an enlarged lymph increases when the outdoor temperature exceeds 25 °C. Dražan (2001) shows the optimum temperature for horses between 8–18 °C. The horse's ability to adapt to cold depends on the duration of the cold weather and on the horse's energy intake. The latter factor, energy intake, is the most critical in determining how readily a horse develops a tolerance for cold. Horses lose weight if they do not eat enough energy to offset the heat loss to the cold surrounding air. Enough feed and good-quality feed are needed to supply adequate energy intake for the horse (Cymbaluk and Manitoba 2001). Hošák (2009) maintains that enough energy in the feed ration is very important for the correct development of the growing organism; on the other hand, a great surplus is not suitable either. Over feeding the growing foals is not desirable because it increases the risk of developmental orthopaedic disorders. Hence the breeder is recommended to allow the foal or weaned young animal sufficient and adequate movements and energy release.

*Table 3 The effect of month of birth and sex on the dependent variable – **Weight gain of the foals**, based on the general linear model*

Source of variability	Probability
Month of birth	0.0698
Sex	0.8996

Legend: Red figures = P < 0.05

Table 3 shows that the month of birth and sex had statistically insignificant effect on the weight gain of the foals.

CONCLUSION

The effect of the outdoor temperature on, their body temperature and incidence of defects (health problems) was statistically significant. The other monitored effects were statistically insignificant. The breeder is recommended the following: the breeder should see to the proper technology of rearing, ensure that the foal has a sufficient supply of roughage and concentrates, provide a sufficiently long period of time on the pasture with corresponding equipment. The stable must be equipped with ventilation to allow air circulation for the high number of animals housed on deep litter. In the summer months the deep litter must be removed every month and after each removal the stable must be disinfected. The farmer should check the animals on a regular basis.

ACKNOWLEDGEMENTS

This work was funded by the Internal Grant Agency at the Mendel University in Brno, Faculty of AgriSciences, under Grant TP 7/2017: Analysis of performance and behaviour of farm animals in relation to ambient temperature variability and possibilities of elimination of its impact.

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SUM OF EFFECTIVE TEMPERATURES AND ITS EFFECT ON YIELD OF CZECH FLECKVIEH-SIMMENTAL

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Abstract: This study is aimed on the effect of sum of effective temperature (SET) on the yield of Czech Fleckvieh-Simmental. The study was made on a private farm in Czech Republic (49°12'36.7"N 16°23'42.1"E) between May and July 2016. The cows were stabled in free-stall permanently open-sided barn. There were 114 cows in total present during the whole duration of experiment. Data were collected via data logger placed in the middle of the barn. For this particular experiment, the section with a largest yield was chosen. The cows in this section were divided into three groups: A (25 and more kg of milk per day), B (20.1–24.9 kg of milk per day) and C (20 and less kg of milk per day). The results shows that there is a significant negative correlation between SET and yield of all cows (r = -0.535). Also for groups A (r = -0.304), B (r = -0.178) and C (r = -0.355) there is a significant negative correlation. Our results could indicate, that SET is more appropriate for assessing a heat stress than a temperature itself.

Key Words: SET, Czech fleckvieh-simmental, heat stress, yield, cattle

INTRODUCTION

Internal and external factors have a big influence on yield and milk quality (Cimen et al. 2010, Bayram et al. 2009). Factors like stage of lactation and parity (Summer et al. 2003) and feed (Davies and White 1958) are also relevant factors of milk production. Temperature of environment belongs between these factors. The high temperature can be a factor, that lowers the quality and quantity of commercially sold animal products (Fuquay 1981, Morrison 1983). According to Purwanto et al. (1990) the cows with bigger yield suffers from heat stress more that those with lower milk production. Fans and showers can be a way to alleviate the effects of high environmental temperature (Her et al. 1988). According to West et al. (2003) hot weather has larger impact on yield in comparing to cold temperature which had a little effect on milk production.

The effective microclimate in barn is essential for achieving the competitive milk production through good animal welfare (Velecká et al. 2014). According to Berman et al. (1985) the amount of heat that animal receives could be affected by factors like housing system, location of animal and social rank. When high humidity and temperature are both present at the same time, the feed intake, reproduction and milk production are affected negatively (Erbez et al. 2012). Vokřálová et al. (2007) claims, that the thermosneutral zone of cattle ranges between -5 °C to +24 °C.

Phase of lactation also alters the level of heat stress toleration. According to Bouček et al. (2009) the ability to cope with temperature stress is worse during the first stage of lactation and right after calving.

Also, the financial losses are present when heats stress in in effect. St-Pierre et al. (2003) claim, that the losses in USA were $728 million annually. That is 43% of all national losses.

Sum of effective temperatures (SET) could be used for many purposes. Lin et al. (2007) it for assessing the seahorse egg development. SET is a sum of temperatures, that are crucial for development, growing and other processes in organisms. Finch et al. (1986) in their work claim that if the intake of heat is greater than heat output, heat can be stored in the body. A number of studies

using SET were made on fruit or insect. But very little is known about SET effects on cattle and milk production. That is why SET calculation was used to assess the effect of SET on milk production of Czech Fleckvieh-Simmental.

MATERIAL AND METHODS

For those work, an experiment was executed on private farm Genagro Říčany (49°12'36.7"N 16°23'42.1"E) in Czech Republic. The experiment lasted three months (May–July) of 2016. The section with the highest milk production was chosen for experiment. According to other authors, the impact on this section should had been the highest. The cows were divided to 3 groups according to yield for experimental purposes: A (25+ kg of milk per day), B (20.1–24.9 kg of milk per day) and C (20 and less kg of milk per day).

In total, there were 114 cows included in this experiment. The feed ration was same for all cows and constant during the experimental period. It included: 22 kg of maize silage, 13 kg of lucerne silage, 0.7 kg of cut straw, 4 kg of sugar beet pulp, 4 kg of molasses, 3 kg of brewing dough, and 8 kg of special mix for high-yield cows mix. This mix is specially designed for the high production cows. It contains 57% of barely and whey, 20% of extracted rapeseed grind, 15% of extracted soybean grind 7% of mineral premix and 1% of feeding urea. Cows were stabled in unusual barn, because the sides of it are opened for whole year.

Temperature data collection was provided by data logger placed in the middle, between the sections in height of withers. Data logger collected temperature data every 30 minutes for duration of whole experiment. Milk yield data were extracted from milk parlor. All data were analyzed in programs STATISTICA 12 and MS Excel 2016. The SET was used to analyze the heat load received above certain level and its cumulation effect. From data we selected temperatures above 23 °C. Some authors claim that a heat stress limit for cattle is 21 °C (Bernabucci et al. 2014, Igono et al. 1992). Nevertheless, it was decided to adhere to the 23 °C limit because this was already proven by other authors (Polák et al. 2011, Večeřa et al. 2016). The SET value was calculated daily according to following formulas:

$$K = T - 23$$

$$SET = K_1 + K_n$$

K Effective temperature

SET Sum of effective temperatures

T Temperature above 23 °C

K_1 First effective temperature of a day

K_n Every other effective temperature of a day

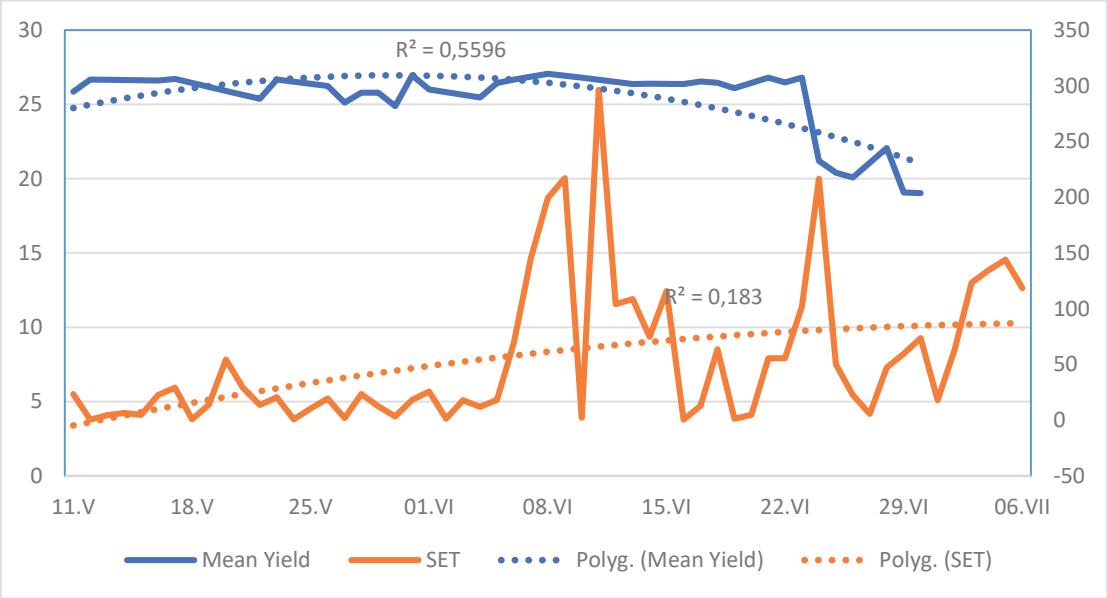
The Monthly SET values were calculated as a mean of whole month.

Data logger also measured a humidity inside of a barn. Zejdová et al. (2014) claim, that the correlation between temperature and temperature-humidity index (THI), which is traditionally used to assess heat stress, is high (r = 0.998). Therefore, in Czech Republic, there is almost no reason to use THI for evaluation of heat stress. This statement is supported by findings of Paldusová et al. (2014), who claim that THI is not ideal for assessing of heat stress in Czech Republic due to weather conditions without sudden changes.

RESULTS AND DISSCUSION

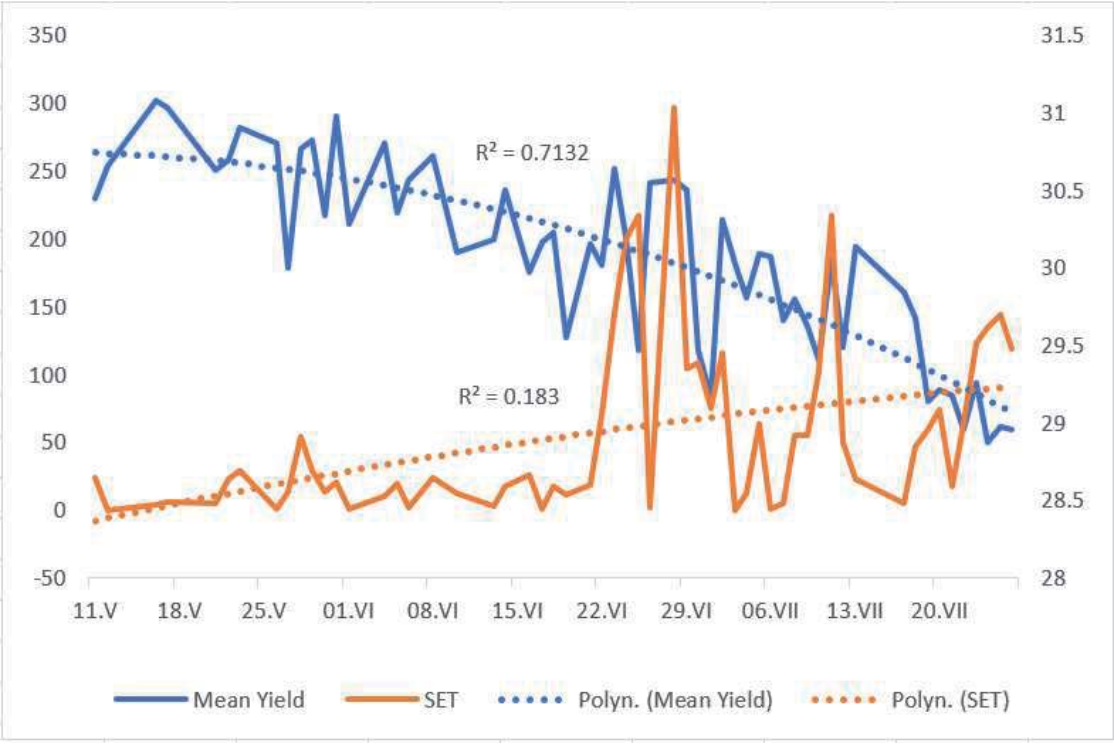
The relationship between mean yield of cows in all groups and SET is presented in Figure 1. As can be seen there is a moderate correlation between yield and SET (r = -0.535). This figure shows that with more heat received above the heat limit (23 °C in case of this experiment) the amount of milk is decreasing. The correlation was highly statistically significant (p = 0.000).

Figure 1 Relationship between set and all groups yield



The relationship between SET and yield of group A (25 and more kg of milk per day) is presented in Figure 2. Correlation was found here as well (r = -0.304, p = 0.021) as same as in the previous figure.

Figure 2 Relationship between SET and yield of group A



The relation between SET and yield of group B (between 20.1 and 24.9 kilograms of milk per day) is presented in Figure 3. As well as in the previous figures, the negative correlation (r = -0.178) between SET and yield is apparent. Nevertheless, this group had a weakest correlation of all. This correlation was not significant (p = 0.185).

The relationship between the SET and yield of group C (20 and less kilograms of milk per day) can be seen in Figure 4. As on all previous figures, the correlation (r = -0.355, p = 0.007) is negative. The correlation is stronger than in Figure 3. This mean, that group C is more affected by SET.

Figure 3 Relationship between SET and yield of group B

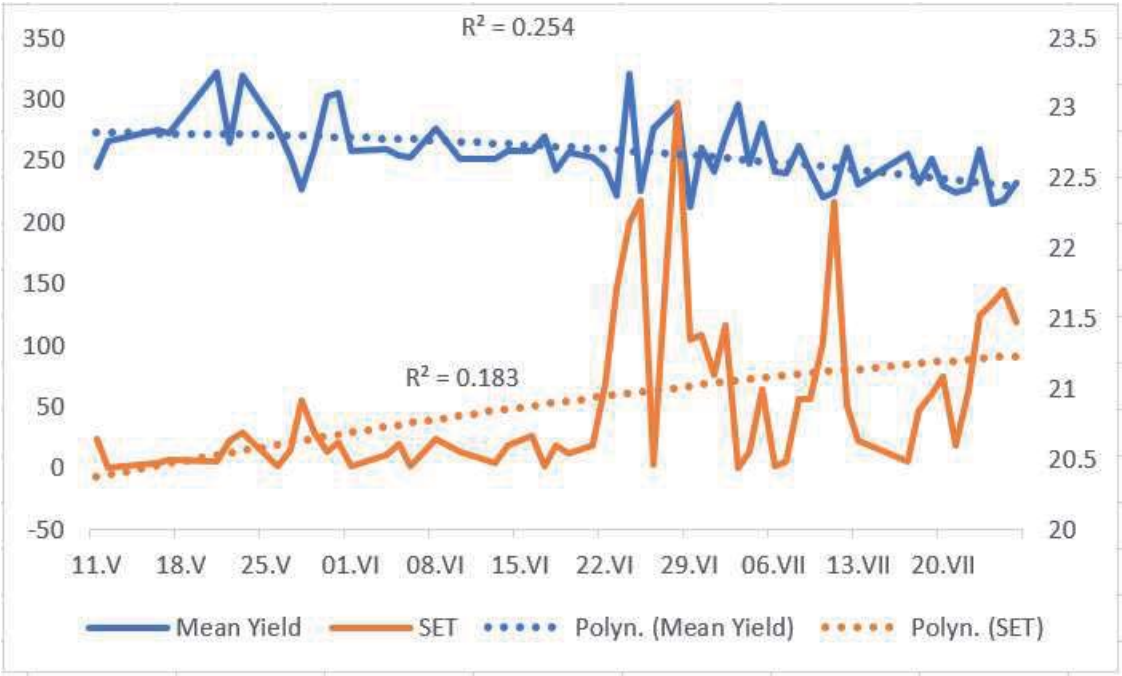


Figure 4 Relationship between SET and yield of group C

On all four Figures 1–4 can be seen that a temperature and SET is in a negative correlation. The group A with a biggest yield is the most affected by a SET changes. Therefore, it can be said, that a cowswith higher yield are affected by SET more. This is also supported by statement of West (2003) and Kadzere et al. (2002) that claims that a cows with bigger yield are affected by temperature more. Purwanto et al. (1990) claims that the sensitivity to a high temperature is conditioned by a higher heat production caused by intensive metabolism. Araki et al. (1984) claim, that a body temperature of cattle is very sensitive to environmental temperature and its changes. This also supports the previous statement. The correlation between SET and yield of group B was lower that the one of group C. This seems like it is in a contrary with our statement, but this correlation was not significant. Toušová et al. (2017) claim, that an overall production in summer is higher. It could be caused by overall higher

production in summer period, but during the heat waves the yield should decrease. This hypothesis could be supported by our findings.

Table 1 Relationship between SET and month

	Mean	Min	Max	S_x	V_x
V.	17.12	0.24	54.25	14.57	372.24
VI.	62.24	0.63	296.42	83.11	356.68
VII.	67.93	0.24	216.6	54.04	400.79

On Table 1 and 2 we can see the relationship of month and SET and yield. As is shown on Table 1, there is a visible difference between SET in each month. Especially between May and rest of the experimental period. Nevertheless, this difference was not significant. Same case is shown on Table 2. There is a difference between yield of groups and each month. Although this difference is perspicuous, it was not significant.

Table 2 Relationship between yield and month of all three groups

	A (25 and more kg of milk)				
Month	Mean	Min	Max	S_x	V_x
V.	30.70	30.00	31.07	0.30	10587.04
VI.	30.24	29.47	30.80	0.37	8046.50
VII.	29.55	28.87	30.31	0.44	6773.26
	B (20.1–24.9 kg of milk)				
Month	Mean	Min	Max	S_x	V_x
V.	22.85	22.42	23.25	0.24	9481.61
VI.	22.68	22.29	23.24	0.20	11239.45
VII.	22.57	22.32	23.03	0.18	12591.31
	C (20 and less kg of milk)				
Month	Mean	Min	Max	S_x	V_x
V.	16.53	14.47	18.48	0.92	1803.83
VI.	15.55	12.49	17.52	1.65	940.03
VII.	16.08	13.20	17.06	0.98	1640.89

ACKNOWLEDGEMENT

The study was supported by the grant project IGA TP 7/2017.

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RUMINAL DEGRADABILITY OF DRY MATTER AND CRUDE PROTEIN IN UNTREATED AND SOLVENT-EXTRACTED SOYBEAN MEAL

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Abstract: The aim of the study was to determine the dry matter and protein degradability in untreated ground soybean (S) and solvent-extracted soybean meal (SSBM) using the in sacco method. The experiment was carried out on two ruminally cannulated sheep that were fed twice a day a diet consisted of hay and a supplemental mixture. The nylon bags with feed samples were incubated in the rumen for 0, 2, 4, 8, 16 and 24 h. The effective degradability (ED) of dry matter (DM) and crude protein (CP) was calculated at outflow rate of 0.05 h. The ED of DM in S was 79.1% and was higher than in SSBM being 73.2%. Higher ED of CP was also observed in S (79.9%) compared to SSBM (69.4%). SSBM had a lower soluble fractions and a lower rates of degradation of DM and CP than untreated soybean (S).

Key Words: in sacco, rumen degradation, protein, untreated soybean, solvent-extracted soybean meal

INTRODUCTION

The main objective of each nutritionist is to estimate the nutritional value of feed for the purpose of properly creating feed ration. In this case, knowledge of the specific chemical composition of feed is a basic requirement. In the area of ruminant nutrition, other findings are also being used, such as kinetics of feed degradation in the rumen, which significantly influences the nutritional value of the feed and is an important factor in creating the diet (Belanche et al. 2014). Many in vitro methods have been developed to determine the feed degradability (Deaville et al. 1997), but the use of rumen fluid is irreplaceable in some cases (Chaudhry 2007, Schadt et al. 2014). The in sacco method still remains the popular method (Hristov 1992, Homolka et al. 2007). Using nylon bags, the feed is placed in the rumen and incubated for a certain time. In the final phase, progressive disappearance of feed is evaluated (Belanche et al. 2014). This method is costly due to the use of cannulated animals, but its results are a source of valuable information which can not be always detected by in vitro methods (Chaudhry 2007).

Soybean is an important component in the diet of ruminants. In terms of nutritional value, it is an important source of protein, but the specific composition varies depending on soybean processing. Soybean can be fed as untreated or in the form of heat-treated soybean components, from which solvent-extracted soybean meal (SSBM) or extruded soybean meal (ESBM) are used most frequently. In the case of SSBM, soybean is deffated, so SSBM differs from ESBM mainly in lower fat content and higher protein content (Radivojević et al. 2011, Giallongo et al. 2015)

The aim of the study was to determine the dry matter and protein degradability in untreated ground soybean and solvent-extracted soybean meal using the in sacco method.

MATERIAL AND METHODS

Samples

Soybean (S), full fat, ground to pass through a 2-mm screen. Solvent-extracted soybean meal (SSBM), by-product of the production of soybean oil, obtained by the solvent extraction of soybean, ground to pass through a 2-mm screen. The chemical composition of these feed samples is given in Table 1.

Table 1 Chemical composition of soybean (S) and solvent-extracted soybean meal (SSBM)

Items	Units	S	SSBM
Crude protein (CP)	g/kg	402.3	482.0
Fat	g/kg	192.5	16.5
Crude fiber (CF)	g/kg	81.4	59.0
Acid detergent fiber (ADF)	g/kg	104.9	89.7
Neutral detergent fiber (NDF)	g/kg	188.8	156.6
Ash	g/kg	56.4	75.0

Animals

All animal procedures were in accordance with the Czech legislation (Approval No. 28987/2017-MZE-17214).

Two ruminally cannulated sheep were used in this study to determine ruminal degradability of dry matter and crude protein using *in situ* method. The sheep were fed twice a day and their daily feed rations consisted of hay (0.95 kg) and supplemental mixture (0.65 kg) containing in g/kg: barley 200, malt sprouts 100, solvent-extracted sunflower meal, unpeeled 300, lucerne meal 300, grain germs 30, molasses 5, feeding salt 5, limestone 25, monocalcium phosphate 20, microminerals and vitamin premix 3, PROBIOSTAN E10 12). The sheep had free access to fresh drinking water and were adapted to the diet for more than two weeks before starting the incubations. Two-gram samples were weighed into bags 5 × 14 cm of 42 µm pore size (Uhelon 130 T, Silk and Progress Moravská Chrastová). The bags with feed were inserted into the rumen and incubated for 0, 2, 4, 8, 16 and 24 h. After rumen incubation, except for the zero incubation interval, all bags were rinsed in cold tap water for 1 min to remove the coarse content of the rumen from the bag surface and subsequently washed three times in a washing machine (without the spinning programme) for 10 min. Zero-hour bags were only pre-soaked in warm water (37 °C, 10 min) and machine washed. After washing the bags were dried at 60 °C for 48 h to determine DM degradability. The residues from the bags were analysed to determine the CP content.

The disappearance parameters and the effective degradability (ED) were calculated as described by Ørskov and McDonald (1979) with rumen particulate outflow rate (k) of 0.05 h. No corrections for microbial contamination were made.

RESULTS AND DISCUSSION

Results presented herein are preliminary results of the project focused on the rumen degradability of isoflavones and amino acids in two soybean feedstuffs, untreated ground soybean (S) and solvent-extracted soybean meal (SSBM).

The content of nutrients of both feedstuffs described in Table 1 is in agreement with the characteristics of these feeds reported by e.g. Sauvant et al. (2004), Akbarian et al. (2014) or Schadt et al. (2014) but lower than values mentioned by Gonzales et al. (2002), Mjoun et al. (2010) or Giallongo et al. (2015).

The effective degradability of DM and CP was calculated at rumen particulate outflow rate (k) of 0.05 based on k values determined for concentrate feedstuffs (Homolka et al. 2007) considered to be representative of medium feed intake levels (ARC 1984).

The degradation kinetics and ED of DM in S and SSBM are presented in Table 2 and Figure 1. The DM of both studied feedstuffs was extensively degraded in the rumen *in situ*. Higher degradability of DM was found in S (79.1%) than in SSBM (73.2%). Compared to our results Wulf et al. (2005) reported lower values of ED (k = 0.05 h) in ground untreated soybean (69%). The degradation parameters in their study were as follows: soluble fraction (a) 34.1%, potentially degradable fraction (b) 61.9% and rate of degradation (c) 0.083 h. Maxin et al. (2013) calculated ED of DM for soybean meal of 72.6% at outflow rate of 0.074. In agreement with Wulf et al. (2005) SSBM in our study had a lower soluble fraction (a) and a lower rate of degradation (c) of DM than untreated soybean (S).

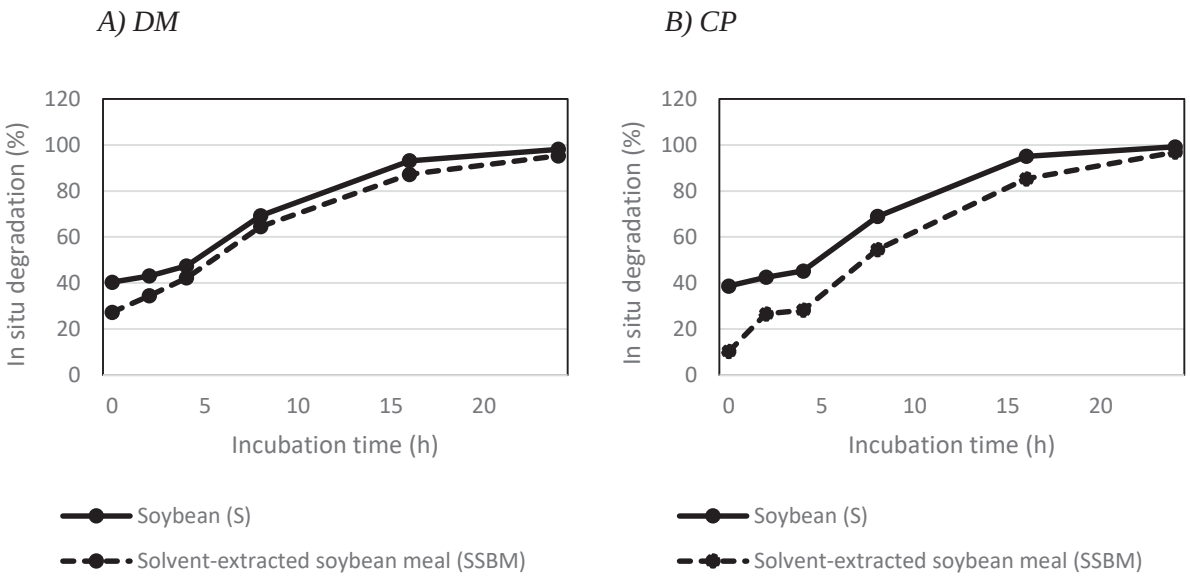
The degradation kinetics and ED of CP in S and SSBM are presented in Table 2 and Figure 1. Rumen degradation of CP was extensive in both samples, however a lower degradability of CP was observed in SSBM reaching 69.4% while the ED found in S was 79.9%. Borucki Castro et al. (2007) reported lower value of ED of solvent-extracted soybean meal, 58%, with an outflow rate of 0.08 h while Maxin et al. (2013) found that ED of CP was 66.0% at outflow rate of 0.074 h. Lower ED (k = 0.06 h) for untreated soybean of 67.4% mentioned Wulf et al. (2005). These discrepancies could be caused by various outflow rates and by different feed particle sizes.

Table 2 Rumen degradation parameters and effective degradability of dry matter (DM) and crude protein (CP) for untreated (S) and solvent-extracted soybean meal (SSBM)

Disappearance parameters	Units	DM		CP	
		S	SSBM	S	SSBM
Soluble fraction, a	%	28.8	21.4	26.6	9.7
Potentially degradable fraction, b	%	69.6	73.8	73.2	87.8
Rate of degradation, c	h	0.13	0.12	0.13	0.11
Effective degradability, ED*	%	79.1	73.2	79.9	69.4

** ED calculated at rumen particulate outflow rate k = 0.05*

Figure 1 Rumen degradation of dry matter (DM) and crude protein (CP) for untreated (S) and solvent-extracted soybean meal (SSBM)



While degradation parameters of CP in S were higher than those calculated by Akbarian et al. (2014), the disappearance parameters a, b and c of CP determined in our study for SSBM are in

agreement with values reported by Borucki Castro et al. (2007) for solvent-extracted soybean meal being a = 9.9%, b = 87.0% and c = 0.105 h and close to values given by Mjoun et al. (2010) for the same feedstuff being a = 8.1, b = 91.9 and c = 0.12 h. On the other hand, values determined by Maxin et al. (2013) were considerably higher. As mentioned above the ED can be influenced by various outflow rates and by different feed particle sizes. Furthermore, the degradation profile can also be affected by variability in the composition of original grain or by variability in technological processing of feedstuff.

CONCLUSION

Preliminary results of our in situ study suggested that ruminal dry matter (DM) and crude protein (CP) degradability was lower for the solvent-extracted soybean meal compared to untreated ground soybean. The solvent-extracted soybean meal had a lower soluble fractions (a) and lower rates of degradation (c) of DM and CP than untreated soybean.

ACKNOWLEDGEMENTS

This study was supported by the Internal Grant Agency (IGA) of the University of Veterinary and Pharmaceutical Sciences Brno project No. 206/2017/FVHE.

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EVALUATION OF THE RESULT RELIABILITY OF BASIC MILK COMPOSITION IN AN AUTOMATED MILKING SYSTEM THROUGH INDIRECT REAL-TIME ANALYSIS

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Abstract: Current and regular knowledge of the milk composition and properties is important for controlling the dairy herd health status, prevention of the occurrence of their production disorders and promoting of milk quality. Therefore, the validation of the result reliability of milk analyses by system of real milk analyses (RTA) in the automated milking system (AMS) was performed. Fat (F), crude protein (P), lactose monohydrate (L), solids-non-fat (SNF) contents and somatic cell count (SCC) were determined. This was done by a direct statistical comparison of the parallel measured results, i.e. RTA and relevant reference – infrared spectroscopy (MIR; F, P, L, SNF) and flow cytometry (FC; SCC) in 30 individual cow milk samples. The results of RTA reliability in AMS as coefficients of determination (R) and correlation (r) of results between methods (RTA and reference) were as follows: r between MIR × RTA methods for F, P, L and SNF 0.524 ($P < 0.01$), 0.744, 0.701 and 0.731 ($P < 0.001$); R explains 27.5, 55.3, 49.2, and 53.5% of the RTA variability by reference method (MIR). For SCC it was 0.758 ($P < 0.001$) and 57.4% variations in RTA values can be explained by FC variability. The reliability of the results of RTA milk analyses in the AMS has been found to be suitable for the above mentioned purposes after appropriate comparison.

Key Words: cow, milk fat, crude protein, somatic cell count, correlation

INTRODUCTION

The regular knowledge of the composition and properties of individual cow milk is essential for managing of animal health preventing the occurrence of their production disorders and ensuring of their milk quality. This is important for the good level of dairy cattle rearing and the operational safety of farmers. Today the various modern automated systems for milking (automated milking system, milking robot, AMS) and milk analyses (indirect real-time analysis methods, RTA) are used. This combination is highly advanced and has been made possible by the development of hardware and software for milking and physico-chemical-analytical techniques (Kawasaki et al. 2008) recently. The reliability of these analytical results of milk composition and properties is important for proper operational decision-making about dairy herd.

Reference (direct, calibration) and routine (indirect, calibrated) dairy analytical methods are now incorporated into a system of relevant standards and nationally and internationally controlled networks (Leray 2007, Hanuš et al. 2011, 2014). In the RTA systems, the fat (F), protein (P), lactose (L) and solids-non-fat (SNF) contents, conductivity and somatic cell counts (SCC) are most often determined on the dairy farms. The RTA method is based on NIR-FT (near infra-red analysis with Fourier transformations; Kukačková et al. 2000, Tsenkova et al. 1999, 2000, Jankovská and Šustová 2003, Šustová et al. 2007) procedure which must cope with unfavourable measurement conditions in the flow analysis, e.g. with milk flow and foaming. The RTA method (AfiLab) and its result reliability in the evaluation of milk quality have been studied by some authors (Ishay et al. 2011, Arazi et al. 2012, Kaniyamattam and de Vries 2014, Hanuš et al. 2016) as well as the practical interpretation of its results (Katz 2007, Katz and Pinski 2008, Karp and Petersson-Wolfe 2010). In the first place, the RTA method has been used and evaluated in classical milking parlours (Katz 2007, Hulová et al. 2014, Hanuš et al. 2016) and now is increasingly combined with AMS (Kamphuis et al. 2008, Kawasaki et al. 2008) to achieve highly sophisticated and effective milk obtaining systems. Therefore, it is also necessary to evaluate this combination of RTA with AMS for the application of the results to control of dairy herds.

The paper aim was to evaluate the result reliability of the indirect determination of cow milk composition in an automated milking system (AMS) using a real time analytical method (RTA) after its calibration to conventional methods of milk recording for the methodological purposes of sample selection to study milk fat composition with respect to its fatty acid profile under FAMAS project conditions.

MATERIAL AND METHODS

Experimental design

One 30 individual milk samples were automatically collected in one day. This dairy farm with free housing currently nurses 57 Holstein cows. These are milked several times a day via a milking robot (AMS, Automatic Milking System, Lely Astronaut, Lely Industries NV, Netherlands). The average daily milk yield of sampled dairy cows per day and AMS visit was: 27.87 ± 7.51 kg/day; 9.54 ± 2.93 kg/visit. Similarly, the average lactating days were 137.3 ± 92.8 and average lactation was 2.3 ± 1.54. The dairy farm (GPS: 16°18′21″ e. l.; 49°31′8″ n. w.) is located at an altitude of 540 m.

The cows are reared in ecology regime for production of organic milk. These were fed in green belt by legume-grain mixture (triticale, pea, clover) with concentrates in the given summer season. Individual milk samples were at the AMS during an accidental milking of cows during one day in July. These were preserved with bronopol (0.03%) and stored in a refrigerator. Then, the samples were transported to the laboratory and analysed. 30 milk samples were collected by one RTA unit (Lely Astronaut). Data on the milk composition were taken from the RTA equipment.

Chemical and statistical analysis

The instrument calibrations (indirect methods) on the F, P, L SNF and SCC in the milk recording laboratory (the LRM Buštěhrad) and at the VÚM Praha (workplace Šumperk) were carried out in accordance with previous works (Hanuš et al. 2007, 2008, 2011). The indirect methods MIR–FT and MIR (F, P, L and SNF; with Fourier transformation (FT) and interferometer and without FT and with optical filter technology; CombiFoss FT + and MilkoScan 133 B, Foss Electric, Hilleröd, Denmark) and flow cytometry (FC; SCC; CombiFoss FT +, Foss Electric, Hilleröd, Denmark; Somacount 300, Bentley Instruments, Chaska, Minnesota, USA) were in the position for creation of reference values for RTA calibration and reference values for RTA validation.

The MilkoScan 133 B used for validation was regularly included in the performance of analytical proficiency testing (PT) with successful results. The combined enlarged uncertainties (CEU) of the measurement results were: ± 2.77% relatively for F (±0.101 for the original units (%); ± 2.59% rel. for P (± 0.085% orig.); ± 2.77% rel. for L (± 0.115% orig.). In both cases, MIR and RTA, the SNF content (%) was determined by the calculation, i.e., P + L + mineral bias (0.61%). The RTA values for validation (F, P, L, SCC) were means from last 5 milk measurements of cow according to relevant

software. The Somacount used for validation was also included in the performance of PT with successful results. The CEU of the measurement results was ± 9.3% at SCC < 900 10^3/ml.

To evaluate the results and compare the methods a linear regression model was used which is central in the evaluation of milk indirect analyses, all in MS Excel (Microsoft, Redmond, USA). With regard to the starting practical application of the RTA in the AMS in the relevant herd the determination coefficients and their relevant correlation coefficients were evaluated for the reliability of the analytical results including variability of individual result differences. These express the relationships between results of methods in right way. Possible average value shifts are easy to solve by calibration.

RESULTS AND DISCUSSION

Comparison of mean differences and relationships between milk analytical methods

The advantage of this evaluation as compared to other papers (Katz and Pinski 2008, Ishay et al. 2011, Hulová et al. 2014, Hanuš et al. 2016) was higher number of samples per RTA unit. This was done by direct comparison of the results measured in parallel arrangement. In previous comparisons more measuring units were included in milking parlours and thus, as a rule, fewer number of validation samples per unit. Reducing the influence of the variability of the measuring units makes it possible to statistically prove the own measurement potential of the method in better way. How the RTA calibration and then also validation were performed in one (Hanuš et al. 2007, 2008, 2011, 2014) laboratory network (Leray 2007) which was important experimental principle. At group and herd level the RTA instrument can be used as a reliable cow nutrition detector (Arazi et al. 2012). Small differences in daily fat and protein content were between RTA and reference data (-0.05 ± 0.28% and +0.01 ± 0.05%).

The mean differences between the RTA—MIR methods (Table 1) were: 0.04 ± 0.6% for F; -0.14 ± 0.32% for P; -0.17 ± 0.12% for L; -0.32 ± 0.32% for SNF. These are significant ($P < 0.001$), however, they can be corrected by calibration and are therefore less relevant for the relationship of methods. The MIR × RTA correlations between methods were: 0.524 ($P < 0.01$); 0.744 ($P < 0,001$); 0.701 ($P < 0.001$). The correlation for the F was slightly lower compared to the previous observation (0.524 and 0.733 or 0.787), comparable for P (0.744 and 0.785 or 0.788) and significantly higher for L (0.701 and without relationship; Hanuš et al. 2016, Hulová et al. 2014). At F, P and L the explanation of RTA variability includes 27.5, 55.3 and 49.2% of the variability of the values of reference methods (MIR). The MIR × RTA correlation for SNF determination was 0.731 ($P < 0.001$). Here the explanation of RTA variability includes 53.5% of MIR variability.

The mean difference between RTA–FC was -9 ± 66 10^3/ml and significant ($P < 0.001$) for SCC. However, the shifts are easily solved by a subsequent constant bias in the next calibration (RTA) and do not substantially interfere with the principle relationship of the methods. Here in RTA a better relationship was to the reference results (0.758, $P < 0.001$) than in the previous observations (Hulová et al. 2014, Hanuš et al. 2016), where was not found. 57.4% of variations in SCC RTA values are explainable by variability in SCC FC values. This correlation can already be compared to the previous correlation results (0.48 bulk milk, $P < 0.01$, 0.78 individual milk, $P < 0.01$) between mastitis test NK and reference SCC (Hanuš et al. 1993). The SCC logarithmic transformation did not improve the relation of the methods. These good results were achieved at relatively low SCC values and their low original variability (69 ± 85 10^3/ml, v = 123%, x_g = 33 x 10^3/ml). In the case of work with a higher SCC mean and variability a better result comparable to the correlation values between other respected direct and indirect SCC methods can be legitimately expected (Hanuš et al. 2011; 0.75 and above, $P < 0.001$).

From the regression evaluation (Hanuš et al. 2016), the following correlation coefficients between RTA and infrared spectroscopy (MIR—FT and MIR) were obtained: for F from 0.733 to 0.743 ($P < 0.001$); for P from 0.785 to 0.787 ($P < 0.001$). Therefore, for F and P the explanation of the variability of the RTA method values includes 53.8 and 61.9% of the variability of the confronted reference (validation) methods. However, the relationship for L was not found.

Table 1 Basic statistical parameters (difference statistic) of validation data set and relationships (correlations) between results of methods MIR × RTA (in automated milking system) at analyse of individual cow milk samples

	Method						Differences		Correlation		
	MIR (n=30)			RTA (n=30)							
	$x \pm s_d x$	x_g	v%	$x \pm s_d x$	x_g	v%	$d \pm s_d d$	*P*	R^2	r	*P*
Fat (%)	3.75 ± 0.47	-	12.5	3.79 ± 0.69	-	18.3	0.04 ± 0.6	***	0.275	0.524	**
Protein (%)	3.18 ± 0.47	-	14.7	3.03 ± 0.26	-	8.7	-0.14 ± 0.32	***	0.553	0.744	***
Lactose (%)	5.02 ± 0.16	-	3.2	4.85 ± 0.07	-	1.4	-0.17 ± 0.12	***	0.492	0.701	***
Solid-non-fat (%)	8.81 ± 0.46		5.2	8.49 ± 0.28	-	3.3	-0.32 ± 0.32	***	0.535	0.731	***
SCC (10^3/ml)	69 ± 85	33	123	60 ± 29	54	47.6	-9 ± 66	***	0.574	0.758	***
log SCC	1.5239 ± 0.5417	-	-	1.7335 ± 0.2025	-	-	0.21 ± 0.467	***	-	-	-

*Legend: n – number of cases, x – arithmetic mean, $s_d x$ – standard deviation of x, x_g – geometric mean, v% – variation coefficient (v% = ($s_d x/x$) × 100), P – probability (** P < 0.01, *** P < 0.001),d – mean difference, $s_d d$ – standard deviation of d, R^2 – determination coefficient, r – correlation coefficient, MIR – infra-red spectroscopy in middle range with technology of optical filters, RTA – real time analyse (NIR infra-red (IR) spectroscopy in range near IR), SCC – somatic cell count, log – decadic logarithm log_{10}*

These values confirm the data mentioned by Karp and Petersson-Wolfe (2010: 64 and 76% for F and 45 and 52% for P), of course, these were obtained over a longer time period. These authors reported for L a variation in the methodological explanation of the variability of the RTA measured values by a determination from 19 to 52%. Overall means ± standard deviations of the monthly correlations between RTA and MIR were 0.59 ± 0.09 for F, 0.67 ± 0.04 for P and 0.46 ± 0.08 for L (Kaniyamattam and De Vries 2014). Hulová et al. (2014) found significant ($P < 0.001$) correlation coefficients between results obtained from RTA and infrared spectroscopy: 0.787 (F); 0.788 (P). However, correlations for L and SCC were not significant ($P > 0.05$). Kawasaki et al. (2008) introduced very significant determination coefficients for prediction of milk components to a specific model of NIR spectroscopy in AMS. These were 0.95 for F, 0.72 for P and 0.83 for L, which were by far the highest. However, the device apparently represented the classic NIR construction more than its low-cost RTA modification here described.

Comparison of variability of individual differences between milk analytical methods

The variability of individual differences (F, P, L) between infrared spectroscopy (MIR and MIR—FT) and RTA methods was much higher than between direct and indirect methods or indirect MIR and MIR—FT methods in this paper. This was similar (Hanuš et al. 2014) more to variability of the differences between MIR or MIR—FT methods and other indirect methods (e.g. ultrasound). From the analytical point of view these variability values of individual differences probably would not meet the standard requirements for milk payment or breeding purposes. This is explicitly confirmed by Katz (2007), Katz and Pinski (2008) or Ishay et al. (2011). However, they are perfectly correct for controlling the milk composition and properties for purposes of herd management, animal health, prevention of their production disorders (mastitis, ketosis) or technological selection of milk as a raw material.

CONCLUSION

The values of the RTA result reliability in the AMS were comparable with these in milking parlours in previous works. The results demonstrated the suitability of the combination of RTA and AMS for controlling the milk composition and properties to produce the results useful for controlling of dairy herds in order to promote animal health, prevent their production disorders and improve milk quality.

ACKNOWLEDGEMENTS

This research was financially supported by the projects MZe NAZV KUS QJ1510336, GAJU 002/2016/Z, "S" grant MSMT CR and RO1417. Authors thank Mr. František Hájek, Mr. Dipl Eng. Jiří Hájek and Mrs. Dipl Eng. Kamila Hájková from farm Lesoňovice for their excellent technical cooperation.

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THE EFFECT OF L-CARNITINE DAILY SUPPLEMENTATION ON QUALITY OF EJACULATE OF DUROC BOARS

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Abstract: The objective of this study was to find out whether the daily supplementation of l-carnitine had an effect on the quality of the ejaculate of duroc boars. L-carnitine was supplemented for 60 days, which were divided into 3 periods (n = 30 days). For the experiment were selected 12 duroc boars and were divided into two groups. Control group (n = 6) was fed by basic feed mixture only. Experimental group (n = 6) was fed by basic feed mixture with the addition of 500 mg of L-carnitine/kg of the basic mixture. The monitored ejaculate parameters included volume of ejaculate, sperm concentration, total rate of sperm, motility and percentage of morphologically abnormal sperm. Amount of L-carnitine in ejaculate was monitored as well.

By the results we confirmed the hypothesis, that L-carnitine has a positive effect on quality of ejaculate. Statistically significant effect was determined in sperm motility and in amount of morphologically abnormal sperm. In sperm motility there was insignificant increase in experimental group, but there was statistically significant difference between groups ($P < 0.05$). In the amount of morphologically abnormal sperm, there was statistically significant increase in experimental group ($P < 0.05$) and as well statistically significant difference between groups ($P < 0.05$).

Keywords: L-carnitine, semen, boar, antioxidant

INTRODUCTION

In literature, there is a lot of information about the positive effect of L-carnitine on spermatogenesis (Jacyno et al. 2007). L-carnitine is the vitamin-like amino acid synthesized from lysine and methionine in liver, kidney and brain (Vaz et al. 2002, Jeulin et al. 1994). L-carnitine plays a very important role in lipid metabolism and cellular energy metabolism (Hoppel 2003). It brings long-chain fatty acids into the mitochondria for beta-oxidation, thus producing the energy (ATP) necessary for proper sperm functioning (Hoppel 2003, Horky et al. 2012). It is also very important for detoxification of the organism, because it eliminates acetyl-CoA from mitochondria, excess of which has a toxic effect and it protects the cell membranes from the oxidative damage caused by peroxidation of polyunsaturated fatty acids (Arrigoni-Martelli and Caso 2001, Kalaiselvi and Panneerselvam 1998, Horky et al. 2014). L-carnitine is absorbed during sperm maturation in the epididymis and its concentration varies in the range of 200–300 nmol × mg^{-1} of protein (Agarwal and Sait 2004, Jeulin et al. 1987). While sperm is passing through the epididymis (1−10 days), they accumulate a high concentration of L-carnitine from the epididymal plasma, thus conferring motility upon the flagellum (Jeulin et al. 1994). L-carnitine improves qualitative parameters of ejaculate, especially an increase of concentration of sperm and motility (Vitali et al. 1995). High concentration, motility and viability of sperm is the key to create more AI doses form one ejaculate.

Artificial insemination clearly augments the rate of genetic improvement, but could be further enhanced by increasing the total number of spermatozoa per ejaculate produced to increase the distribution of genetic material from superior boars. Currently, a single 80–100 ml dose of extended semen contains 2–3 × 10^9 spermatozoa (Krueger et al. 1999). Total volume of an ejaculate ranges from

75–400 ml containing 20–100 × 10^9 spermatozoa (Leman and Rodeffer 1976). Therefore, a single boar ejaculate yields approximately 6–33 AI doses. It would be beneficial to the swine industry to maximize the number of AI doses produced by boars. The increase in usage of artificial insemination will fuel the demand for quality semen from boars trained to mount artificial sows for semen collection (Kozink et al. 2004).

MATERIAL AND METHODS

The experiment was running in the boar insemination station in Velké Meziříčí (N 49° 23.46667', E 15°52.70135'). The experiment lasted for 60 days, which were divided into 3 periods (period 1 = day 0, period 2 = day 30, period 3 = day 60). 12 boars of the Duroc breed, weighing 255 ± 20 kg and 2 ± 0.3 years old, were selected for the experiment. The boars were housed individually in pens (2.5 × 2.5 m). The feed mixture (MEp 12.6 MJ/kg) was fed at a dose of 3.5 kg; the boars had ad libitum access to water. The boars were divided into two groups, where the control group (n = 6) was fed by the basic feed mixture only and the experimental group (n = 6) was fed by the basic feed mixture with addition of 500 mg of L-carnitine per kg of the feed ration.

The ejaculate was taken once a week by using a jump phantom. Methodology of ejaculate analysis was determined by Lovercamp et al. (2013).

Determination of ejaculate volume – volume of the ejaculate was determined by weighing each ejaculate, with 1 g to 1 ml conversion.

Determination of sperm concentration – concentration of the sperms was determined using a self-calibrating photometer (SpermaCueTM, Minitube of America, Verona, WI).

Determination of sperm motility – motility was determined using the Sperm Vision™ software (Minitube of America, Verona, WI) with digital camera connection to a contrast microscope (Olympus microscope IX 71 S8F-3; Tokyo, Japan).

Prior to analysing, 500 µl of each sample was diluted with 500 µl of Androhep diluent and incubated for 30 minutes at 37 °C.

Determination of morphologically abnormal sperms – the phase contrast microscope (Zeiss, Germany) was used for determination of the morphologically abnormal sperms).

Subjective assessment was performed by a single qualified person.

Determination of the total sperm count – determined by calculation (sperm concentration x ejaculate volume).

Biochemical analysis – determination of free L-carnitine in ejaculate: One sample was taken once a month from all boars for the biochemical analysis and frozen for later analyses. 250 µl of pH = 7 phosphate buffer was added to 250 µl of defrosted boar sperm and the solution was poured over by 2 ml of liquid nitrogen. The mixture was homogenized for 2 minutes at 3000 rpm in ice. 1 ml of phosphate buffer was added and the mixture was shaken for 30 minutes at 4 °C and centrifuged at 6000 rpm for 15 minutes afterwards. After centrifugation the supernatant was removed, which 500 µl of 10% TFA was added to. The sample was re-centrifuged and the supernatant taken for analysis using AAA 400.

Statistics – the data were statistically analysed using STATISTICA.CZ version 10.0 (Czech Republic). The results were expressed as the mean ± standard variance. Statistical significance was observed between the groups using ANOVA and Scheffe's test – the two-factor analysis (the first factor was the animal group, the second one – the sampling factor) for parameters of L-carnitine ejaculate volume, sperm concentration and motility, percentage of pathological sperms. The difference ($P < 0.05$) was considered as significant.

RESULTS AND DISCUSSION

Table 1 shows the effect of L-carnitine supplementation on qualitative ejaculate parameters of each group and in each period of the experiment. From the results it's obvious, that addition of 500 mg of L-carnitine had insignificant effect on volume of ejaculate, concentration of sperm and total rate of sperm. On the contrary, the control group reached better values of these parameters than the experimental group. The difference between groups reached a peak in the 3. period in concentration of

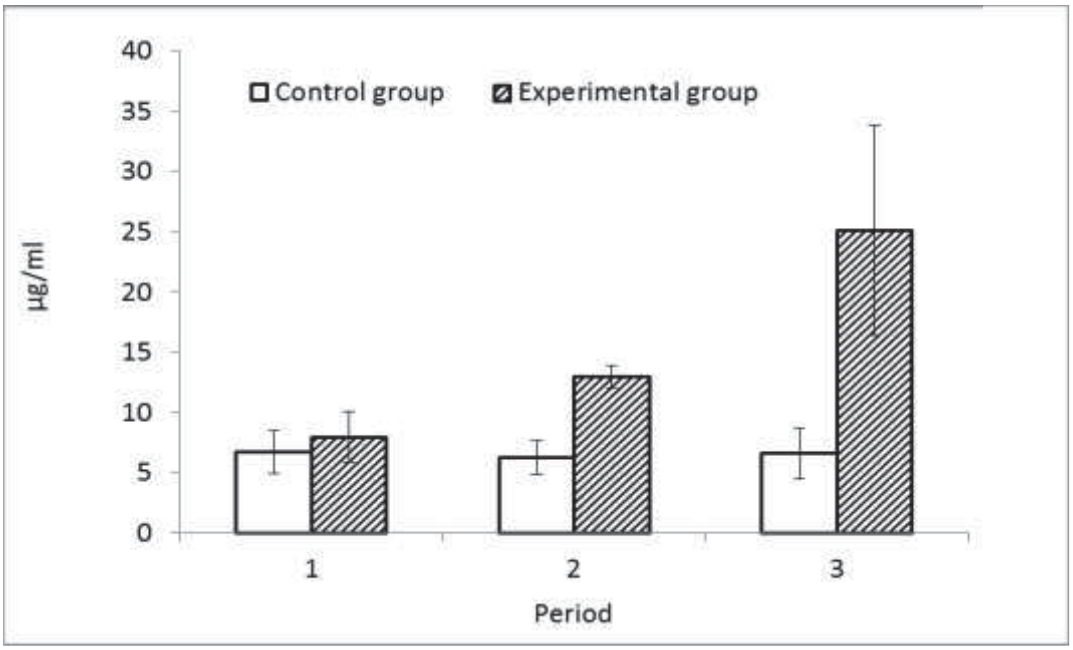
sperm (about 131 × 10^6/ml) and total rate of sperm (about 26.06 × 10^9), but these results are not statistically significant. Statistically significant effect of supplementation of L-carnitine has been proven in motility and the amount of morphologically abnormal sperm. Already after 30 days of supplementation there was statistically significant difference between groups in motility of sperm (about 6.46%), ($P < 0.05$). In the 3. period there were significant differences in motility (8.54%) and the amount of morphologically abnormal sperm (about 9.25%), ($P < 0.05$).

Table 1 Average values of analysed parametres in each period

Period	1		2		3	
	Control group	Experimental group	Control group	Experimental group	Control group	Experimental group
Volume of ejaculate	**168.33** ± 41.76	**150** ± 41.44	**192.22** ± 29.34	**173.06** ± 35.07	**186.42** ± 45.38	**175.42** ± 37.72
Concentration of sperm	**515.83** ± 67.26	**492.5** ± 46.37	**514.86** ± 98.36	**464.31** ± 31.36	**594** ± 102.36	**463** ± 135.23
Total rate of sperm	**85.26** ± 22.21	**76.78** ± 24.86	**97.52** ± 10.85	**81.87** ± 21.09	**110.11** ± 25.43	**84.05** ± 23.45
Motility	**71.67** ± 4.21	**71.67** ± 5.24	**67.85*** ± 1.82	**74.31*** ± 3.12	**66.46*** ± 2.21	**75*** ± 3.19
Morphologically abnormal sperm	**9.83** ± 2.69	**7.17** ± 2.70	**9.22** ± 2.32	**7.83** ± 2.36	**17.83*** ± 3.23	**8.58*** ± 2.97

After biochemical analysis, it was found that statistically significant increase of L-carnitine concentration in experimental group was occurred already after 30 days (about 5.02 µg/ml) and after 60 days (about 12.12 µg/ml) of supplementation ($P < 0.05$). Statistically significant differences between groups were occurred after 30 days (about 6.78 µg/ml) and 60 days (about 18.55 µg/ml) of supplementation ($P < 0.05$) (Figure 1).

Figure 1 Concentration of L-carnitine in ejaculate



Cerovsky et al. (2009) in his research states that the addition of L-carnitine has no positive effect on the quality of the ejaculate of boars. On the other hand, Jacyno et al. (2007) recorded improvement of qualitative markers of ejaculate after supplementation of 500 mg of L-carnitine in Pietrain boars, especially in decrease of the amount of morphologically abnormal sperm and in increase of the total

sperm count in the ejaculate. In our experiment, we have recorded a decrease of amount morphologically abnormal sperm as well, the difference between groups was about 9.25% ($P < 0.05$). Vitali et al. (1995) in their experiment argue that addition of L-carnitin into boars diet positively affected the motility and the sperm concentration. We could not approve the influence of L-carnitine on concentration of sperm, but in motility we have recorded a statistically significant increase (difference between groups about 8.54%), ($P < 0.05$). Baumgartner (1998) claims that after supplementation of L-carnitine it could be produced more insemination doses form one ejaculate. By the increase of volume of ejaculate and total amount of sperm it is possible to prepare more insemination doses (Jacyno et al. 2007). The increase of total number of sperm in ejaculate could be due to the fact, that L-carnitine is protecting the sperm cells and less dead sperm is absorbed in epididymis (Jeulin and Lewin 1996). As we have recorded no significant increase of volume of ejaculate and concentration sperm, we cannot stand that L-carnitine can uplift the total amount of sperm in ejaculate.

CONCLUSION

The aim of the study was to confirm the hypothesis, that daily supplementation of L-carnitine have a positive effect on quality of ejaculate. We confirmed it in observed parameters: motility of sperm, the amount of morphologically abnormal sperm and concentration of L-carnitine in ejaculate. In volume of ejaculate, concentration of sperm and total rate of sperm there weren't noticed any positive changes during the experiment.

ACKNOWLEDGEMENT

This project was funded from grants IGA IP 038/2017: Effect of supplementation of L-carnitine on qualitative and quantitative parameters of ejaculate of duroc boars.

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ACARICIDAL ACTIVITY OF PLANT ESSENTIAL OILS AGAINST POULTRY RED MITE (*DERMANYSSUS GALLINAE*)

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Abstract: The main objective of the performed experiments was to monitor acaricidal activity of selected plant essential oils against poultry red mites (*Dermanyssus gallinae*) and the second objective was to define the lethal doses (LD_{50} a LD_{90}) of selected essential oils. Effect of six plant essential oils as acaricides against poultry house-collected red mites was examined using direct contact method – by glass vial bioassay. All used essential oils caused mortality of poultry red mites in all their stages of development. Essential oils derived from clove buds and cinnamon have been shown to be effective acaricides against the poultry red mites at concentrations 0.5 and 0.25 µL/cm^2, when tested over a 24 h period. The average mortality in the negative control was 2%. Clove bud, lavender and cinnamon essential oils in merit further study as potential poultry red mites control agents.

Key Words: natural botanical pesticides, plant essential oils, poultry red mites, *Dermanyssus gallinae*

INTRODUCTION

The poultry red mite (*Dermanyssus gallinae*) is a huge economic problem in domestic poultry flocks all across the Europe, USA, Japan and China. It is haematophagous ectoparasite of birds (Kim et al. 2004).

Its abundance in poultry houses is a major source of irritation and disturbs egg-laying hens, which are more aggressive feather-pecking themselves and have cannibalistic behaviors. Infestation by this parasite can lead to anemia and in some cases there is causal association with host death syndrome. Infestation can lead to decrease in egg production, egg quality (through shell thinning and spotting) and egg venality (Dohnal 2009). The poultry red mite may also serve as a possible vector of a variety of poultry pathogens. The cost expends on fight with poultry red mite and production losses are calculated on 130 million EUR per year only in the EU (Sparagano et al. 2014).

Poultry red mite's life cycle is very fast and it is becoming increasingly resistant against commercial acaricides, for this reasons the fight with it is very difficult. These problems have highlighted the need for the development of selective poultry red mite control alternatives. It is also necessary to monitor infestation on poultry houses (Sparagano et al. 2014, Mul et al. 2009). Plant essential oils may be an alternative source of materials for mite control because they constitute a rich source of bioactive chemicals and they have little or no harmful effects on non-target organisms (Isman 2006).

MATERIAL AND METHODS

The acaricidal activity of six plant essential oils against poultry red mites was examined using direct contact method – by glass vial bioassay.

The essential oils and all other chemicals were of reagent grade. There were used six essential oils derived from clove bud, lavender, cinnamon, rosemary, skin of Brazilian oranges and peppermint. The active ingredient in clove bud is eugenol ($C_{10}H_{12}O_2$), in lavender is linalool ($C_{10}H_{18}O$),

in cinnamon is cinnamaldehyde (C_9H_8O), in rosemary is eukalyptol ($C_{10}H_{18}O$), in skin of Brazilian oranges is limonen ($C_{10}H_{16}$) and in peppermint is menthol ($C_{10}H_{20}O$). Oils were dissolved in methanol or distilled water or Tween 85 with water. Each of the essential oils was applied on two circles of filter paper (Whatman No. 3; 22 mm diameter) at the bottom of the glass vial at concentrations 0.5; 0.25; 0.12; 0.06; 0.03; 0.015 μL/cm^2 in amount of 100 μL solution. The solvent of solution was then evaporated. Always eleven glass vials per each concentration and oil and negative control were used. Negative control contained no essential oil on filter paper.

Colonies of poultry red mites (*Dermanyssus gallinae*) were collected from crevices of poultry houses in the Czech Republic in 2016 and 2017. For trapping these parasites were used plastic containers filled with rolled cardboard attached to the constructions in poultry houses. The plastic containers with collected poultry red mites were closed by lids and transferred to the laboratory. Poultry red mites were tested within two days after collection (Kim et al. 2004). Twenty movable poultry red mites all their stages of development were placed on the filter paper impregnated by essential oil at the bottom of the glass vials. Each glass vial was then closed with lid. The mortality of the poultry red mites in the glass vials was measured 24 h after the treatment. Poultry red mites were considered dead if their appendages did not move when parasites were prodded with a fine pin. Data were processed using software MS Excel, XLSTAT (XLSTAT-Dose) and Unistat 5.1 (Unistat Ltd, ENGLAND) (Hubert et al. 2015).

RESULTS AND DISCUSSION

Influence of selected essential oils on mortality poultry red mites

Comparison of the acaricidal activity of six plant essential oils at a dose of 0.5; 0.25; 0.12; 0.06; 0.03; 0.015 μL/cm^2 with solvents of methanol, distilled water and Tween 85 with water, against poultry red mites to all their stages of development is shown in Table 1–3. Out of used essential oils, three gave more than 90% mortality against poultry red mites (clove bud, lavender and cinnamon with all solvents at concentration 0.5 μL/cm^2), it was measured after 24 h.

Rosemary essential oil with distilled water at all concentrations caused less than 50% mortality, therefore this oil was tested with methanol and Tween 85 with water only at the highest concentration (0.5 μL/cm^2). Essential oils derived from skin of Brazilian oranges and peppermint were tested only with distilled water at concentration 0.5 μL/cm^2 because of low mortality of poultry red mites. For this reason rosemary, skin of Brazilian oranges and peppermint essential oils were not tested at other concentrations with methanol and water and Tween 85. The average mortality in controls was 2%.

Many essential oils are known to possess various bioefficacies such as ovicidal, repellent, antifeeding and biocidal activities against various arthropod pests. Essential oils are volatile, so their environmental performance is limited (Pavela 2011). Chemical differences between seemingly similar oils can result from variations in a number of factors, including environmental conditions, harvesting regimens, and extraction protocols. This problem might be resolved by isolating active components from plant products and developing them for use as acaricides; geraniol and several forms of cinnamaldehyde are toxic to poultry red mites (Isman 2006). As a benefit of plant pesticides would be that the numerous active compounds in essential oils would make development of pest resistance to any essential oil-based product extremely difficult (Miresmailli et al. 2006).

Influence of essential oils diluted with distilled water on mortality of poultry red mites

Statistically significant the most effective essential oil with dissolved water as solvent was clove bud, which caused 99% mortality of poultry red mites at concentrations of 0.5 and 0.25 μL/cm^2 and more than 95% mortality at concentration of 0.12 μL/cm^2 and at 74.5% mortality still at concentration 0.06 μL/cm^2, which was statistically significantly the highest mortality at the same concentration from all tested oils ($P < 0.001$). At concentrations 0.12 and 0.06 μL/cm^2 the mortality was significantly the highest at using clove bud essential oil ($P < 0.001$).

Lavender and cinnamon caused higher than 99% mortality only at concentrations 0.25 and 0.5 μL/cm^2. At these concentrations, there were significant difference ($P < 0.001$) between the efficiency of these three oils in comparison with the efficiency of rosemary, skin of Brazilian oranges and peppermint essential oils.

Excepting clove bud all oils caused less than 50% mortality at concentrations 0.12; 0.06 and 0.03 μL/cm^2 (here clove bud too). At these concentrations, there were significant differences between the oils ($P < 0.001$, see Table 1).

Essential oil derived from skin of Brazilian oranges caused 55.5% mortality at concentration of 0.5 μL/cm^2, other concentrations caused lower than 50% mortality. Rosemary as well as peppermint caused less than 50% mortality even at the highest concentration (see Table 1).

Table 1 Influence of essential oils with distilled water to mortality poultry red mites

Essential oil + ddH_2O	Average mortality poultry red mites ± SE (%)				
	Dose (μL/cm^2)				
	0.5	0.25	0.12	0.06	0.03
Clove bud	99.5 ± 0.5^b	100.0 ± 0.0^b	98.2 ± 1.0^c	74.5 ± 7.3^b	34.5 ± 7.0^c
Lavender	99.5 ± 0.5^b	99.5 ± 0.2^b	25.0 ± 6.1^{ab}	17.5 ± 2.8^a	9.0 ± 2.1^{ab}
Cinnamon	100.0 ± 0.0^b	99.5 ± 0.5^b	30.5 ± 3.4^b	18.0 ± 2.5^a	23.3 ± 5.1^{bc}
Rosemary	45.0 ± 4.8^a	34.5 ± 8.1^a	10.4 ± 2.1^a	3.5 ± 1.0^a	16.6 ± 4.2^{ab}
Skin of Brazilian oranges	55.5 ± 12.2^a	25.0 ± 3.9^a	19.8 ± 3.4^{ab}	4.2 ± 1.5^a	0.0 ± 0.0^a
Peppermint	46.6 ± 8.3^a	37.6 ± 7.0^a	27.1 ± 6.6^{ab}	12.5 ± 2.2^a	7.2 ± 2.1^{ab}
P	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001

a, b – between the values marked with different letters is statistically significant difference between the essential oils at specific concentration; SE – standard error.

Influence of essential oils diluted with methanol on mortality of poultry red mites

Significantly the most effective essential oil with methanol as solvent was clove bud, which caused 99% mortality of poultry red mites at concentrations 0.5; 0.25 and 0.12 μL/cm^2 and more than 90% mortality at concentration 0.06 μL/cm^2 and 73.5% mortality at concentration 0.03 μL/cm^2.

Lavender and cinnamon essential oils caused more than 90% mortality at concentrations 0.5 μL/cm^2 and 0.25 μL/cm^2. The lavender caused 69.5% mortality at concentration 0.03 μL/cm^2 and cinnamon more than 50% mortality at concentration 0.06 μL/cm^2.

At concentrations 0.25; 0.12; 0.06 and 0.03 μL/cm^2 there was no significant difference between the efficiency of clove bud and lavender. At concentration 0.015 μL/cm^2 there was significant difference in the efficiency of clove bud and lavender oils ($P < 0.05$) (see Table 2).

Rosemary essential oil caused only 14.4% mortality at concentration 0.5 μL/cm^2, for this reason it was no tested at other concentrations. At concentration 0.5 μL/cm^2 there was significant difference ($P < 0.001$) between the efficiency of clove bud, lavender and cinnamon considering to the efficiency of rosemary oil.

Table 2 Influence of essential oils with methanol to mortality poultry red mites

Essential oil + CH_3OH	Average mortality poultry red mites ± SE (%)				
	Dose (μl/cm^2)				
	0.5	0.25	0.12	0.06	0.03
Clove bud	100.0 ± 0.0^b	99.5 ± 0.3^a	99.1 ± 0.6^b	91.6 ± 3.1^b	73.5 ± 9.8^a
Lavender	98.2 ± 0.8^b	91.4 ± 5.1^a	85.5 ± 4.5^{ab}	69.5 ± 10.9^{ab}	69.5 ± 9.7^a
Cinnamon	99.7 ± 0.2^b	99.5 ± 0.2^a	75.6 ± 6.8^a	56.5 ± 8.5^a	-
Rosemary	14.4 ± 2.6^a	-	-	-	-
P	P < 0.001	P > 0.05	P < 0.01	P < 0.05	P > 0.05

a, b – between the values marked with different letters is statistically significant difference between the essential oils at specific concentration; SE – standard error.

Influence of essential oils diluted with Tween 85 with water on mortality of poultry red mites

The most effective essential oils with Tween 85 with water as solvent were clove bud and cinnamon, which caused more than 99% mortality of poultry red mites at concentrations 0.5; 0.25; 0.12 and 0.06 μL/cm^2. Cinnamon caused more than 90% mortality and clove bud caused 81.8%

mortality at concentration 0.03 μL/cm^2, there was no significant difference ($P < 0.05$) between these two oils. Both oils were significantly more effective considering to the efficiency of lavender at concentrations 0.25; 0.12; 0.06 and 0.03 μL/cm^2.

Lavender essential oil caused more than 99% mortality at concentration 0.5 μL/cm^2. At concentration 0.5 μL/cm^2 there was no significant difference between the efficiency of lavender, cinnamon and clove bud. Lavender caused less than 50% mortality at other concentrations.

Rosemary essential oil caused only 22.1% mortality at concentration 0.5 μL/cm^2, for this reason it was not tested at other concentrations. At concentration 0.5 μL/cm^2 was activity of this oil significantly the lowest ($P < 0.001$).

At concentration 0.15 μL/cm^2 there was significant difference between the efficiency of clove bud and lavender, however both caused less than 10% mortality (see Table 3).

Table 3 Influence of essential oils with Tween 85 with water to mortality poultry red mites

Essential oil + Tween 85	Average mortality poultry red mites ± SE (%)					
	Dose (μl/cm^2)					
	0.5	0.25	0.12	0.06	0.03	0.015
Clove bud	100.0 ± 0.0^b	100.0 ± 0.0^b	100.0 ± 0.0^b	100.0 ± 0.0^b	81.8 ± 7.0^b	8.6 ± 1.2^b
Lavender	100.0 ± 0.0^b	38.6 ± 7.5^a	19.5 ± 2.3^a	2.3 ± 1.2^a	10.9 ± 2.9^a	3.6 ± 1.9^a
Cinnamon	100.0 ± 0.0^b	100.0 ± 0.0^b	100.0 ± 0.0^b	99.4 ± 0.5^b	97.5 ± 1.9^b	-
Rosemary	22.1 ± 5.7^a	-	-	-	-	-
P	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.05

a, b – between the values marked with different letters is the statistically significant difference between the essential oils at specific concentration; SE – standard error.

Lethal doses of the essential oils for poultry red mites

Table 4 shows the concentrations of the essential oil (μL/cm^2) necessary for 50 and 90% mortality of poultry red mites. R^2 coefficient (Nagelkerke) is in the range between 0 and 1 and shows how well the model fit data. Significant differences were observed in essential oils toxicity and used solvents. For the fitted model parameters, i.e. LD_{50} and LD_{90} are given fit 95% confidence interval in round brackets (Hubert et al. 2015).

Clove bud essential oil was the most effective with Tween 85 with water as solvent for the lethal doses LD_{50} and LD_{90} of poultry red mites, when there was necessary to be applied on the filter paper for LD_{50} 0.02 μL/cm^2 and for LD_{90} 0.03 μL/cm^2. On the other hand clove bud was necessary to be applied on the filter paper in the biggest concentration with distilled water, for LD_{50} 0.04 μL/cm^2 and for LD_{90} 0.08 μL/cm^2.

Lavender essential oil was the most effective with methanol as solvent for the lethal doses LD_{50} and LD_{90} of poultry red mites, when there was necessary to be applied on the filter paper for LD_{50} 0.02 μL/cm^2 and for LD_{90} 0.19 μL/cm^2. On the other hand lavender was necessary to be applied on the filter paper in the biggest concentration with Tween 85 with water for LD_{50} 0.55 μL/cm^2 and for LD_{90} 6.20 μL/cm^2.

Cinnamon essential oil was the most effective with Tween 85 with water as solvent for the lethal doses LD_{50} and LD_{90} of poultry red mites, when there was necessary to be applied on the filter paper for LD_{50} 0.004 μL/cm^2 and for LD_{90} 0.02 μL/cm^2. On the other hand lavender was necessary to be applied on the filter paper in the biggest concentration with distilled water for LD_{50} 0.11 μL/cm^2 and for LD_{90} 0.28 μL/cm^2.

Rosemary essential oil, essential oil derived from skin of Brazilian oranges and peppermint essential oils were tested only with distilled water as solvent. From their comparison ensue, that the most effective was skin of Brazilian oranges for the lethal doses LD_{50} and LD_{90} of poultry red mites, when there was necessary to be applied on the filter paper for LD_{50} 0.45 μL/cm^2 and for LD_{90} 1.82 μL/cm^2. On the other hand rosemary was necessary to be applied on the filter paper in the biggest concentration, for LD_{50} 0.66 μL/cm^2 and for LD_{90} 5.93 μL/cm^2.

Table 4 Dependence of the amount of the essential oil (µL/cm²) on the mortality of poultry red mites

Essential oil	Solvent	R^2	LD_{50}	LD_{90}
Clove bud	ddH_2O	0.57	0.04 (0.04/0.04)	0.08 (0.07/0.09)
	CH_3OH	0.64	0.03 (0.02/0.03)	0.05 (0.05/0.06)
	Tween 85	0.8	0.02 (0.02/0.02)	0.03 (0.03/0.04)
Lavender	ddH_2O	0.71	0.13 (0.12/0.17)	0.22 (0.17/0.38)
	CH_3OH	0.23	0.02 (0.01/0.02)	0.19 (0.15/0.24)
	Tween 85	0.17	0.55 (0.39/0.89)	6.20 (3.03/17.91)
Cinnamon	ddH2O	0.57	0.11 (0.10/0.12)	0.28 (0.25/0.32)
	CH_3OH	0.45	0.03 (0.03/0.03)	0.07 (0.07/0.09)
	Tween 85	0.19	0.004 (0.00001/0.01)	0.02 (0.002/0.03)
Rosemary	ddH_2O	0.15	0.66 (0.50/0.98)	5.93 (3.02/18.23)
Skin of Brazilian oranges	ddH_2O	0.32	0.45 (0.39/0.53)	1.82 (1.35/2.72)
Peppermint	ddH_2O	0.17	0.49 (0.40/0.66)	4.97 (2.89/10.90)

For the fitted model parameters, i.e. LD50 and LD90 are given fit 95% confidence interval in round brackets.

Figure 1 Dependence of the concentration of the lavender essential oil with methanol as solvent for the lethal doses of poultry red mites on cm²

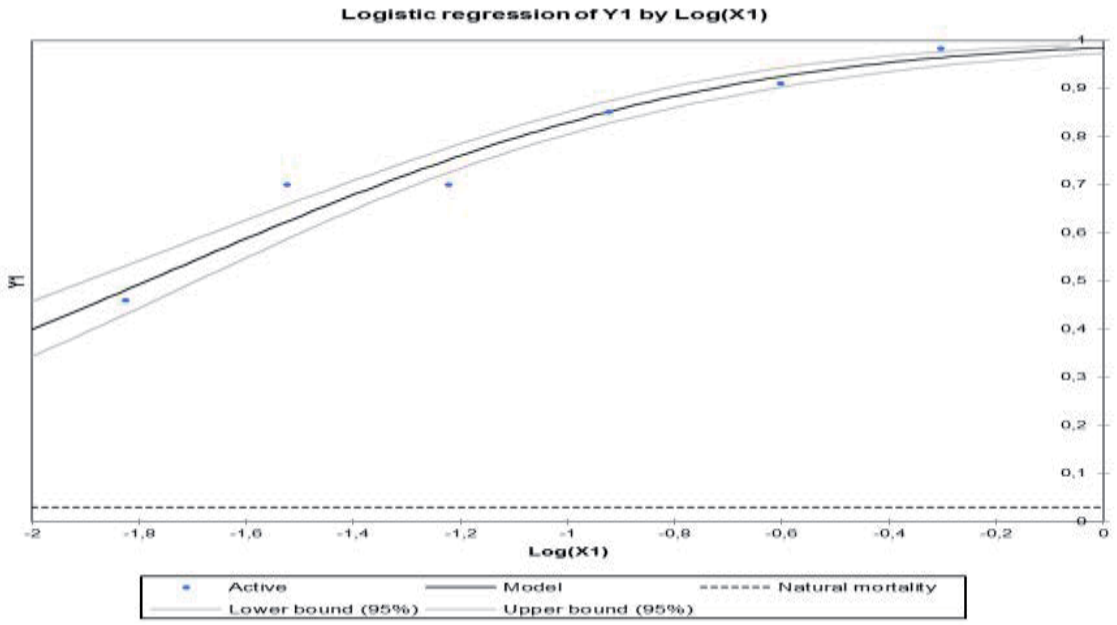

Figure 1 shows the correlation between the amount of lavender essential oil with methanol as solvent (µL/cm²) and the mortality of the poultry red mites population, which is represented by a black curve in the graph. Gray curves show confidence intervals and blue points measurements. It was necessary to be applied on the filter paper for LD_{50} 0.02 µL/cm² and for LD_{90} 0.19 µL/cm².

CONCLUSION

The results of these experiments suggest that certain essential oils may make effective natural botanical pesticides against poultry red mites. The highest mortality was observed with clove buds and cinnamon essential oils. For practical usage may enough exerting more than 50% mortality. Plant essential oils have little or no harmful effects on non-target organisms. Perhaps the greatest constraint to the use of essential oils like effective natural botanical pesticides in poultry red mites control is their relative lack of standardization and consequent inconsistent efficacy.

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THE INFLUENCE OF FEEDING WHEAT WITH BLUE ALEURONE ON BIOCHEMICAL PARAMETERS, ANTIOXIDANT ACTIVITY AND PERFORMANCE OF BROILER CHICKENS

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Abstract: Blue wheat contain the higher content of anthocyanins than common wheat cultivars. The aim of this study was to evaluate the influence of feeding wheat with blue aleurone on broiler chicken's biochemical parameters, antioxidant activity and performance parameters. The study was conducted on 60 hybrid broiler chickens Ross 308. They were fattened on deep litter system. The experimental period lasted from day 12 to day 36 of chickens ages. The broilers were randomly divided on 2 groups. The first group (n = 30) was fed experimental feed mixtures containing 38.2% of blue wheat UC66049 and second group (n = 30) was fed control feed mixtures containing 38.2% of common wheat Vánek (C). At the end of the experiment was collected the blood samples (n = 8 in the both groups). In the blood were determined the enzymes activities (aspartate aminotransferase, gamma-glutamyl transferase, alanine aminotransferase, lactate dehydrogenase), concentrations of albumin, total protein, cholesterol, triglycerides, bilirubin, uric acid, urea, and antioxidant activity. During the trial were not observed significant differences ($P > 0.05$) between both groups in blood biochemical parameters, antioxidant activity, feed intake, feed conversion, weight, and carcass indicators.

Key Words: anthocyanins, poultry nutrition, metabolism, liver enzymes, poultry nutrition

INTRODUCTION

The colours of wheat grains are caused different pigments. Pigments include for example carotenoids and anthocyanins. The carotenoids and anthocyanins have a positive effect on human health (Donatella et al. 2014). Blue colour is caused mainly by delphinidin (Abdel-Aal and Hucl 2003). In cultivars with blue grains content of anthocyanins ranges within the range 106–153 μg/g (Chňapek et al. 2010). A positive influence on the organism is a reason to think about including non-traditional wheat cultivating human food as a functional food. Functional food is a food that has a demonstrably positive effect on real health human or animals it also helps the good physical and mental state of an individual (Kalač 2003). Many sources describea anthocyanins that they have antibacterial and anti-carcinogenic effects, they report antioxidative activity which is prevention of retinal degeneration and heart disease (Abdel-Aal and Hucl 2003; Donatella et al. 2014, Kalač 2003). Anthocyanins are involved in the repair of proteins in trombocytes wall (Ďuračková 2008). Anthocyanins increase the resistance of hepatocytes to oxidation (Mazza 2000). They are possible bind the free radicals (Li et al. 2005). The aim of this study was to evaluate the influence of feeding wheat with blue aleurone on broiler chicken's biochemical parameters, antioxidant activity and performance parameters. The assumption was whether the intake of anthocyanins contained in coloured wheat positively/negatively affects the metabolism and the monitored biochemical parameters, or the performance parameters of the chickens.

MATERIAL AND METHODS

The 60 chickens of hybrid Ross 308 were fattened on deep litter system. The first 10 days of age chickens were fed by commercial starter. The broilers were adapted to experimental diets for 2 days. The trial was conducted from day 12 to day 36 of age of chickens. Room temperature and humidity was set and controlled by technological instructions Ross 308 (Aviagen group 2014). Lighting system was 16 hours light and 8 hours dark with a maximum light intensity of 20 luxuries. The broilers were randomly divided on 2 groups. The first group (n = 30) was fed experimental feed mixtures containing 38.2% of blue wheat UC66049 (Blue wheat) and second group (n = 30) was fed control feed mixtures containing 38.2% of common wheat Vánek (Control).

Chemical analyses of used experimental wheats are shown in Table 1. The compositions of experimental rations are shown in table 2. The feed mixtures were calculated according to the recommended nutrient content in feed mixture for poultry (Zelenka et al. 2007). Table 3 show chemical composition of experimental feed mixtures.

Table 1 Chemical compositions of diets

	Control	Blue wheat
Dry Matter (g/kg)	886.6	886.8
Crude Protein (g/kg)	169.5	165.8
Crude Fat (g/kg)	19.4	14.3
Crude Fiber (g/kg)	18.4	22.4
Crude Ash (g/kg)	13.3	19.5
Cyanidin-3-glucoside (mg/kg)	5.1	47.6

Table 2 Composition of feed mixture (%)

Component	Control (%)	Blue wheat (%)
Wheat	38.2	38.2
Maize	27.2	24.7
Soybean extruded	19.0	19.0
Soyabean meal	9.5	10.5
Premix VBR3*	3.0	3.0
Rapeseed oil	2.0	2.0
Monocalciumphosphate	0.7	0.7
Limestone milled	0.4	0.4
Wheat gluten	0.0	1.5

**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; phylochinon 350 mg; thiamine 140 mg; B2 230 mg; B6 200 mg; cobalamine 1000 mg; biotin 7 mg; niacinamid 1200 mg; folic acid 57 mg, calcium pantothenate 450 mg; choline chloride 6000 mg; salinomycin sodium 2333 mg.*

Table 3 Chemical composition of feed mixture (in dry matter)

	Control	Blue wheat
Crude protein (%)	21.29	21.63
AME (MJ/kg) *	12.81	12.87
Ether extract (%)	8.09	8.15
Crude ash (%)	5.89	6.33
Crude fibre (%)	3.27	3.03

**AME – apparent metabolizable energy (calculated value)*

The chickens were fed *ad-libitum.* Health status was evaluated daily and live weight measured every week during the trial. Body weight gain was measured individually. At the end of the experiment 8 birds were selected randomly from each group, weighed and slaughtered by decapitation. 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. At the end of the experiment six birds were selected randomly from each group, weighed and slaughtered by decapitation. Feathers were removed and chickens were eviscerated. Carcass yield was calculated. Breast and thigh meats without skin were separated from carcasses after cooling. All visible external fat was removed from sample meats. The breast and thigh meat was weighed and their percentage of live body weight was calculated.

The biochemical indicators of blood plasma were analysed with the use of Ellipse (AMS Spa, Italy) analyser. Blood 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); ALT – alanine aminotransferase (ALT/GPT 500); LD – lactate dehydrogenase (LDH–L 100); cholesterol (CHOL 250); TG – triglycerides (TG 250), and by company Randox, UK: Urea (Urea, cat. No. UR 107). Total antioxidant capacity was determined from blood plasma samples by the method FRAP (Ferric reducing/antioxidant power assay) described in the publication by Benzie and Strain (1996). The FRAP reagent containing 2,4,6-tripyridyltriazine (TPTZ)/ferric chloride/acetate buffer was prepared by mixing ten volumes of acetate buffer (300 mM, pH 3.6) with one volume of TPTZ (40 mM dissolved in 40 mM HCl) and one volume of ferric chloride (20 mM in water). For determination of the antioxidant capacity, 96-well microtiter plate was used. To each well, 10 µl of sample was added to 200 µl of FRAP reagent. The mixture was vortexed and incubated for 8 min at 37°C. Then the absorbance at 593 nm was measured using Tecan Infinite M200 PRO microplate reader (Tecan; Mannedorf, Switzerland). All samples were run in triplicate. Results were calculated using the standard curve with ascorbic acid. All chemicals were purchased from Sigma-Aldrich (Prague, Czech Republic). 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 $P < 0.05$ as regarded as statistically significant difference.

RESULTS AND DISCUSSION

Results of the biochemical analysis are presented in table 4. The analysed parameters AST, GGT, ALT, ALP and LD showed liver tissue status. The TG and concentrations of cholesterol characterized fat metabolism. The concentrations of urea, TP, and albumin indicate nitrogen metabolism of chicken's organism. The activities of most analysed enzymes were higher in the control group but differences are not significant. Our expectation was that the content of anthocyanins in blue wheat UC66049 may affect on liver enzymes and other biochemical indicators. The table 4 showed that this effect was not discovered in our trial and differences between groups were non-statistically significant ($P > 0.05$).

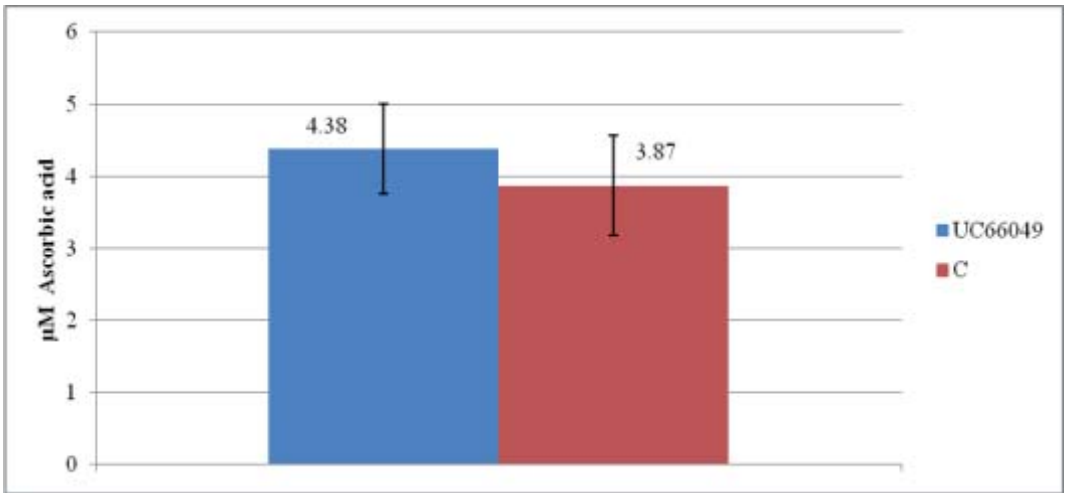
Table 4 Biochemical parametres

	Control	Blue wheat
Parameters	n = 8 Mean ± standard error	n = 8 Mean ± standard error
AST (µkat/l)	5.7 ± 0.59	4.4 ± 0.27
GGT (µkat/l)	0.4 ± 0.05	0.4 ± 0.03
ALT (µkat/l)	0.2 ± 0.02	0.3 ± 0.04
LD (µkat/l)	28.2 ± 4.14	22.5 ± 1.47
Bilirubin (µmol/l)	1.4 ± 0.22	1.2 ± 0.16
Uric acid (mmol/l)	235.8 ± 27.90	212.98 ± 38.58
Urea (mmol/l)	1.2 ± 0.15	1.1 ± 0.08
Chol (mmol/l)	2.7 ± 0.18	2.7 ± 0.09
TG (mmol/l)	0.5 ± 0.03	0.5 ± 0.04
TP (g/l)	33.3 ± 1.73	31.9 ± 1.30
Albumin (g/l)	12.3 ± 0.66	12.4 ± 0.31

These results may be due to short life of broiler chickens. The same result of biochemical analysis is reported in study by Abadi et al. (2014), Šťastník et al. (2016). On the other side, significant differences were showed in study with blue wheat in rats by Šťastník et al. (2016). In their study was analyzed lower plasma cholesterol concentration in experimental rats group.

Figure 1 show results of antioxidant activity analysis. There were observed no significant differences ($P > 0.05$), but the UC6609 group tends to have higher antioxidant activity.

Figure 1 Antioxidant activity (FRAP method)



There were no statistically significant differences between groups, the same as in one study by Karásek et al. (2016). In another study Karásek et al. (2014) describes a higher antioxidant activity in experimental group which was fed colour cultivar wheat. Similar results are discussed by Mrkvicová et al. (2016) on feeding purple wheat Konini. The positive effect of blue wheat Skorpion and UC66049 wheat is described by Šťastník et al. (2017). Comparing the results of individual studies assumed to the feeding coloured cultivars wheat does not have a negatively affect on our monitored parameters during chickens fattening and have positively effect on health parameters organism of chickens.

The mean bodyweight of chickens during the trial is present in table 5. The differences in the body weight were not significant ($P > 0.05$), but in the control group has body weight tendency to higher value. The statistically significant ($P < 0.05$) difference between groups was determined only in 29 days of age but no difference was found at 36 days of age. Many previous studies had the same results (Šťastník et al. 2014, Karásek et al. 2016, Mrkvicová et al. 2016).

Table 5 Body weight of chickens during the experiment and total increment in grams

	Control	Blue wheat
	n = 30	n = 30
Day of age	Mean ± standard error	Mean ± standard error
8 day	171.8 ± 1.97[a]	169.6 ± 1.62[a]
12 day	289.6 ± 3.46[a]	284.5 ± 2.70[a]
15 day	475.9 ± 6.77[a]	464.6 ± 5.39[a]
22 day	957.3 ± 72.03[a]	938.1 ± 15.94[a]
29 day	1578.0 ± 18.60[a]	1475.1 ± 33.33[b]
36 day	2333.5 ± 19.91[a]	2232.1 ± 47.50[a]
BWG *	2043.9 ± 18.15[a]	1947.7 ± 46.82[a]
Feed consumption *	3320.0	3480.0
Conversion *	1.63	1.79

BWG – body weight gain = Total body weight gain for trial period. [a,b] – the values with different letters are statistically significant difference ($P < 0.05$). Feed consumption * = mean feed consumption on chicken per experimental period (12–36 days of age). conversion * = mean feed consumption on unit of increment per experimental period (12–36 days of age).*

The chickens had a lower mean body weight at the 12th day of age in our experiment opposite body weight in manual for chicken's hybrids Ross 308 by Aviagen (2014). The slaughter indicators show table 6. The highest leg meat (Table 6) was found in the experimental group (15.66 ± 0.79) but differences between groups were not significant (P > 0.05). Our result of breast meat yield is consistent with statement that in normal broilers the yield of breast muscle is about 20% and in elite breeds ranging from 21 to 24% (Zelenka 2014).

Table 6 Slaughter indicators of chickens in 36 day of age calculated as a percentage of live weight

	Control	
	n = 6	n = 6
indicator	Mean ± standard error	Mean ± standard error
Live weight (grams)	2356.7 ± 88.27	2298.5 ± 104.80
Carcass (%)	76.0 ± 1.41	75.6 ± 0.99
Breast meat (%)	23.7 ± 0.52	22.6 ± 0.94
Leg meat (%)	15.6 ± 0.57	15.6 ± 0.79

Differences between groups are not statistically significant (P > 0.05).

CONCLUSION

In conclusion, based on results of analyzed biochemical characteristics and antioxidant activity the blue wheat UC66049 feeding at dose 38.2% does not show positive or negative effect on animal's health. In addition, was not observed any influence on performance parameters of broilers in our experiment.

ACKNOWLEDGEMENTS

The project was supported by the project by NAZV QJ1510206. Authors are grateful to Ing. Petr Martinek, CSc. for experimental wheat.

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EFFECTS OF PROTEIN SUPPLEMENT ON GROWTH PERFORMANCE AND BLOOD PARAMETERS OF HOLSTEIN-FRIESIAN CALVES

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Abstract: The objective of this study was to determine the effects of feeding protein-iron chelates complexes in milk replacers for cattle. Milk replacer formulation with addition of protein-mineral chelates for were used in nutrition of Holstein-Friesian calves aged between of 7 and 35 days. Preparation of the protein complex involved enzymatic hydrolysis of milk casein and binding iron chloride. There were created two experimental groups (lower and higher dose of the complex) and the control group. Addition of protein-mineral chelate admitted to higher body weight gain by lower feed conversion rates. Nevertheless, the dose, the complex has not a significant impact on carbohydrate-lipid parameters. Changes of activity of enzymes resulted from the age of the cattle, not from applied supplementation.

Key Words: calves; chelates; blood parameters, growth

INTRODUCTION

Improvement of properties of milk replacers might be achieved by using a number of functional supplements, to which there can be include: prebiotics, probiotics, non-protein nitrogenous compounds, essential amino acids, nucleotides, lactoferrin, immunoglobulin, sodium butyrate, organic acids, chelates or selected herbs (Hill et al. 2008, Fokkink et al. 2009, Kupczyński et al. 2017). Meta-analysis shows that growth of neonatal dairy calves appears to be more controlled by nutrient intake and their interactions than by surrogates for health status of the calves or environmental temperature (Beteman et al. 2012). Application of various supplements is associated with not only with influence on increase of body mass, but also affection on immune system and metabolism. Bioactive peptides originating from cow milk, due to their specific properties (not only immunomodulatory, but also antibacterial, antiviral and antifungal) can be applied for humans, but also in animals breeding (Szwajkowska et al. 2011). The objective of this study was to determine the effects of feeding protein-iron chelates complexes in milk replacers for cattle.

MATERIAL AND METHODS

The study was carried on 18 Polish Holstein-Friesian calves of black-white variety. The study was carried out with the consent of the 2nd Local Ethical Committee for Experiments on Animals in Wroclaw (No 63/2013). Animals were put into randomized groups taking into account the age (7 day of age), body weight and sex. Division into groups included the applied supplementation of protein-iron chelates complex:

- Control group: fed with standard milk replacer (n = 9);
- Treatment group: receiving a supplement of protein - mineral chelates in the amount of 1% of dry matter (n = 9).

The calves were kept during the experiment in the individual pens of 2.3 sqm area. Each pens was provided in automatic water bowl and container for starter forage. The calves were fed by milk-

replacer preparation (Polmass, Poland) and *ad libitum* full portion, granulated feed (Cargill Poland Sp. z o.o.). Method of production of preparation and its chemical composition was described in earlier surveys (Kupczyński et al. 2016). Vitality, dehydration and fecal score were described on the basis of clinical trial and observations conducted in 7th, 14th, 28th i 35th day of life according to methodology given by Sunderland et al. (2003). From all calves the blood was taken from the external jugular vein (*vena jugularis externa*) in 7th, 14th, 28th i 35th day of age. Seven days of age was treated as the starting point of the experiment. In all the terms the blood was taken into a tube containing K2EDTA, a tube containing sodium heparin, and into a tube without anticoagulant (Sarstedt, Warsaw, Poland). The blood samples for serum and plasma were centrifuged at 3000 × *g* for 10 min at a room temperature (2 hour from collection), and the serum samples were frozen (-20 °C) until the analysis. The laboratory analyses in blood serum were done using Pentra 400 biochemical analyser (Horiba ABX, France). The following parameters were estimated:

- biochemical parameters of the blood: glucose, triglycerides (Tg), non-esterified fatty acids (NEFA), β-Hydroxybutyric acid (BHBA), total protein (TP), albumins, total cholesterol;
- asparagine aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamylotranspephethydase (GGT).

During the experiment period production parameters such as: feed intake, average daily gain (ADG), growth rate (GR) and feed conversion rates (FCR) were investigated (Szymanska-Czerwinska i Bednarek 2011). Measurement of the body mass was performed in 7th, 14th, 28th i 35th day of calves' life. The above mentioned production parameters were calculated in accordance with the following formulas:

$$ADG = (Bk - Bo) / (Wk - Wo) \times 1000$$

$$FCR = Px(Bk - Bo)$$

$$GR = (Bk - Bo) / ½ (Bo + Bk) \times 100\%$$

where: P – quantity of feed intake (kg), Bo – initial body mass, Bk – final body mass (kg), Wk – final age of animal, Wo – initial age of animal.

Results obtained were further subjected to statistical analysis using STATISTICA 10.0 software (Statistica, Tulsa, OK, USA). Data were analysed using a general linear model for repeated measures ANOVA with dietary treatments (*D*) and sampling time (*T*) as fixed effects and their interactions (*D* × *T*). Differences between treatment group means were analysed for significance ($P < 0.05$) using the *t*-student test. Production and health data were subjected to the nonparametric Wilcoxon test. The data are presented as average values and accompanied by standard error of the means.

RESULTS AND DISCUSSION

Casein protein, as by-products of dairy industry, can be used as a low-cost source of protein for food fortification or as animal supplements (Kupczyński et al. 2016, 2017). In our study, protein supplementation increase average daily gain in comparison to the control group (Table 1). Increased body weight in response to higher dose of protein feeding was observed by Brown et al. (2005), Quigley et al. (2006) and Niwińska and Bilik (2012). In our study, protein supplementation did not significantly increase of GR and FCR in comparison to the control group.

During the experiment period the number of digestive system diseases was similar in experimental groups and presented decreasing tendency in the course of the survey. In the control group the number of calves with diarrhoea syndrome was the lowest at the end of experiment. Dehydration in the control group was highest (1.00) at the day of completion of the survey and this result has significantly distincted to the value obtained at the beginning of the survey (7th day). Experimental groups have not shown statistically significant differences between the day of commencement and completion of the survey. Parameters describing the general condition, i.e. the number of breaths and inside temperature have not represent significant differences between particular stages of the survey. Many authors (Diaz et al. 2001, Ballard et al. 2004, Brown et al. 2005, Bednarski

et al. 2015) point that feeding additional may increase number of cases of diarrhoeas in prevented calves which was not observed in our study.

Table 1 Mean of average daily gains, feed conversation rate (FCR) and growth rate (GR) of calves

Item	Feeding groups		SEM[5]	P – value[4]		
	Control	Treatment		D	T	D x T
ADG[1] (g/day)	583.90	632.02	16.31	0.07	0.01	0.35
GR[2] (%)	1.97	1.82	0.11	0.43	0.18	0.27
FCR[3] (kg/kg)	1.59	1.45	0.07	0.14	0.25	0.48

[1]Average daily gain, [2]Growth rate, [3]Feed conversation rate, [4]Significant effect of experimental diet (D), time on diet (T), and their interaction (D × T), [5]Standard error of the means.

Table 2 Mean values of selected parameters in calf blood

Item	Parameters	Groups		SEM[2]	P – value[1]		
		Control	Treatment		D	T	D x T
Lipid and carbohydrate parameters	Glucose (mmol/L)	5.72	5.21	0.45	0.01	0.01	0.01
	NEFA (mmol/L)	0.24	0.27	0.13	0.45	0.12	0.08
	BHBA (mmol/L)	0.10	0.12	0.23	0.05	0.05	0.15
	Cholesterol (mmol/L)	2.32	2.15	0.36	0.28	0.01	0.34
	TG (mmol/L)	0.29	0.31	0.27	0.21	0.70	0.19
Protein indicators	TP (g/L)	52.20	60.15	0.27	0.01	0.05	0.01
	Albumin (g/L)	27.60	29.75	0.68	0.34	0.41	0.36
	Urea (mmol/L)	1.89	2.23	0.45	0.01	0.10	0.10
Enzymes	AST (IU/L)	50.89	54.02	1.72	0.18	0.05	0.11
	AST (IU/L)	10.23	10.43	0.32	0.23	0.13	0.35
	GGT (IU/L)	65.20	60.02	8.34	0.23	<0.01	0.72

[1]Significant effect of experimental diet (D), time on diet (T), and their interaction (D × T), [2]Standard error of the means.

Plasma glucose concentrations was lower in calves fed protein supplements (Table 2) and was affected by a day x treatment interaction. More, there were differences in the ages of the animals at which blood samples were collected. Differences in glucose uptake in calves are largely due to the type of diet. With milk replacers (MR) feed vs. colostrum or milk, insufficient glucose uptake was observed (Hammon et al 2013, Kupczyński et al. 2017). Quigley et al. (2006) observed the opposite trend: glucose in calves receiving higher protein dose was significantly higher in comparison to the control group, which suggests that some part of protein was used for energy. Similar to other study (Quigley et al. 2006) plasma concentration of NEFA were unaffected by dietary treatment.

Our investigations showed a statistically significant increase of BHBA in the in the groups where the protein supplement was administered. Concentration of blood BHBA increased as starter intake increased and then increased and was related rumen development (Quigle 1999, Suárez et al. 2006).

Significant differences were observed in total protein concentration between groups for the entire study period, but there were differences in the ages of the animals at which blood samples were collected. The urea concentration showed similar dependence. TP is used to affected by dietary treatment, can increase in dehydration or decreased in prolonged diarrhoea (Bednarski et al. 2015a, 2015b). In other study (Quigley et al. 2006) TP were unaffected by dietary treatment, while protein dose in feed correlates with higher urea concentrations. Higher urea concentration in protein rich feeding related to deamination process. This is used to break down amino acids for energy and is used to related to increase of glucose blood concentrations, which was not observed in current study, and previous one (Kupczyński et al. 2017).

No statistically significant differences were observed in mean AST, ALT and GGT activity between groups. These parameters are commonly used to evaluate liver function and their levels

indicate the degree of dysfunction of this organ like intoxication, inflammation, cholestasis etc. (Mohri et al. 2007). These results indicate no toxic effect of protein supplement.

CONCLUSION

Supplementation with protein - mineral chelates had a benefit of the growth rate of the calves. BHBA concentration in blood confirms the better development of rumen of experimental calves in compare to the control group. Activity of surveyed enzymes has not significantly distincted between the groups (experimental vs. control), what indicates lack of negative impact of applied preparations to the liver' functions. In the future the surveys shall be orientated on estimation of particular doses of preparation applied for calves.

ACKNOWLEDGEMENT

Project POIG.01.03.01-02-080/12 "*Y. lipolytica* and *D. hansenii* yeast, enzymes and toxin killers used for preparation of preparations useful in industry and agrotechnics" co-financed by the European Regional Development Fund under the Operational Program Innovative Economy 2007–2013. Project supported by Wroclaw Centre of Biotechnology, programme The Leading National Research Centre (KNOW) for years 2014–2018.

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PHOSPHORUS RETENTION FROM BARLEY-TYPE DIETS WITH DIFFERENT LEVELS OF ENDO-PHYTASE IN BROILERS

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Abstract: The efficacy of low-phosphorus diets with 0.20% of available phosphorus and two levels of phytase on growth rate, feed conversion ratio and phosphorus retention was examined in broiler chickens using eight replicates per treatment. The diets contained 40% of spring barley with 201 or 305 phytase activity units per kg. Chromic oxide was included in the diets as an indigestible marker. Excreta were collected during four consecutive three-day balance periods from the 12th to the 23rd day of age. No difference was observed for body weight gain between the dietary treatment groups but feed conversion ratio was better ($P < 0.05$) when higher phytase barley was used. The contents of total phosphorus was 4.56 and 4.72 g/kg in low and high phytase diets, respectively. In spite of the higher level of phosphorus, the coefficient of apparent phosphorus retention was higher ($P < 0.01$) in higher than in lower phytase group. The retention of phosphorus increased ($P < 0.01$) with duration of feeding in both low-phosphorus dietary groups.

Key Words: plant phytase, low-phosphorus diet, chicken

INTRODUCTION

Phytate, i. e. myo-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate serves as a storage form of phosphorus (P) in plant seeds (Reddy et al. 1982). About 70% of the P in barley is bound as phytate (Lott et al. 2000).

Phytase is a naturally occurring enzyme that can degrade phytate to yield inositol and P (Liu et al. 1998). One unit (1 U) of phytase activity is defined as the amount of enzyme which releases 1 μmol of inorganic phosphate from sodium phytate per minute at pH 5.5 and 37 °C. Feedstuffs of plant origin contain their own endo-phytases which can hydrolyze a part of total plant phosphorus. It was demonstrated that the plant phosphorus availability for young chicks, estimated by bone mineralisation, was rather high (between 50 and 70%) in wheat, triticale and barley but less than 25% in corn, leguminous grains, soya meal and rapeseed meal (Hoshii and Yoshida 1977, Sauveur 1983). Li et al. (2001) reported that in their experiment the bioavailability of phosphorus in barley was between 28 and 49%.

Denbow et al. (1995) studied the supplements of phytase to broiler diets containing different levels of P. They observed improved body weight gain ($P < 0.01$) at all P levels, but the magnitude of response was greatest at low dietary level. Feed efficiency was unaffected by phytase addition.

The aim of the present experiment was to study the effect of a low-P barley-soyabean meal-maize diets containing two different cultivars of barley with different content of phytase activity on growth rate, feed efficiency and phosphorus retention in broiler chickens.

MATERIALS AND METHODS

The effects of phytase content and time of feeding upon the apparent P retention of diet were investigated in balance experiments with chickens during the fattening from the 12th to the 23rd day of age.

The animal procedures were reviewed and approved by the Animal Care Committee of Ministry of Education, Youth and Sports of the Czech Republic. The first 8 days of age chickens were fed by

commercial starter. Eight-day-old male Ross 308 broiler chickens were distributed according to body mass into two dietary treatments with eight replicates per treatment. There were 8 chickens per replicate pen. The animals were kept in balance cages in an air-conditioned room. Heating and lighting programmes were in accordance with the Ross Broiler Management Handbook (2014). The birds were adapted to cages and experimental diets for 3 days. On day 12, four subsequent three-day balance periods started, during which excreta were collected daily. In each period, weight gain individually and feed consumption per pen were recorded. The coefficients of apparent P retention were estimated using the chromic oxide indicator method.

During the whole experiment, all chickens were fed on a non-pelleted grower diet (Table 1) containing 13.20 MJ nitrogen-corrected metabolisable energy and 210 g crude protein per kg. Except for phosphorus, two isocaloric barley-soybean meal-maize diets (40-31-15) were formulated to be in line with the Ross Nutrition Supplement (2009). To enable sensitive detection of changes in phosphorus retention, the diets were calculated to contain 0.20% of available phosphorus rather than the recommended value of 0.45%. The diets comprised 40% spring barley, either with a low (LPB) or a high (HPB) phytase activity. The diets were supplied *ad libitum*.

Table 1 Composition of diet (as fed basis)

Ingredients, %	
Barley	40.00
Soybean meal 48	31.40
Maize	15.00
Soybean oil	9.90
Calcium carbonate	1.97
Monocalcium phosphate	0.33
Sodium chloride	0.33
DL-Methionine 99%	0.27
L-Lysine.HCl 78% Lys	0.13
L-Threonine 98%	0.07
Supplementary premix[1)]	0.30
Chromic oxide	0.30
Calculated composition	
AME_n (MJ/kg)	13.20
Crude protein, %	21.00
SID Lysine, %	1.10
SID Met+Cys, %	0.84
SID Threonine, %	0.73
SID Valine, %	0.84
SID Isoleucine, %	0.75
Ca, %	0.90
Available P, %	0.20

SID standardised ileal digestibility; [1)]The premix supplied (mg/kg diet): retinyl acetate 4.13; cholecalciferol 0.128; DL-α-tocopherol acetate 56; menadione 3; thiamine 3; riboflavin 6; pyridoxine 4.1; hydroxycobalamine 0.015; niacin amide 50; pantothenic acid 18; biotin 0.2; folic acid 1.7; choline chloride 240; betaine 100; Narasin 70; copper 17; iron 50; zinc 80; manganese 100; iodine 1; cobalt 0.4; molybdenum 0.5; selenium 0.3; Endo-1,3(4)-beta-glucanase, Endo-1,4-beta-xylanase

The activity of barley phytase was determined according to ISO 30024:2009 (Animal feeding stuffs – Determination of phytase activity). The content of chromic oxide in food and freeze-dried excreta was estimated iodometrically (Mandel et al. 1960). Phosphorus was estimated after wet mineralization by sulphuric acid and hydrogen peroxide spectrophotometrically as vanadate yellow using Unicam 8625 UV/VIS Spectrophotometer (LabX, Midland, ON, Canada) at a wavelength of 442 nm.

The activity of phytase in LPB was 201 U/kg, i. e. 34% lower than that in HPB (305 U/kg). The intrinsic phytase activity in soybean meal used to prepare diets was not detected (heat treatment destroy phytase), and in maize it was 8 U/kg. The contents of total phosphorus was 4.56 and 4.72 g/kg in LPB and HPB diets, respectively.

Experimental data were analyzed as a completely randomized block design using ANOVA procedure of Statgraphics Plus package (Version 3.1, 1994). When a significant value for treatment effect ($P < 0.05$) was observed, the differences between means were assessed by Tukey HSD test. The experimental unit was a replicate pen.

RESULTS AND DISCUSSION

The average body weights (mean ± standard error of the mean) of HPB and LPB chickens at 11 days of age were 252 ± 2.3 g and 257 ± 1.8 g, respectively. In contrast to the findings of Denbow et al. (1995) with chickens fed on 0.26% of available P and 0 or 200 U/kg diet of added phytase in the first 3 weeks of age, in this experiment when animals were fed with LPB, weight gains were slightly lower and the feed conversion ratio was significantly ($P < 0.05$) worse when compared with HPB (Table 2).

Table 2 Effect of phytase on weight gains and feed conversion ratio

Parameter	Barley	11–14 d	15–17 d	18–20 d	21–23 d	11–23 d
Weight gain (g)	HPB	108.4[a]	140.2[a]	159.4[a]	158.9[a]	566.8[a]
	LPB	111.2[a]	132.2[a]	152.1[a]	145.5[b]	540.0[a]
Feed conversion ratio	HPB	1.607[a]	1.491[a]	1.555[a]	1.534[a]	1.553[a]
	LPB	1.586[a]	1.578[b]	1.624[a]	1.686[a]	1.647[b]

[a,b] Means of HPB and LPB not sharing a common superscript were significantly different (Tukey HSD test, P < 0.05).

Juanpere et al. (2004) used a low-P diet with untreated (191 Phytase U/kg) or autoclaved barley in an experiment with broilers. Destruction of endogenous phytase had only a little negative influence ($P > 0.05$) on the total P retention coefficient. Similar results with different intrinstic phytase activity of cereal grains (16–99 U/kg) was reported by Leytem et al. (2008). In contrast, the effect of endogenous phytase was significant ($P < 0.05$) in the present study. An average apparent phosphorus retention in the LPB and HPB diets was 38.6% and 45.1%, respectively. In spite of slightly higher level of phosphorus in the HPB group, the coefficient of apparent P retention was relatively 16.8% and absolutely 6.5% higher than in the LPB group (Table 3).

Table 3 Effect of phytase on phosphorus retention

Parameter	Diet	11–14 d	15–17 d	18–20 d	21–23 d	11–23 d
P-retention (% of intake)	HPB	37.81[a]	41.28[a]	48.98[a]	52.51[a]	45.14[a]
	LPB	31.00[b]	37.97[b]	38.16[b]	47.29[b]	38.61[b]

[a,b] Means of HPB and LPB not sharing a common superscript were significantly different (Tukey HSD test, P < 0.05).

The effect of duration of feeding of low-phosphorus diet on apparent phosphorus utilization was highly significant ($P < 0.01$). The feeding of the low-phosphorus diet resulted in a linear increase in P retention after equations

$$y_{HPB} = 13.19 + 1.727x;\ R^2 = 0.612$$
$$y_{LPB} = 8.35 + 1.635x;\ R^2 = 0.629$$

i. e. by 1.6–1.7% for each day. These results demonstrate that the organism adapts itself gradually to an insufficient supply of this element.

McDowell (1992) reported that the utilisation of P could be increased through active transport. In our previous experiment (Zelenka and Fajmonová 2001) on chickens from 1st to 21st day of age fed a diet with sufficient level of P, coefficients of apparent retention of P highly significantly decreased as the phosphorus requirement decreased with increasing age. On the other hand, the utilisation of P from a diet with a suboptimum level of P increased gradually with the time of feeding. Similarly, Olukosi et al. (2007) found in a 21-day experiment with a diet marginally deficient in P that the apparent total tract retention of P increased ($P < 0.05$) with the time of feeding as the chicks grew from 1 to 3 weeks old.

The ability of the chicks to extract P increased by 120% from week 1 (15.7%) to 2 (35.1%) but the increase from week 2 to 3 (48.1%) was only 40%.

CONCLUSION

In our experiment the higher level of plant endo-phytase in barley was very efficacious for improving phosphorus utilization in fattening chickens. Retention of P from the diet with the suboptimum level of P increased gradually with the duration of feeding.

ACKNOWLEDGEMENT

The research was supported by IGA FA MENDELU grant, no. TP_4/2017.

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VARIATION OF BIOCHEMICAL PARAMETERS OF ENERGY AND LIVER METABOLISM IN PERIPARTURIENT GOATS

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Abstract: The goal of the study is to monitor the biochemical parameters of energy metabolism in goats in periparturient period and evaluating their correlation with biochemical parameters of liver function. The study is conducted on 10 pregnant white shorthaired goats. Blood samples are collected before parturition (3, 2 and one week), on the day of parturition (day 0) and after parturition (2nd day and 1st, 2nd, 3rd and 4th week). In the blood samples selected biochemical parameters are analysed (glucose – GLU; triglycerides – TGs; cholesterol – CHOL; non-esterified fatty acids – NEFA; beta-hydroxybutyrate – BHB; total bilirubine – TBIL; aspartate aminotransferase – AST; gamma-glutamyl transferase – GGT). The results show that negative energy balance – NEB (characterized by increasing NEFA and BHB concentration and decreasing of TGs concentrations) developed in the postpartum period. The NEB was accompanied by the alteration of liver function parameters indicated by an increase of AST and GGT activity and TBIL concentration. The concentration of CHOL was not significantly changed. On the day of parturition, enormous increase of glucose was followed by a rapid decrease in the following days. The results of the correlation analysis showed a significant correlation between the liver damage parameters (TBIL, AST, and GGT) and the parameters of energy metabolism (TGs, NEFA, BHB) in goat's blood plasma.

Key Words: ketosis, negative energy balance, liver, peripartum period, biochemical profile

INTRODUCTION

Ketosis is an acute metabolic disorder of carbohydrates metabolism that is caused by the inadequate intake of energy. Significant signs of ketosis are hyperketonemia, hyperketolactia, hypoglycaemia and liver steatosis (Hofirek et al. 2009).

During the increase of milk production and reduced feeding of energy, body fat reserves are mobilized to support milk production. Lipids are degraded into non-esterified fatty acids (NEFA) and glycerol. The increase of these parameters show disproportion between intake and output of energy. In normal course, glycerol is transported to the liver by the blood and reused for gluconeogenesis. At the beginning of lipomobilisation, NEFA can be utilised for synthesis of milk fat. But most of components of the lipids degradation go to liver, where they are metabolised on acetyl-CoA as a result of beta oxidation. If there is a small amount of oxalacetate in hepatocytes, ketones as acetoacetate, beta-hydroxybutyrate (BHB) and acetone are made (Murray et al. 1998). These products when produced in excess such that organism is not able to utilise proves to be toxic. Hence it should be possible to examine liver function by monitoring the energy metabolism parameters. The current study aims to prove this hypothesis.

MATERIAL AND METHODS

Animals

The study was performed on white shorthaired pregnant goats (n = 10) kept at stalls in groups with straw litter. None of these animals showed any clinical signs of illness. They were fed with the identical ration of feed. The daily dose of feed contained 1.2 kg of meadow hay and 0.6 kg of

supplementary diet per animal. They had ad libitum access to water and minerals in the form of salt block. The supplementary diet was composed of barley (30%), wheat (20%), Lucerne meal (18%), unpeeled sunflower extracted meal (10%), wheat bran (10%), corn (5%), malt sprouts (5%), dicalcium phosphate (1.1%), sodium chloride (0.7%) and calcium carbonate (0.2%). The addition of 0.15 to 0.5 kg of oat was given to goats after kidding. The amount of kids ranged from one to three.

Clinical biochemistry

The blood samples of 10 chosen animals were collected in regular intervals for 7 weeks. The first and last samplings were made 4 weeks before parturition and third week after parturition, respectively. Every week the blood was collected from each. The blood was also collected on the day of delivery. The blood was taken from *vena jugularis* and collected into heparinized tubes. Within 2 hours, the samples of whole blood were centrifuged at 3000 revolutions per minute for 10 minutes. The biochemical parameters determining energy and liver metabolism (glucose – GLU; triglycerides – TGs; cholesterol – CHOL; non-esterified fatty acids – NEFA; beta-hydroxybutyrate – BHB; total bilirubine – TBIL; aspartate aminotransferase – AST; gamma-glutamyl transferase – GGT) were calculated from the blood samples according to the standard biochemical sets.

Statistical analysis

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. The relationship of the set parameters was tested by correlation analysis. For the relationship of values, the correlation coefficient (r) was calculated.

RESULTS AND DISCUSSION

The results of biochemical evaluation of goat's plasma are presented in tables 1 and 2. The relationship between individual biochemical parameters are presented in table 3.

According to the results presented in table 1, glycaemia is relatively constant (3 weeks before parturition (b.p.) 3.15 ± 0.45, 2 weeks b.p. 3.13 ± 0.44, 1 week b.p. 3.14 ± 0.3 and 1 week after parturition (a.p.) 2.96 ± 0.35, 2 weeks a.p. 2.87 ± 0.34, 3 weeks a.p. 3.38 ± 0.46, and 4 weeks a.p. 3.32 ± 0.59 mmol/l). A notable peak was recorded on the day of delivery. The highest average concentration of glucose was 12.75 mmol/l on the day of parturition and the lowest was 1.75 mmol/l on the next day. Since the second week after parturition, glycaemia rises slowly in reference range until the last day of the trial. Similar observations are reported in Sadjadian et al. (2013) and Manat et al. (2016). Moreover, the level of glucose rises in organisms due to the stress of parturition (Doležel et al. 2000). Insulin is necessary for utilisation of glucose for the peripheral tissue. It stimulates enzymes to gluconeogenesis and lipogenesis in the liver and inhibits glycogenolysis. As a consequence of that, glycaemia is decreased whereas glucagon plays the opposite role (Reece 1998).

Table 1 Biochemical profile (mean ± standard deviation) in goats (n = 10) during peripartum period – glucose (GLU), total bilirubine (TBIL), AST, GGT

Days relating to parturition	GLU [mmol/l]	TBIL [µmol/l]	AST [µkat/l]	GGT [µkat/l]
-21	3.15 ± 0.45 ^{a}	4.79 ± 0.82 ^{b}	1.45 ± 0.22 ^{bc}	0.65 ± 0.11 ^{a}
-14	3.13 ± 0.44 ^{a}	5.33 ± 1.13 ^{bc}	1.38 ± 0.19 ^{b}	0.62 ± 0.10 ^{a}
-7	3.14 ± 0.33 ^{a}	6.42 ± 1.10 ^{abc}	1.39 ± 0.19 ^{b}	0.63 ± 0.12 ^{a}
0	12.75 ± 4.22 ^{b}	7.17 ± 1.06 ^{ac}	1.33 ± 0.24 ^{b}	0.62 ± 0.16 ^{a}
+2	1.75 ± 1.00 ^{a}	6.03 ± 2.35 ^{abc}	1.95 ± 0.25 ^{a}	0.65 ± 0.10 ^{a}
+7	2.96 ± 0.35 ^{a}	7.66 ± 1.20 ^{a}	1.89 ± 0.30 ^{ac}	0.72 ± 0.11 ^{ac}
+14	2.87 ± 0.34 ^{a}	7.76 ± 0.88 ^{a}	2.04 ± 0.32 ^{a}	0.89 ± 0.13 ^{bc}
+21	3.38 ± 0.46 ^{a}	7.89 ± 0.90 ^{a}	2.18 ± 0.32 ^{a}	0.97 ± 0.16 ^{b}
+28	3.32 ± 0.59 ^{a}	7.91 ± 0.78 ^{a}	2.16 ± 0.35 ^{a}	0.99 ± 0.14 ^{b}

$^{a, b, c}$ *– different letter in column means statistically significant difference P > 0.05*

An alteration of the ketones (BHB) in blood after parturition was established. The average value of BHB was observed to increase since the second week after delivery. The values progressively grew with increasing lactation and rising negative energy balance. The highest value of BHB was noticed in the blood sample of last day. The observed value of BHB (1.26 ± 0.55 mmol/l) is much higher than the reference value (under 0.6 mmol/l) (LABOKLIN 2014). According to the study of the Sannen goats (Albay et al. 2014), the reference range of values is wider. The signs of pregnancy toxaemia are seen in the goats with BHB concentration over 0.86 mmol/l. The animals with BHB value that is equal or lower than mentioned number are classified as having subclinical ketosis. The lowest concentration was recorded during parturition (0.39 mmol/l). That may be due to the highest level of glucose. In view of ongoing lipolysis, low level of the triglycerides and high level of non-esterified fatty acids were observed. The main three parameters that attest to lipolysis are triglycerols, ketone bodies (like BHB) and NEFA. These represent important indicators of health condition in ruminants. The correlation of these parameters can be seen in table 3. An increased NEFA value indicates starving, lipolysis or disruption of liver function.

In ketones the highest significant difference was observed between the level of BHB before and during parturition (0.44 ± 0.12; 0.46 ± 0.19; 0.39 ± 0.09 mmol/l) and the last blood collection done at the 4th week after delivery (1.26 ± 0.55 mmol/l). NEFA are significantly lower before parturition than during parturition and in the first week after parturition. The decrease of the NEFA values after birth is in agreement with the report by Manat et al. (2016).

As seen in table 3, the observed values of liver enzymes (AST, GGT) and the level of total bilirubine (TBIL) testify presence of liver damage. With increasing level of NEFA and BHB (the signs of lipolysis and follow-up ketosis) liver damage rises too. That is seen with increasing level of TBIL, AST and GGT. On the other hand the level of triglycerides goes down. Bilirubine concentration was observed to increase almost for whole duration of the trial. At the beginning of the experiment it was on the top of the references. Since the second week the level of bilirubine is over reference value with the positive trend. Liver enzymes like AST and GGT rises in the postpartum period. The highest concentrations of these parameters are noticed in the 2nd, 3rd and 4th week after delivery.

The level of triglycerides (TGs) is mildly rising to the parturition (0.53 ± 0.25, 0.63 ± 0.20 and 0.74 ± 0.25 mmol/l). On the day of delivery there is rapid decrease in TGs level (from the value 0.74 ± 0.25 to 0.17 ± 0.11 mmol/l). As it can be seen, the observed TAG levels are over the reference values in the prepartum blood samples. After the parturition, the values rapidly dropped under the influence of lipolysis due to supply of energy for milk synthesis. This is different than the observations of Manat et al. (2016), where a mild decline of TGs was reported, however with levels over the reference value too.

Table 2 Biochemical profile (mean ± standard deviation) in goats (n = 10) during peripartum period - triglycerides (TGs), cholesterol (CHOL), non-esterified fatty acids (NEFA), beta-hydroxybutyrate (BHB)

Days relating to parturition	TGs [mmol/l]	CHOL [mmol/l]	NEFA [mmol/l]	BHB [mmol/l]
- 21	0.53 ± 0.25 ^{b}	2.11 ± 0.34 ^{a}	0.21 ± 0.07 ^{a}	0.44 ± 0.12 ^{a}
-14	0.63 ± 0.20 ^{b}	2.14 ± 0.25 ^{a}	0.11 ± 0.03 ^{a}	0.46 ± 0.19 ^{a}
-7	0.74 ± 0.25 ^{b}	2.02 ± 0.20 ^{a}	0.16 ± 0.09 ^{a}	0.54 ± 0.16 ^{ab}
0	0.17 ± 0.11 ^{a}	1.91 ± 0.27 ^{a}	0.94 ± 0.25 ^{b}	0.39 ± 0.09 ^{a}
+2	0.16 ± 0.17 ^{a}	1.85 ± 0.27 ^{a}	0.59 ± 0.16 ^{ab}	0.53 ± 0.18 ^{ab}
+7	0.16 ± 0.06 ^{a}	2.18 ± 0.27 ^{a}	0.91 ± 1.03 ^{b}	0.51 ± 0.26 ^{ab}
+14	0.19 ± 0.06 ^{a}	2.12 ± 0.32 ^{a}	0.40 ± 0.16 ^{ab}	1.14 ± 0.59 ^{bc}
+21	0.20 ± 0.12 ^{a}	2.32 ± 0.36 ^{a}	0.45 ± 0.33 ^{ab}	1.15 ± 0.59 ^{bc}
+28	0.12 ± 0.04 ^{a}	2.44 ± 0.55 ^{a}	0.34 ± 0.15 ^{ab}	1.26 ± 0.55 ^{c}

$^{a, b, c}$ *- different letter in column means statistically significant difference P > 0.05*

Cholesterol levels in prepartum period were at the bottom of reference value with a negative trend to the delivery. The lowest level of cholesterol was measured on the 3rd day of the kidding (1.85 mmol/l). Afterwards the levels of cholesterol increase with the highest value in the 3rd week after

parturition (2.44 mmol/l). This is in agreement with results of Sadjadian et al. (2013). The low level cholesterol during the last week of pregnancy can be explained with need of the foetus and also the utilisation of cholesterol for the synthesis of steroid hormones.

Table 3 Correlation (r) in all blood samples (n = 90)

Correlation	GLU	TBL	AST	GGT	TGs	CHOL	NEFA	BHB
GLU		0.06	-0.36**	-0.10	-0.14	-0.06	0.25*	-0.18
TBIL			0.61**	0.45**	-0.46**	0.27**	0.42**	0.39**
AST				0.51**	-0.47**	0.37**	0.25*	0.45**
GGT					-0.31**	0.14	-0.02	0.67**
TGs						-0.04	-0.36**	-0.25*
CHOL							0.01	0.16
NEFA								-0.15
BHB								

**P < 0.05; **P < 0.01*

CONCLUSION

The results of this research testify that a development of negative energy balance commenced in goats in postpartum period. This was accompanied by weight loss and production of keton bodies. Ketosis development was also followed by increasing liver enzyme activity and concentration of total bilirubine. The research also proves the existence of correlation between ketosis and lipomobilisation in goats and liver cell damage. These biochemical parameters imply that they are suitable for monitoring of energy metabolism as well as diagnosis of liver damage. However, no significant influence of negative energy balance on glucose concentration is observed. Hyperglycaemia on the day of parturition is related to stress load which is a consequence of parturition.

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THE EFFECT OF SELENIUM NANOPARTICLES ON THE ANTIOXIDANT POTENTIAL OF LABORATORY RATS

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Abstract: The aim of the experiment was to determine the influence of different forms of selenium (sodium selenite, selenium nanoparticles) on the antioxidant status of laboratory rats. The male of Wistar albino rats strain were sorted into 4 groups. The first group (n = 5) served as control with no selenium (Se) administration. The second group was fed with mixture containing 1.2 mg/kg/diet of sodium selenite (Na_2SeO_3). The third (n = 5) and the fourth group (n = 5) were fed with different forms of selenium nanoparticles containing 1.2 mg Se/kg/diet. Selenium nanoparticles were modified by poly(vinylalcohol) (PVA 49 kDa, PVA 100 kDa). After 30 days of experiment, the rats were slaughtered and the total content of selenium in liver and blood tissue and also changes in concentration of reduced (GSH) and oxidized (GSSG) form of glutathione were measured. In liver samples, a statistically significant increase in the amount of selenium was found only in the Na_2SeO_3 group (by 59%), although the increase occurred in all of the experimental groups. In the blood, the amount of selenium increased for Na_2SeO_3 and Se-100. These results were statistically significant. GSH and GSSG were statistically significant only in blood samples where GSH was decreased in all the experimental groups (Na_2SeO_3 by 60%, Se-49 by 56%, Se-100 by 83%) and increased only in the Na_2SeO_3 group (by 96%).

Key Words: rats, nanoparticles, liver, blood, antioxidant status

INTRODUCTION

Selenium (Se) is an essential microelement influencing the state of health of animals, humans and plants and is used in the prevention and treatment of the disease (Klusonova et al. 2015). It is part of the antioxidant defense system of the liver and is important as protection against oxidative stress during which cells and tissues may suffer damage (Fernandez et al. 2008, Horky et al. 2014, Zhai et al. 2017). Epidemiological and laboratory studies observed an inverse relationship between Se status and the incidence of various disease. This has led to the hypothesis that Se supplementation may be useful in the prevention of disease (Weekly et al. 2013). However, the narrow range between the effective and toxic dose limits its use (Zhai et al. 2017). The concentration of Se in plants is dependent from its content in the soil. In European Union the Se content is relatively low, therefore it is necessary to supplement it into the diet of farm animals. (Horky et al. 2014, Kursa et al. 2010, Polakova 2010).

Selenium in organic form is poorly soluble in water and easily converts to a grey analoque that is thermodynamically stable but biologically inert. By increasing the specific surface area and reducing size by appropriate nanotechnology, the solubility of an insoluble substance can be greatly improved (Zhai et al. 2017). By transforming the material into a nanomaterial, there changes in its chemical and biological properties and catalytic activity. Selenium nanoparticles are referred to as compounds with excellent antioxidant properties and lower toxicity compared to other selenospecies (Estevez et al. 2014). It should be noted that informations on the action of nanoparticles in the antioxidant system are still incomplete, especially the relationships between the microstructures and biological activities of whole system in vitro and in vivo (Zhai et al. 2017).

MATERIAL AND METHOD

Animals

The feeding experiment was carried out in the experimental facility of Department of Animal Nutrition and Forage Production of Mendel University in Brno, in accordance with the act on the protection of animals against cruelty No. 246/1992 Coll. Throughout the whole experiment, microclimatic conditions were measured and controlled at 23 ± 1 °C at constant humidity of 60%. The light regime was maintained at 12 hour of light and 12 hours of darkness with a maximum illumination of 200 lx.

Laboratory rats of the outbreed strain Wistar albino were selected as model animals in number of 20 pieces with an average initial weight of 150 ± 5 g. The rats were divided into 4 groups of 5 pieces. The first group was a control with no addition of selenium in their feed. The second group was supplemented with selenium in the form of Na_2SeO_3 at a dose of 1.2 mg/kg/diet. The third and fourth group were fed with selenium in form of Se-49 and Se-100 nanoparticles at a dose of 1.2 mg/kg/diet. All groups were fed with monodiet containing 0.03 mg Se/kg/diet. The experiment duration was 30 days. The animals had an access to feed and drinking water ad libitum. At the end of the experiment, the animals were sacrificed and samples of blood and liver were collected and subjected to biochemical analyses. Reduced and oxidized glutathione was determined using high performance liquid chromatography with electrochemical detection (HPLC-ED) and total content of selenium was measured by atomic absorption spectrometry (AAS).

Preparation of selenium nanoparticles

Se-49

Poly(vinyl alcohol) (PVA 49 kDa) (0.19 g) was added to a solution of 1.88 ml Na_2SeO_3 ×$5H_2O$ (2.63 g/50 ml) in water (80 ml). Cysteine (9 mg/1 ml) was added with mixing and left for 2 h. The colour turned to light orange and water was added to final 100 ml volume.

Se-100

The preparation was the same as in previous case, only Poly(vinyl alcohol) (PVA 100 kDa) was used. Undissolved PVA was filtered off. After addition of cysteine, the colour turned to orange and water was added to 100 ml.

Preparation of samples for HPLC analysis

Liver: Two grams of samples from each variant were homogenized in a fritted bowl with the addition of liquid nitrogen and 1.5 ml of water. After homogenization, each sample was sonicated using an ultrasound needle for 2 minutes, shaken for 10 minutes, and centrifuged for 20 minutes at 25 000 g and at 4 °C. 100 µl of supernatant was taken from each sample and mixed with 100 µl of 10% trifluoroacetic acid and centrifuged again for 20 minutes at 25 000 g and 4 °C. After the centrifugation, the supernatant was taken and analyzed by HPLC.

Blood: Sample processing was performed by pipetting 200 µl of sample from each variant, placing it into liquid nitrogen for 2 minutes and adding 500 µl of water. Each sample was sonicated with an ultrasound needle for 2 minutes, shaken for 1 minute, and centrifuged for 20 minutes at 25 000g and at 4 °C. 200 µl of supernatant was taken from each sample and mixed with 200 µl of 10% trifluoroacetic acid. The samples were again centrifuged for 20 minutes at 25 000g and 4 °C. After centrifugation, the supernatant was analyzed by HPLC.

Preparation of samples for AAS analysis

Samples of liver weighting 0.3 g and samples of blood weighting 0.5 g were disintegrated by dry method in a muffle furnace (LAC, Czech Rep.) and mineralized in 2.5 ml concentrated nitric acid Suprapure.

Determination of reduced and oxidized glutathione

The chromatographic system consisted of two solvent delivery pumps operating in the range of 0.001–9.999 ml/min (Model 582 ESA Inc., Chelmsford, MA, USA), Zorbax eclipse AAA C18 (150 × 4.6; 3.5 µm particle size; Agilent Technologies, Santa Clara, CA, USA) and a CoulArray electrochemical detector (Model 5600A, ESA). The electrochemical detector includes three flow cells

(Model 6210, ESA). Each cell consists of four working carbon porous electrodes, each one with auxiliary and dry Pd/H_2 reference electrodes. The sample (20 µl) was injected using autosampler (Model 542 HPLC, ESA). Samples were kept in the carousel at 8 °C during the analysis. The column was thermostated at 35 °C. Mobile phase consisted of 80 mM TFA (A) and methanol (B). The compounds of interest were separated by the following linear gradient: 0 → 14.5 min (4% B), 14,5 → 16 min (5% B), 16 → 22 min (98% B), 22 → 31 min (4% B). Mobile phase flow rate was of 1 ml/min, working electrode potential 900 mV. Time of analysis was 31 min (Kominkova et al. 2015).

Determination of selenium by atomic absorption spectrometry

Selenium was determined on 280Z Agilent Technologies atomic absorption spectrometer (Agilent, USA) with electrothermal atomization. Selenium ultrasensitive hollow cathode lamp (Agilent) was used as the radiation source (lamp current 10 mA). The spectrometer was operated at 196.0 nm resonance line with spectral bandwidth of 1.0 nm. The sample volume 20 µl was injected into the graphite tube. The flow of argon inert gas was 300 ml/min. Zeeman background correction was used with field strength 0.8 Tesla. Selenium was determined in the presence of palladium chemical modifier.

Statistics

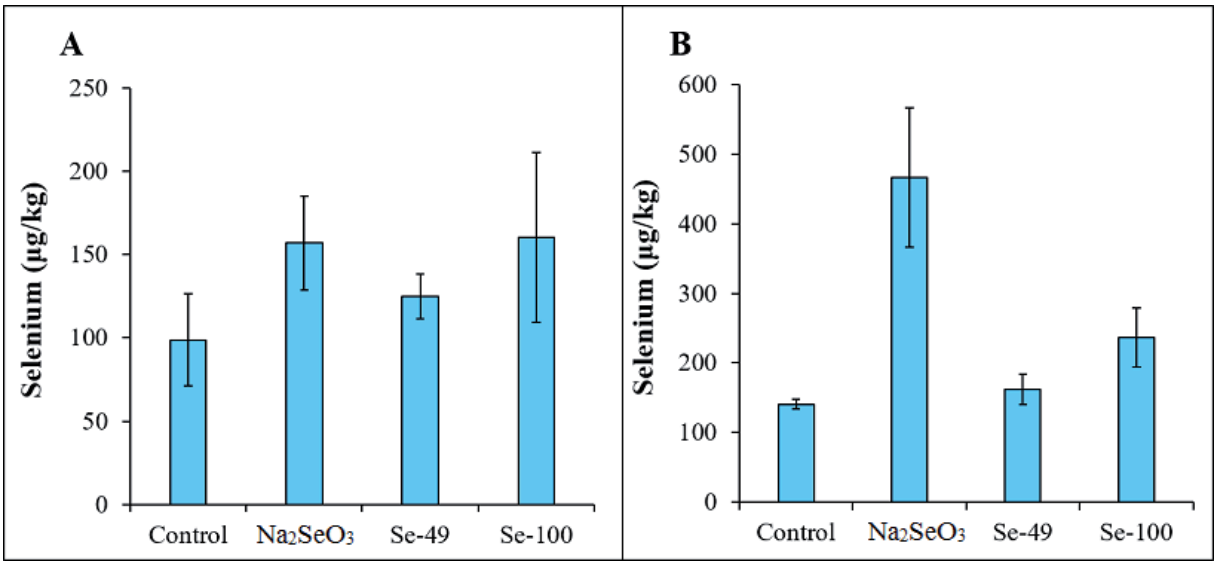
The data were processed statistically using STATISTICA.CZ, version 10.0. Results were expressed as mean from 3 measurements ± standard deviation. Statistical significance was determined by examining the basic differences among groups using ANOVA and Scheffé's test for the parameters GSH; GSSG; Se. Differences with $P < 0.05$ were considered significant.

RESULTS AND DISCUSSION

In the experiment the effect of two forms of selenium (Na_2SeO_3, Se NPs), included in the feed ration for rats on the antioxidant status of organism was monitoring. In blood and liver samples the total selenium level was determined. Another measured value was concentration of glutathione as one of the markers of oxidative stress in the body.

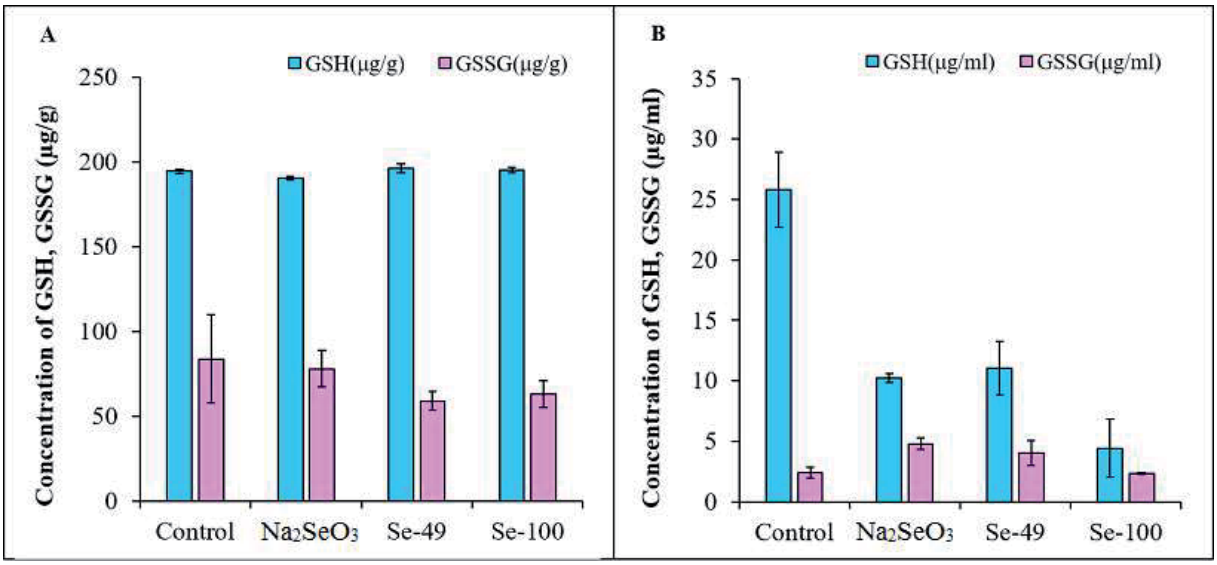
The level of selenium in liver was increased in all experimental groups compared with control (Figure 1A). For the Na_2SeO_3 group, the increase (by 59%, $P < 0.05$) was statistically significant. In the blood samples increasing amount of selenium was measured, especially in the Na_2SeO_3 group, where the level was nearly twice as high, and in the Se-100 group, which increased by 68% ($P < 0.05$) (Figure 1B). Both of these differences showed statistical significance. These results are in agreement with results of Horky et al. (2016) and show that the addition of selenium to the feed dose has an effect on increasing the amount of selenium in the liver and blood tissues.

Figure 1 Influence of sodium selenite and selenium nanoparticles on selenium concentration in (A) liver, (B) blood



The level of GSH and GSSG is a parameter which has a direct correlation with selenium and indicates antioxidant potential of organism. In the Figure 2A are shown a concentrations of GSH and GSSG in liver. No statistically significant difference were detected. The GSH level was almost equal for all groups and the GSSG showed a slight decrease in groups with selenium nanoparticles (Se-49, Se-100). The concentration of GSH and GSSG in blood is shown in the Figure 2B. The significant decrease of GSH was observed in all experimental groups (Na_2SeO_3 by 60%, Se-49 by 56%, Se-100 by 83%, $P < 0.05$). On the other hand the increase of GSSG by 96% ($P < 0.05$) in the Na_2SeO_3 group with statistical significance was measured. Kominkova et al. (2015) states that the optimum ratio of GSH and GSSG is 90 : 10. The results of our experiment were in contradiction with the results of Horky et al. (2016), which was probably caused due to the intentional use of higher selenium doses and stress.

Figure 2 Influence of sodium selenite and selenium nanoparticles on GSH and GSSG concentration in (A) liver, (B) blood



CONCLUSION

The experiment was focused on the influence on selenium nanoparticles and sodium selenite on the antioxidant status of animals. Total content of selenium and the concentration of reduced and oxidized glutathione in a liver and blood was observed. Both forms of selenium had the effect of the level of monitored indicators. The concentration of selenium was increased in liver and also in blood compared with the control group. The results shows that selenium nanoparticles may be an alternative to dietary selenium for organism. However, it would be advisable to test these sources of selenium even at lower concentrations in order to avoid potential toxicity.

ACKNOWLEDGEMENT

The study was supported by IGA MENDELU TP 1/2016.

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THE EFFECT OF ZINC ON THE CONCENTRATION OF REDUCED AND OXIDIZED GLUTATHIONE IN THE LABORATORY RATS ORGANISM

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Abstract: The aim of the experiment was to determine the effect of zinc nanoparticles (Zn NPs) on the antioxidant status of rats. The male of outbred Wistar albino rats were used in this experiment. The rats were divided into four groups. In each of group were stabled 5 males. The first group (Control) of rats served as control one without zinc administration. The second group (Zn-Phe) and the third group (Zn-Tyr) were administrated by zinc nanoparticles. In the fourth group of rats zinc oxide (ZnO) was dosed. After 30 days of the experiment, rats were sacrificed and the samples of blood and liver were analysed. Reduced (GSH) and oxidized (GSSG) form of glutathione was determined using high performance liquid chromatography with electrochemical detection (HPLC-ED) and total content of zinc was determined by atomic absorption spectrometry (AAS). In the analysis of liver, decrease of GSSG in all groups was observed. Statistically significant was decrease by 54% ($P < 0.05$) for ZnO group compared with control. The level of GSSG and total content of zinc in liver was without significant difference. In the whole blood, a significant decrease of GSH by 94% in the ZnO and by 65% in the Zn-Phe group was observed, compared with control group. The level of GSSG and total content of zinc in blood was without significant difference.

Key words: zinc, antioxidant status, rats, blood, liver

INTRODUCTION

Zinc (Zn) is an important essential trace mineral found in numerous enzymes, structural proteins, transcriptions factors and ribosomal proteins. It is involved in many physiological processes such as protein synthesis, carbohydrate metabolism and other biological reactions, which affects cellular functions (Zhao et al. 2016). Zinc deficiency is considered to cause an increased oxidative stress that leads to damage of biomolecules including DNA (Stenclova et al. 2016). The most often source of zinc is zinc oxide (ZnO). Due to its wide application, in cosmetics (UV-protection in sunscreens), in paints or as anticorrosive, antibacterial and antifungal agents, further increase of Zn nanoparticles use can be anticipated (Vankova et al. 2016).

Compared with ZnO, Zn NPs has a stronger chemical activity, oxidation reactions and the permeability on Zn NPs can help avoid adverse gastrointestinal reactions (Zhao et al. 2014). It may be used at lower doses in animal feed to provide better results than conventional Zn sources (Swain et al. 2016). Due to their small size, Zn NPs are readily absorbed and easily cross biological barriers, which make them promising candidates as diet additives. However, some studies have reported that Zn NPs cause toxicity, therefore, their safety and potency as diet additives for farm animals should be established (Zhao et al. 2016).

MATERIAL AND METHODS

Animals

The experiment was carried out in experimental facility on the Department of Animal Nutrition and Forage Production, Faculty of AgriSciences Mendel University in Brno. All tests were done in accordance with the act to protect animals against cruelty (No. 246/1992). Microclimatic conditions in

laboratory limited by temperature were measured using DATALOGER S 3120 (Comet system, Czech Republic). The temperature was kept at 23 ± 1 °C. The same device was used for the monitoring of constant humidity. The air conditioning unit was set at a level of 60%. The photoperiod was driven according to scheme: 12 h per day and 12 h per night with maximal intensity 200 lx. The male of outbred Wistar albino rats strain were used in this experiment. The average weight of each animal was 235 ± 3 g.

The experimental animals were stabled on plastic cages with grates. The rats had free access to food and water ad libitum. The rats were sorted out to four groups. In each group were stabled 5 males. The first group (Control) of rats served as control one without zinc administration. The second group (Zn-Phe) and the third group (Zn-Tyr) were administrated by zinc nanoparticles (200 mg/kg of diet). In the fourth group of rats, ZnO (200 mg of zinc/kg of diet) was dosed. All groups were fed with monodiet (kibbled wheat) containing 32.2 mg of zinc per kg of body weight per day. The rats were sacrificed after 30 days of the experiment. The samples of whole blood and liver were obtained from animals and immediately subjected to the appropriate sampling analysis.

Preparation of liver and blood samples

Liver: A sample (2 g of liver, fresh weight) was deeply frozen by liquid nitrogen and 1.5 ml water. After that, sample was homogenized and vortexed for 10 min and centrifuged at 16 400 rpm (20 min, 4 ºC). A volume of 100 µl of supernatant was taken and mixed with 100 µl of 10% trifluoroacetic acid (TFA). Subsequently the sample was centrifuged (20 min, 16 400 rpm, 4 ºC). Supernatant was used for analysis.

Blood: A sample (200 µl of blood, fresh weight) was deeply frozen by liquid nitrogen and 500 ml water. Sample was vortexed for 1 min and centrifuged at 16 400 rpm (20 min, 4 ºC). A volume of 200 µl was taken and mixed with 200 µl of 10% trifluoroacetic acid (TFA). After that, samples were centrifuged (20 min, 16 400 rpm, 4 ºC). Supernatant was used for analysis. Samples were stored on the ice all the time.

Determination of reduced and oxidized glutathione

Reduced and oxidized glutathione was determined using high performance liquid chromatography with electrochemical detection (HPLC-ED). The chromatographic system consisted of two solvent delivery pumps operating in the range of 0.001–9.999 ml/min (Model 582 ESA Inc., Chelmsford, MA, USA), Zorbax eclipse AAA C18 (150 × 4.6; 3.5 µm particle size; Agilent Technologies, Santa Clara, CA, USA) and a CoulArray electrochemical detector (Model 5600A, ESA). The electrochemical detector includes three flow cells (Model 6210, ESA). Each cell consists of four working carbon porous electrodes, each one with auxiliary and dry Pd/H_2 reference electrodes. Both the detector and the reaction coil/column were thermostated. The sample (20 µl) was injected using autosampler (Model 542 HPLC, ESA). Samples were kept in the carousel at 8 °C during the analysis. The column was thermostated at 35 °C. Mobile phase consisted of 80 mM TFA (A) and methanol (B). The compounds of interest were separated by the following linear gradient: 0 → 14.5 min (4% B), 14.5 → 16 min (5% B), 16 → 22 min (98% B), 22 → 31 min (4% B). Mobile phase flow rate was of 1 ml/min, working electrode potential 900 mV. Time of analysis was 31 min (Kominková et al. 2015).

Determination of zinc

A 10 µl of blood were pipetted into digestion vials and 10 mg of homogenized liver were weighed into digestion vials. Nitric acid suprapure and hydrogen peroxide (30%) were used as digestion mixture. A 500 µl of volume of digestion mixture was used (300 µl HNO_3 and 200 µl H_2O_2). The samples were digested by Microwave 3000 (Anton Paar GmbH, Austria) rotor MG-65. Microwave power was 100 W in the main part of the programs (for 30 min, 140 ºC). Zinc was determined by ContrAA 700 (Analytik Jena, Germany) atomic absorption spectrometer for flame and hydride technique. The spectrometer was operated at 213.83 nm resonance line (Horky et al. 2016, Horky et al. 2013).

Statistic

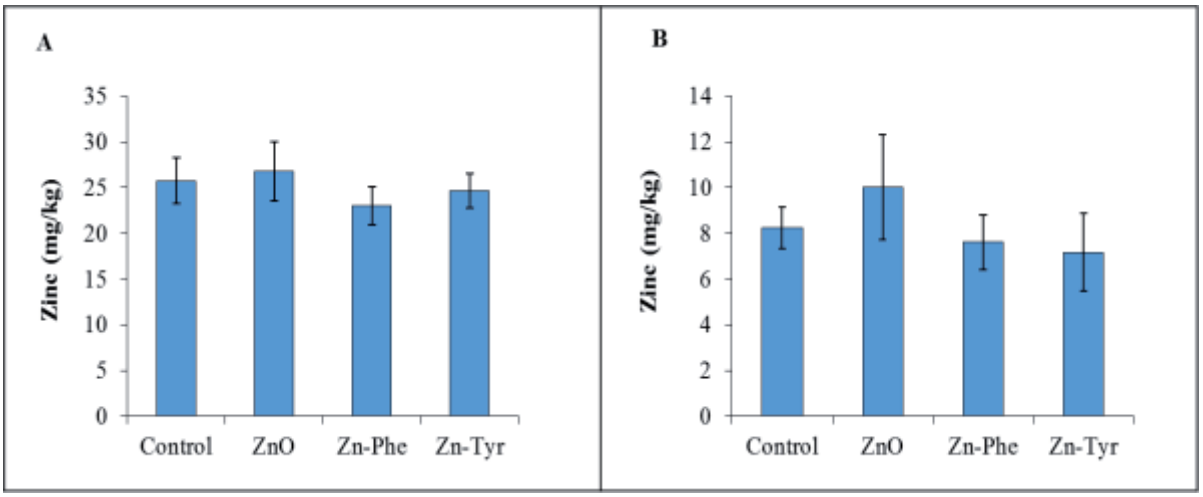
The data were processed statistically using STATISTICA.CZ, version 10.0 (Czech Republic). Significance was determined by examining the basic differences among groups using ANOVA and

Scheffé's test for the parameters GSH; GSSG; Zn. Differences with $P < 0.05$ were considered significant.

RESULTS AND DISCUSSION

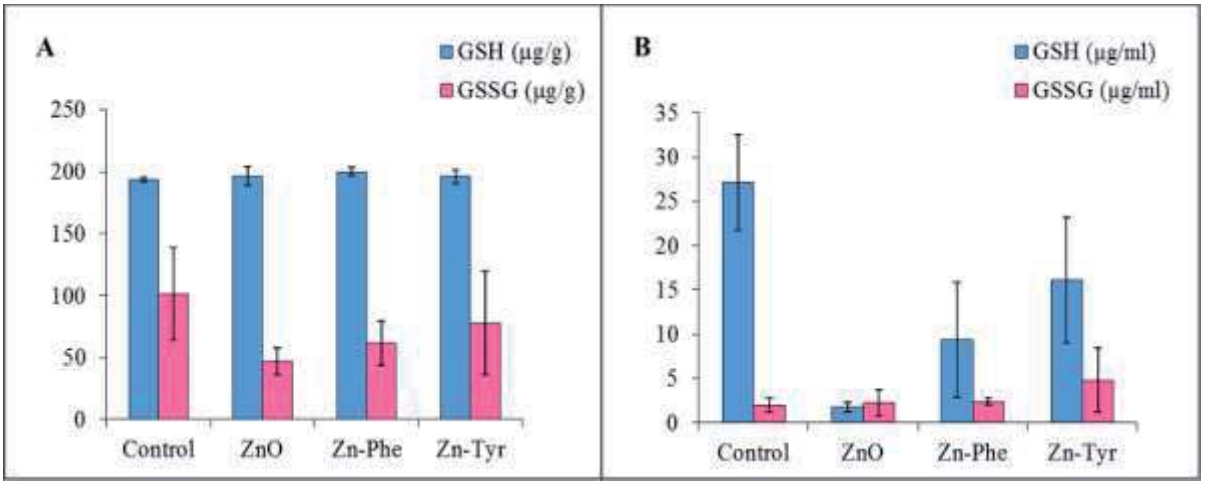
In the experiment, the influence of different forms of zinc (ZnO, Zn-Phe NPs, Zn-Tyr NPs) on the antioxidant status of rats was observed. Samples of whole blood and liver were analysed. The first observed parameter was the total level of zinc. No statistical significant differences were detected. In the liver, concentration of zinc was similar in all groups (Figure 1A). In the whole blood, there was increase by 21% in ZnO group and both groups with Zn NPs were decreased compared with control group (Figure 1B). The results of liver samples are in agreement with Horky et al. (2016) and Stenclová et al. (2016), but in their experiments, the content of zinc in blood was decreased in groups with Zn NPs. This could be caused by the use of different zinc modifications and shorter time of experiment.

Figure 1 The influence of Zn nanocomplexes in (A) liver, (B) blood on zinc concentration.



Another parameter, which indicates the antioxidant potential of the organism, was level of GSH and GSSG. In the liver a significant increase in the GSH level by 3% ($P < 0.05$) was found in the Zn-Phe group compared with control. In the case of GSSG, decrease by 54% in ZnO group with significant evidence was observed. On the other hand a decrease was observed for all experimental groups (Figure 2A).

Figure 2 The influence of Zn nanocomplexes in (A) liver, (B) blood on the level of reduced (GSH) and oxidized (GSSG) form.



In the analyses of whole blood, a significant decrease of GSH was measured in the ZnO group (by 94%, $P < 0.05$) and Zn-Phe group (by 65%, $P < 0.05$) compared with control. Lower level of GSH was also observed in the Zn-Tyr group (by 41%) but without statistical significance. The whole blood

samples showed increase in the GSSG concentration but not statistically significant (Figure 2B). Kominková et al. (2015) indicates the optimal GSH : GSSG ratio is 90 : 10%. In our experiment, in liver, increase of GSH and decrease of GSSG in both groups with zinc nanoparticles was measured.

CONCLUSION

The experiment was focused on the use of zinc nanoparticles and zinc oxide in the diet of rats. The antioxidant status was observed by GSH and GSSG determination. Both forms of zinc had the effect on the level of reduced and oxidized glutathione in a whole blood and the liver. The total level of zinc was decreased in liver and also blood samples, but without significant difference.

ACKNOWLEDGEMENT

The study was supported by TP IGA MENDELU 1/2017.

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THE EFFECT OF HIGH BARN TEMPERATURE ON THE BEHAVIOUR IN HOLSTEIN DAIRY COWS

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Abstract: The aim of this study was to determinate the effect of high barn temperature on animal behaviour in Holstein dairy cows. The experiment was conducted on University's farm in Žabčice from 27th June to 27th July 2017 at an average outside air temperature of 25.9 °C and 25.5 °C temperature inside the barn. Two groups of dairy cows (72 animals each group) were included in the experiment. Dairy cows were housed in free stall bedded barn. Ethological manifestations (presence of dairy cows inside the stall – lying down or standing, presence of dairy cows outside the stall – in corridor or in feeding area) were recorded for each group three times a day (in the morning, at the noon, before leaving for the parlor), two consecutive days a week. The bioclimatic parameters were measured concurrently – outside and inside the barn (in the living area of both groups). Monitored dairy cows were fed during the entire monitoring period identical TMR. Results of our study demonstrated the effect of high barn temperature on dairy cows behaviour. Overall, dairy cows were standing more than lying down, by 4%. However, while sections 1 and 9 did not have a more significant impact on ethological manifestations of dairy cows (dairy cows in both section acted the same way – they were lying down more than standing, except for slight variations), we found out significant differences in dairy cows behaviour in assessing the preferences of the individual rows of stalls. The highest number of dairy cows were in the middle row (B) compared to the row A (row closer to the feeding area) and the row C (row at the perimeter wall). Nevertheless, the proportion of individual dairy cow ethological manifestations in the individual rows (A, B, C) was comparable.

Key Words: barn temperature, behaviour, dairy cows, heat stress, Holstein

INTRODUCTION

Nowadays, when the prognoses of constant global warming are not very positive, it can be assumed that the heat stress in livestock will be an increasing problem that farmers will have to deal with even more intensely (Doležal 2010). According to the latest findings, the thermoneutral zone of dairy cows ranges from 0 to 20 °C (Prýmas 2006), but as soon as the temperature exceeds 20 °C, dairy cows begin to show symptoms of heat stress (Prýmas 2006, Michal 2006). Such symptoms can be, for example, reduced feed intake (Novák and Rožnovský 2008), reduced rumination (Colturato 2012) and, among other things, searching for places with greater air flow, reduced lying in boxes (Kadečka 2012) and especially reduced time spent lying down (Doležal 2010). The exact value of thermoneutral zone cannot be said, because it always depends on the actual performance of dairy cow, their individuality, health status and also values of other microclimatic parameters – relative air humidity, cooling velocity, airflow velocity and others (Zejdová 2014). Cattle farmers are more and more aware of the fact that heat stress caused by high ambient temperatures is one of the mojer factors influencing the economics of their farm, however, despite a great deal of information on the negative impact of heat stress, the choice of a suitable method of protection against the heat stress is still a big problem (Doležal 2010). It's important to realize that while the effect of high temperature on reproduction and production indicators shows up with certain time offset, the effect of heat stress on overall cow behaviour can be seen in very short time.

MATERIAL AND METHODS

For the purposes of this experiment, ethological monitoring of high producing Holstein dairy cows was carried out form 27th June to 27th July on the University's farm in Žabčice. Two groups of dairy cows were included in the experiment (72 animals each group). Ethological manifestations (presence of dairy cows inside the stall – lying down or standing, presence of dairy cows outside the stall – in corridor or in feeding area) were recorded for each group three times a day (in the morning, at the noon, before leaving for the parlor), two consecutive days a week. The bioclimatic parameters were measured concurrently – outside and inside the barn (in the living area of both groups). Specifically, these were the parameters: air temperature (°C), refrigeration quantity (W/m^2), air flow (m/s) and light intensity (lx). Monitored dairy cows were fed during the entire monitoring period identical TMR. Dairy cows were housed in free stall barn with three rows of boxes – A (close to the feeding area), B (in the middle), C (at the perimeter wall). All bioclimatic parameters were always measured on the day of observation, three times a day, concurrently with the recording of the ethological manifestation. The results of the ethological monitoring were processed by common mathematical and statistical methods.

RESULTS AND DISCUSSION

The influence of bioclimatic parameters on the behaviour of dairy cows is shown in Table 1. The table shows that the average barn temperature was 25.5 °C, wherein the temperature difference between the sections not being demonstrated. Other bioclimatic parameters in the barn had values: air flow 0.7 (m/s), wherein the difference between sections 1 and 9 being 0.2 (m/s), light intensity 2811 (lx) with difference between sections of 52 (lx) and refrigerating quantity 75.2 (W/m^2), difference between sections being 10.2 (W/m^2). Overall number of standing cows was 52%. As for lying cows it was 48%. The difference between the number of standing and lying cows in the sections is, except for negligible variations (1%), the same as the difference between the number of standing and lying cows in the barn.

Table 1 The effect of bioclimatic parameters on behaviour in dairy cows

Bioclimatic parameters	$\bar{x}$				
	Outdoor	In the stall	Section		Difference
			1	9	1 x 9
Temperature (°C)	25.9	25.5	25.3	25.3	0
Air flow (m/s)	1.0	0.7	1.0	0.8	0.2
Light intenzity (lx)	39046	2811	1598	1650	52
Refrigerating quantity (W/m^2)	73.5	75.2	70.2	80.4	10.2
Etological parameters	$\bar{x}$ (%)				
	∑	Section			
	In the stall	1		9	
Standing cows – total number	52	53		53	
Lying cows – total number	48	47		47	
∑ Standing + Lying cows	100	100		100	
Cows in the box	68	68		67	
Cows in the box – standing	20	20		20	
Cows in the box – lying	48	48		47	
Cows outside the box	32	32		33	
Cows in the corridor	15	16		15	
Cows in the trought	17	16		18	
∑ Cows in + outside the box	100	100		100	

This fact is being confirmed by (Doležal 2010, Zejdová 2014). The difference between the sections in the case of standing and lying did not uccur, which could be assumed with the same average temperatures in sections 1 and 9. Both overall and also in each section (1 and 9), dairy cows were more inside the box than outside (in the corridor or in the feeding area) and they were lying down more than standing despite the fact that the air temperature was high. According to Doležal (2010) and Zejdová (2014), dairy cows should be standing more than being inside the box in these conditions. However this barn was enriched with the fans and thanks to this it can be assumed that dairy cows were cooled better and therefore they were more inside the box and lying down more (Michal 2006, Kadečka 2012).

In Table 2 the preference of row of box in dairy cows for the whole barn is displayed. In Table 3 the preference of row of box in dairy cows is displayed for each section (1, 9). In both tables we can see that in all three rows dairy cows were lying more than standing despite the high average temperature in barn (25.5 °C) and in sections (25.3 °C). Even in this case we must not forget the aforementioned facts, that the barn has been enriched with fans, so it can be assumed that dairy cows were cooled better and thanks to this they were lying down more. This is being confirmed by the finding of Kadečka (2012), Michal (2006), Kic (1996), Knížková et al. (2007). From the table it is clear that in the context of the preference of each row of boxes in dairy cows, they generally preferred boxes of B – row. As mentioned several times, barn was equipped with fans. These fans were situated above the B – row of boxes and therefore it can be assumed that the largest air flow was there and therefore dairy cows in that area were cooled the best. This is also confirmed by Michal (2006), Kadečka (2012), Zejdová (2014), Doležal (2010), Novák and Rožnovský (2008), Chloupek and Suchý (2008).

Table 2 Preference of line of box in dairy cows – in the stall

$\bar{x}$ (%)				
Line of box	Cows - total number	Standing cows	Lying cows	∑
A	30	30	70	100
B	43	29	71	100
C	27	28	72	100
∑	100			

Table 3 Preference of line of box in dairy cows – in the section

$\bar{x}$ (%)					
Section	Line of box	Cows - total number	Standing cows	Lying cows	∑
1	A	30	30	70	100
	B	44	31	69	100
	C	26	31	69	100
	∑	100			
9	A	31	31	69	100
	B	42	30	70	100
	C	27	27	73	100
	∑	100			

CONCLUSION

From the results of our research it can be stated that the influence of high barn temperature on the behaviour of dairy cows was proved. In this barn, which has been enriched with fans, dairy cows were standing (52%) more than lying (48%). When comparing the preference of individual row of boxes, both in the whole barn and in sections (1, 9), it was found that dairy cows preferred the B – row the most. For the whole barn it was 43% in the B – row, compared to 30% in the A – row and 27% in the

C – row. In section 1 the preference in dairy cows was 44% in the B – row, 30% in the A – row and 26% in the C – row. In section 9 compared to section 1 the values between the individual rows were only slightly different. This was in the B – row by 2%, in the A – row by 1% and in the C – row also by 1%.

ACKNOWLEDGEMENTS
The research was financially supported by the project by TP IGA MENDELU 7/2017.

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USE OF HERBAL ADDITIVE TO ELIMINATE THE NEGATIVE EFFECTS OF HEAT STRESS ON BROILERS

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Abstract: The aim of this study was to evaluated addition of herbal extracts on performance, quality of meat and blood parameters in broilers affected by heat stress. Both sexes (135 broilers) of COBB 500 hybrids, were used in the experiment. Broilers were divided into two groups with six replications; control group (CG) and experimental group (EG). Both groups were fed the same diets till 28 days of age. From this age CG fed control diet and EG fed the same diet only supplemented with 0.1% herbal additive based on Baical Skullcap root (*Scutellaria baicalensis* L.) containing glycoside baicalin. Both groups were fed *ad libitum.* At the age 30 days temperature was increased to 27 °C and kept the same till the end of experiment at 34 days of age. Live body gain and live body weight were higher in EG, but the difference were no significant. Feed conversion ratio was better in EG, but there was no significant difference between the groups and feed intake was almost the same in both groups. Weight of abdominal fat, liver, bursa fabricii and spleen was the same in both groups. Weight of heart was significantly higher in EG ($P < 0.05$). pH of meat after 1 hour and 24 hours after slaughter were almost the same in both groups. Drip loss was slightly higher in EG. Blood parameters such as alkaline phosphatase, alanine transaminase, aspartate transaminase, glucose, gamma-glutamyl transpeptidase, glutathion peroxidase and concentration of urea were not influenced by herbal additive added to the diet.

Key Words: herbal additive, heat stress, broilers performance, baicalin, *Scutellaria baicalensis* L.

INTRODUCTION

Negative impacts of heat stress on broilers are objects of interest for breeders and researchers. The danger of heat stress has increased every year. The growth performance of broilers is steadily increasing too. On one hand rapid metabolism produces a lot of heat and on the other hand broilers do not have sweat glands. These reasons cause sensitivity to heat stress in broilers, which increases with the age of birds. Heat stress is a serious problem on broilers farms, especially during the summer months.

A negative influence on the growth performance was demonstrated by several studies (Quinteiro-Filho et al. 2010, Azad et al. 2010, Moura et al. 2015). Heat stress also impairs the conversion of feed and reduces carcass yield (Jahejo et al. 2016). Qualitative indicators of meat are also negatively affected by heat stress. Heat stress before the slaughter affects the metabolism of the muscles and disturbs the integrity of the membranes (Tang et al. 2013). There is also a decrease in pH of meat and may cause PSE incidence (Fernandes et al. 2016). The immune system is also negatively affected. Heat stress increases blood corticosterone levels (Sohail et al. 2012). An increase in plasma corticosterone concentration induced lymphoid tissue involution, thereby reducing the weight of thymus and bursa fabricii (Yang et al. 2016). Heat stress also has a deleterious effect on the organism by producing free radicals and reactive oxygen species (ROS). Increased production of free radicals and ROS compounds can cause damage to the constituents of various biological tissues including lipids, proteins and deoxyribose nucleic acid (Jahejo et al. 2016).

The large number of negative impacts of heat stress on broilers stimulate researchers to eliminate its negative impacts through feed additives. Nowadays, the use of various herbs and herbal extracts as feed additives is very actual. The aim of this study was to investigate the effect of the addition of herbal

additive based on Baical Skullcap root (*Scutellaria baicalensis* L.) containing glycoside baicalin to eliminate the negative effects of heat stress in broilers.

MATERIAL AND METHODS

Both sexes (135 broilers) of COBB 500 hybrids were fed in 12 littered floor boxes. They were divided into two groups with six replications: control group (CG) and experimental group (EG). The average weight of day old broilers was 42 g and broilers were fed till 34 days of age, when the experiment was finished. Temperature in the room was 30 °C at the start of experiment and gradually decreased to 20 °C according to guidelines for the hybrid. The light regime was as follow: in the first week of experiment 23 hours of light and 1 hour of darkness and after the first week 18 hours of light and 6 hours of darkness.

The water intake was supplied with nipple drinkers and feeding was manually submitted from tube feeders. The water and feed were available *ad libitum.* The hybrids were fed on broiler diets meeting the requirements for COBB 500. Starter BR 1 (crumble pellets) was fed from 1 to 10 days of chicken age, grower BR 2 (pellets) from 10 to 28 days. Composition of BR 1 and BR 2 were the same in both groups on maize, wheat, soybean extraction meal, soybean toasting meal, expanded rapeseed meal, calcium carbonate, calcium dihydrogen phosphate, animal fat and sodium chloride. Finisher BR 3 (pellets) was fed from 28 days to 34 days. Composition of BR 3 was based on crushed wheat, maize, soybean extraction meal, soybean toasting meal, expanded soybeans, wheat, rapeseed, animal fat, calcium carbonate, sodium chloride, monocalcium phosphate and sodium sulfate. Content of nutrients in the diets is shown in the Table 1. Both groups fed the same diets till 28 days of age. From this age CG fed control diet BR3 and EG fed the same diet BR3 only supplemented with 0.1% herbal additive Axion feedstim based on Baical Skullcap root (*Scutellaria baicalensis* L.) containing glycoside baicalin.

The broilers were marked individually by the wing tags and weighted at 10, 17, 24, 28 and 34 day of experiment. The amount of feed was recorded and feed conversion ratio was evaluated on weighting days.

Table 1 Content of nutrients in the diet

Content of nutrients [g/kg]	BR 1	BR 2	BR 3
Crude protein	196	187.0	198
Crude oils and fats	40	48	85
Crude fiber	29	33	35
Crude ash	53	43	46
Lysine	11.7	10.9	11.8
Methionine	4.9	4.7	3
Calcium	9	6.0	5.1
Phosphorus	5.5	4.4	4.4
Sodium	1.4	1.3	1.6

At the age 30 days temperature was increased to 27 °C and kept the same till the end of experiment, consequently exposure to heat stress ran from 31 to 34 days. Temperature humidity index (THI) was as follow: 28 day: 66; 32 day: 74–75; 33 day: 73–74; 34 day: 74–75 (Burgos-Zimbelman and Collier 2011).

At the age 28 days there were no significant difference in weight, weight gain and feed conversion ratio between CG and EG ($P < 0.05$). At 28th day, blood was collected from 20 broilers (10 from EG, 10 from CG) and alkaline phosphatase, alanine transaminase, aspartate transaminase, glucose, gamma-glutamyl transpeptidase, glutathion peroxidase and content of urea were recorded. On the 34th day, blood was collected again from the same 20 broilers and the same analysis was performed. Subsequently, the broilers were slaughtered and the weight of the abdominal fat, heart, liver, bursa fabricii and spleen was recorded. At 1 and 24 hours after slaughter pH of breast muscles from 20 broilers (10 from EG, 10 from

CG) were recorded. Water holding capacity was determined by a modified method (Grau and Hamm 1952) expressed as drip loss.

The observed characteristics were analyzed using one way ANOVA and LSD-test using the software package Unistat 5.1 (UNISTAT Ltd, ENGLAND).

RESULTS AND DISCUSSION

Live weight, daily feed intake and feed conversion ratio of broilers at the beginning of the experiment (28th day) are shown in Table 2.

Table 2 Broilers performance till 28 days of experiment

	Live weight [g]	Feed intake [g]	Feed conversion ratio
Experimental group	1699	168	1.625
Control group	1707	169	1.575

Performance parameters of broilers are shown in Table 3. At the end of experiment live body weight was slightly higher in experimental group, live weight gain was also slightly higher in experimental group, but there were no significant differences between experimental and control group. Feed conversion ratio (FCR) was almost the same in both groups. The daily feed intake was the same in both groups.

There was no significant difference in qualitative parameters of meat between the groups. pH of meat 1 hour and 24 hours after slaughter was same and drip loss was slightly higher in experimental group (Table 4).

Between experimental and control group was no significant difference in weight of abdominal fat, liver, bursa fabricii and spleen, but weight of heart was significantly higher ($P < 0.05$) in experimental group (Table 5).

The results of blood tests are shown in Table 6. No significant difference between the experimental and the control group was demonstrated in all parameters.

Table 3 Parameters of broiler performance

Parameter	Experiment		Control	
	Mean ± SE*	v_x** [%]	Mean ± SE*	v_x** [%]
Live body weight [g]	2298^a ± 28.54	10.32	2282^a ± 27.90	9.86
Live weight gain [g]	600^a ± 15.62	21.61	579^a ± 12.16	16.94
Feed conversion ratio	1.628^a ± 0.04	6.08	1.637^a ± 0.02	3.46
Daily feed intake [g]	155^a ± 2.84	4.49	153^a ± 2.11	3.36

a, b – different letters on one line – statistically significant differences ($P < 0.05$); SE – standard error; v_x** – coefficient of variation*

Króliczewska et al. (2008) evaluated addition of *Scutellaria baicalensis L.* extract (SBE) on growth performance of Hubbard Hi-Y broilers. In contrast of our results their results shown significantly higher live body weight and weight gain in experimental groups. Feed conversion ratio was slightly better in experimental groups. Feed intake was higher only in experimental group with highest concentration of SBE in diet. In their experiment broilers were not exposed to heat stress.

Positive effect on weight gain and feed conversion ratio with feeding SBE was also demonstrated by Park et al. (2016), in their study. They also studied effect on blood parameters like aspartate transaminase, alanine transaminase, gamma-glutamyl transpeptidase, alkaline phosphatase and glucose and they also did not found significant differences between control and experimental groups. Drip loss increased linearly in experimental groups, so feeding with SBE caused worse water holding capacity.

Króliczewska et al. (2017) studied the effect of the addition of SBE on selected organs of broilers (liver, bursa fabricii and spleen). Addition of SBE to the diet did not influence on relative weight of the

liver, but caused linear decrease in the relative weight of spleen and bursa fabricii with increasing SBE dose.

Table 4 pH of meat 1 hour and 24 hours after slaughter and drip loss of meat

Parameter	Experiment		Control	
	Mean ± SE*	v_x** [%]	Mean ± SE*	v_x** [%]
pH 1	$6.21^a \pm 0.07$	3.97	$6.29^a \pm 0.04$	2.25
pH 24	$5.97^a \pm 0.05$	2.78	$6.06^a \pm 0.04$	2.58
Drip loss [%]	$18.56^a \pm 0.55$	2.36	$16.88^a \pm 0.83$	3.58

a, b – different letters on one line – statistically significant differences (P < 0.05); SE – standard error; v_x** – coefficient of variation*

Table 5 Weight of selected organs and abdominal fat (g)

Parameter	Experiment		Control	
	Mean ± SE*	v_x ** [%]	Mean ± SE*	v_x ** [%]
Abdominal fat	$20.4^a \pm 0.98$	27.70	$20.9^a \pm 1.27$	36.35
Heart	$12.8^a \pm 0.34$	15.47	$11.5^b \pm 0.27$	14.16
Liver	$45.2^a \pm 1.03$	13.16	$44.4^a \pm 1.27$	17.11
Bursa fabricii	$3.2^a \pm 0.15$	28.04	$3.5^a \pm 0.16$	27.82
Spleen	$1.8^a \pm 0.08$	25.33	$1.7^a \pm 0.09$	32.06

a, b – different letters on one line – statistically significant differences (P < 0.05); SE – standard error; v_x** – coefficient of variation*

Table 6 Selected blood parameters of broilers from 28th and 34th days of experiment

Parameter	28th day		34th day	
	Experiment	Control	Experiment	Control
Alkaline phosphatase [ukat/l]	89.88^a	80.59^a	107.29^a	58.76^a
Alanine transaminase [ukat/l]	0.02^a	0.04^a	0.11^a	0.04^a
Aspartate transaminase [ukat/l]	3.94^a	4.32^a	0.44^a	2.10^a
Glucose [mmol/l]	9.76^a	9.62^a	8.19^a	8.41^a
Gamma-glutamyl transpeptidase [ukat/l]	0.24^a	0.26^a	0.25^a	0.26^a
Glutathion peroxidase [ukat/l]	26.2^a	26.4^a	25.9^a	24.78^a
Urea [mmol/l]	0.48^a	0.36^a	0.53^a	0.5^a

a, b – different letters on one line – statistically significant differences (P < 0.05)

CONCLUSION

The addition of herbal additive to the diet of broilers subjected to heat stress had no significant effect on live body weight, live body gain, feed conversion ratio and daily feed intake. Meat quality parameters was not affected by feeding herbal additive. Weight of abdominal fat, liver, bursa fabricii was almost the same in both groups and weight of heart was significantly higher (P < 0.05) in experimental group. Blood tests did not show significant differences between the groups in observed parameters. The results of the experiment show that the addition of 0.1% herbal additive does not reduce negative impacts of heat stress neither negatively affect meat production. The recommendation is to increase the dose of herbal additive.

ACKNOWLEDGEMENTS

The results were obtained with financial support by the project of MENDELU, Faculty of AgriSciences IGA No. TP 7/2017: Analysis of performance and behaviour of farm animals in relation to ambient

temperature variability and possibilities of elimination of its impact. The experiment was done thanks equipment financed by project OP VaVpI CZ.1.05/4.1.00/04.0135.

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FISHERIES AND HYDROBIOLOGY

DRAGONFLY (INSECTA: ODONATA) ASSEMBLAGE OF THREE TYPES OF HABITATS IN THE SOUTH OF CENTRAL SLOVAKIA

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Abstract: Several field works between 2016 and 2017 were undertaken on the south of Central Slovakia. Three different types of biotopes based on their species richness and abundance (flooded quarry, fishpond and peat bog) were distinguished and compared in Cerová vrchovina Upland and Juhoslovenská kotlina Lowland. The most diverse locality was flooded quarry with 23 species, followed by fishpond with 13 species, while the lowest diversity had peat bog with 11 species. A total of 27 species were observed. Among them, 4 lestids, 1 platycnemid, 8 coenagrionids, 3 aeshnids, 1 cordulid and 10 libellulids were presented. At 16 of them larval stage was recorded. According to the actualised Czech Republic´s Red List of dragonflies 4 species belongs to Critically endangered, 2 to Endangered, 4 to Vulnerable and 6 to Near Threatened category. New additions to the Cerová vrchovina Upland´s species list were added, i.e. *Coenagrion pulchellum*, *Anaciaeshna isoceles*, *Aeshna grandis* and *Libellula fulva*. New locality of *Epitheca bimaculata* in the Special Protected Area Poiplie is given. The most significant result is record of *Sympetrum depressiusculum* after almost two decades in Slovakia.

Key words: water insects, SPA Cerová vrchovina – Porimavie, SPA Poiplie, conservation

INTRODUCTION

The main problem of the wetlands is the meliorations in the 20th century and the desiccation in the 21th century. It's clear, inadequate management approaches, large-scale land conversion in conjunction with growing population have synergetic effects on freshwaters with its breakable wildlife (Kalkman 2010). Degradation of wetlands directly affects wetland-dependent species, because of the decreased biotope availability and competition for limited resources. On the contrary, sensitive organisms such as dragonflies help us to indicate the quality and integrity of freshwater ecosystems (WWF 2016).

Dragonflies are well suited for bioassesment for many reasons. They often require special habitat conditions, they can be relatively easily recognized in the field and they constitute keystone species, because they are already the best studied group among water insects (Oertli 2008).

There are not too many dry regions in Slovakia such as Cerová vrchovina Upland and Juhoslovenská kotlina Lowland. Water dams and artificial canals constitute the dominant wetlands in this region with uniform species assemblage. Valuable habitats are often situated under competence of special protected areas, where the management efforts could come through. Only a few studies were conducted on the fauna in this part of the country and just a couple of them pertain to wetlands (Adamec et al. 2011, Burai et al. 2014). Thus, the knowledge of water invertebrates in these regions still remains practically unknown.

At first we must obtain adequate data to establish desired management measures for wetland – dependent species protection. In case of dragonflies, the valuable data considers exuviae (discarded exoskeleton that is left after a larva has moulted) or larvaes. For that reason, intention of this work was to clarify the species richness at each studied locality mostly based on their larval stage and then estimate their abundance.

MATERIALS AND METHODS

Sampling

This study was conducted on Cerová vrchovina Upland and Juhoslovenská kotlina Lowland (Figure 1), which are the parts of warm climatic region with moderate dry climate and cold winter, where the numbers of summer days are over 50. Annual average precipitation is 550–600 mm, and the annual average temperature ranges from 7–10 °C (ŠOP SR 2015). An entomological net was used along 100 m transect to capture adult insects. Observations were made by capturing an exemplar and determined using Waldhauser and Černý (2016). While for larvae a kick net was used, exuviae were collected manually. Specimens difficult to identify were determined in the laboratory through Brochard et al. (2012). Shannon-Wiener index was used to calculate the diversity and evenness. Dominance was evaluated according to Losos et al. (1984). At present the national red list of dragonflies is out of date, consequently the Red list of Dragonflies of Czech Republic was adapted (Dolný et al. 2016). Physicochemical parameters were measured by HI98129 tester. Collected materials were deposited at the author's collection.

Study area

Site 1 – Flooded quarry, Konrádovce (N 48°17'37.272", E 19°53'23.270"; 360 m a.s.l.; pH – 7.43, µS – 505; ppm – 255). On the present, inactive basalt quarry, where the depressions are filled with rain water. The rare *Typhetum laxmanni* (Ubrizsy 1961) Nedelcu 1968 association can be found here. Substratum is composed with fine mud, rocks and blocks (Figure 2).

Site 2 – Fishponds, Holiša (N 48°18'29.619", E 19°45'27.754"; 175 m a.s.l.; pH – 8.49; µS – 495; ppm – 248). Large permanent eutrophic still waters with reed beds on mud, gravel and rock substrates.

Site 3 – Pokoradzské jazierka peat bogs, Vyšná Pokoradz (N 48°25'43.27", E20°01'44.85"; 420 m a.s.l.; pH – 7.06; µS – 235; ppm – 125). Shallow small areas with still waters overgrown by *Batrachium* sp., *Carex* sp., and *Phellandrium aquaticum* (L.) Poir. (1798) drying in late summer.

Figure 1 Localities investigated in this study

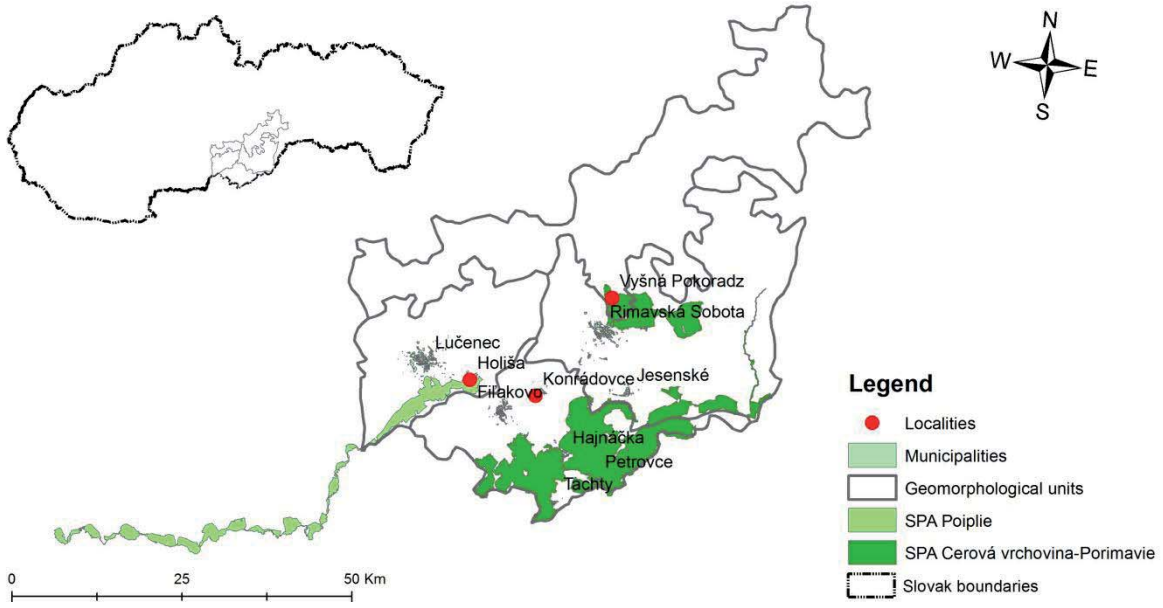


Figure 2 Photos of studied localities (1 – site 1, 2 – site 2, 3 – site 3)

RESULTS

Altogether, 27 species belonging to 15 genera and 6 families (13 Zygoptera and 14 Anisoptera), were recorded in 2016 and 2017 at three types of lentic biotopes (Table 1).

Table 1 List of recorded species at each site during years 2016–2017

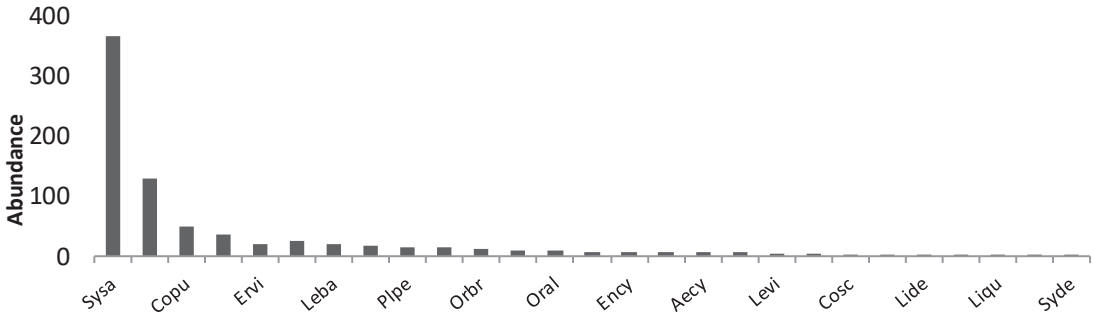
		Site 1			Site 2			Site 3			
			H´	1.714		H´	2.184		H´	1.502	DS
Zygoptera			E.	0.547		E.	0.851		E.	0.626	
Lestidae			D.	Div.		D.	Div.		D.	Div.	
Chalcolestes viridis	*Chvi*	+	Re	0.058				+	Re	0.045	L, A
Lestes barbarus	*Leba*	+	Su	0.086				+	Do	0.197	A
Lestes virens	*Levi*	+	Sr	0.012				+	Su	0.125	L, A
Sympecma fusca	*Syfu*	+	Sr	0.037				+	Su	0.125	E, A
Platycnemididae											
Platycnemis pennipes	*Plpe*	+	Re	0.064	+	Do	0.157				L, E, A
Coenagrionidae											
Coenagrion puella	*Copu*	+	Do	0.187				+	Eu	0.260	L, E, A
Coenagrion pulchellum	*Copu*	+	Sr	0.012							A
Coenagrion scitulum	*Cosc*	+	Sr	0.021							A
Erythromma najas	*Erna*	+	Sr	0.021	+	Do	0.231				L, A
Erythroma viridulum	*Ervi*				+	Eu	0.270				A
Enallagma cyathigerum	*Ency*	+	Re	0.064							A
Ischnura elegans	*Isel*	+	Su	0.086	+	Eu	0.300				A
Ischnura pumilio	*Ispu*	+	Sr	0.012				+	Re	0.045	A
Anisoptera											
Aeshnidae											
Aeshna cyanea	*Aecy*	+	Re	0.058							L, E, A
Aeshna affinis	*Aeaf*	+	Sr	0.012	+	Su	0.097	+	Do	0.146	E, A
Anax imperator	*Anim*	+	Sr	0.021	+	Su	0.097				L, E, A
Cordulidae											
Epitheca bimaculata	*Epbi*				+	Sr	0.034				L
Libellulidae											
Libellula quadrimaculata	*Liqu*	+	Sr	0.012							A
Libellula depressa	*Lide*	+	Sr	0.021							L, E, A
Orthetrum cancellatum	*Orca*	+	Sr	0.012	+	Re	0.058				L
Orthetrum albistylum	*Oral*	+	Sr	0.037	+	Su	0.114				A
Orthetrum brunneum	*Orbr*	+	Su	0.081							A
Crocothemis erythraea	*Crer*				+	Do	0.222				A
Sympetrum sanguineum	*Sysa*	+	Eu	0.343	+	Eu	0.340	+	Eu	0.313	L, E, A
Sympetrum depressiusculum	*Syde*							+	Re	0.045	L
Sympetrum vulgatum	*Syvu*	+	Su	0.129	+	Sr	0.034	+	Su	0.125	E
Sympetrum striolatum	*Syst*	+	Eu	0.329	+	Eu	0.231	+	Su	0.076	L, E, A
		23			13			11			

Legend: DS – Development stage, L – larvae, E – exuviae, A – adult, H´ – Overall diversity/site, E. – Evenness, D. – Dominance (Eu – Eudominant, Do – Dominant, Su – Subdominant, Re – Recedent, Sr – Subrecedent), Div. – Diversity

An additional 3 new species for these areas were observed, but in this comparison were omitted because of the insufficient field data (see chapter Discussion). On the whole, the development process was confirmed at 16 species. The most common encountered species at each locality was *Sympetrum sanguineum* with total 365 individuals (46.92%) (Figure 3). On the second place at site 1 was its congeneric species *Sympetrum striolatum* (21.14%) and at the sites 2 and 3 were the commonest Zygoptera species *Ischnura elegans* (16.75%) and *Coenagrion puella* (12.50%) respectively. A further 14.81% of all species occurred in low abundance (< 1%) and were considered as rare ones.

The hatching period of *Sympetrum sanguineum* and *S. striolatum* was recorded. The first detected specimens have been found on the 2nd July 2016, the last on 28th August 2016. The biggest numbers of exuviae (51) of *Sympetrum sanguineum* were found on 5th July 2016, while on 23rd June 2016 was collected the mostest (16) exuviae of *S. striolatum*.

Figure 3 Overall dominance of dragonflies at each 3 sites



Regarding to zoogeographical elements 10 species belongs to Holomediterranean realm, 6 to Ponto-Mediterranean, 3 to Ponto-Caspian, 3 to Siberian, 2 to Eurosiberian and 3 species individually to Afrotropical, East-Mediterranean and Antlanto-Mediterranean realms. The most diverse locality was flooded quarry (23 species), probably due to advanced decay of succession with almost zero pressure of fish predation. Fishpond´s species list (13) should increase with not recorded, but in Central Europe most common species, such as *Chalcolestes viridis*, *Coenagrion puella*, *Pyrrhosoma nymphula*, *Enallagma cyathigerum*, *Aeshna cyanea* and *Libellula depressa*. The species diversity list at site 3 with 11 species has seemed to be complete yet, but there has still been a chance that another species (*Leucorrhinia pectoralis*) might appear. According to Dragonfly Biotic Index adapted from Dolný et al. (2016) the site 1 has the highest DBI values (39). Despite of site 3´s lower diversity, the total DBI was higher (31) than at site 2 (23), due to recorded ecosozologically important species such as *Lestes virens*, *Aeshna affinis* and *Sympetrum depressiusculum*.

DISCUSSION

Comments to some rare species

In view of odonatological exploration, Slovakia doesn't belong to the well-researched countries. There are still many regions where surveys are not conducted. It is the case of this study, which focuses of the southern parts of Central Slovakia. Between years 2012–2014 first faunistical research have been done on Cerová vrchovina Upland (Balázs et al. 2016). This work supplements previous research with additional first findings to complete species richness of Cerová vrchovina Upland and now, Juhoslovenská kotlina Lowland as well.

Three species of dragonflies were discovered at three different types of biotopes, which have never been observed in these regions. It was a *Coenagrion pulchellum* at site 1, where a single adult male was caught on 31th August 2017 as a new species for Cerová vrchovina Upland. It is also not common species on the other parts of the country. Bigger populations have been found only on the southern parts of Slovakia lengthwise of Danube, Ipeľ and Latorica rivers. Surveys conducted at higher elevations on the northern part of the country didn´t confirm any adults just larvaes. At adjoining Protected Landscape Area Karancs-Medves in Hungary it is known only from few localities. Only from here is known the also rare *Coenagrion scitulum*. Several post-mining quarries

are present in these regions, with the right decisions of recultivation, valuable habitats could arise (not only) for rare species of dragonflies.

The second newly recorded species is *Epitheca bimaculata*. It was found on 16th August 2017 at site 2 as larvae, which rested on submerged vegetation. Nevertheless, additional investigations for larvaes at this locality were unsuccessful. The locality where the specimen was found corresponds with the ecological demands of the species, furthermore in the surroundings a few suitable sites were recognized. This species is one of the rarest dragonfly in western Palearctic realm (Boudot 2010). It is protected by the law of the Act on Nature Protection no. 543/2003 Coll and listed in Annexes 4B and 6B. In the updated Red List of Dragonflies of Czech Republic is ranked in Critically endangered (CR) category (Dolný et al. 2016). In the past, specimens were found mostly on the Podunajská nížina and Východoslovenská nížina Lowlands. However, this poor knowledge most likely does not reflect to actual abundance of this species in Slovakia.

Finally, at site 3, *Sympetrum depressiusculum* was confirmed on 15th June 2016. Unrevised materials from 2000 and 2001 are the last known records of this habitat specialist from Slovakia (Kúdela 2000, Šíbl et al. 2001, 2002). Confirmed materials involve individuals only from the 50s and 60s of the 20th century (Trpiš 1957, 1969). Further fieldworks have to be done in the future to confirm its status at these sites. Closest localities of this extremely rare dragonfly are in the north of Moravia in Czech Republic and at the Hortobágy National Park in Hungary (Gőri 2007). At this point it is important to note, appropriate conservation measures are required for these peat bogs where rare animals braving against huge eutrophic pressure, overgrowing and desiccation.

Additional first findings for Cerová vrchovina Upland

Three new species were caught for Cerová vrchovina Upland, which were not included in this study, but were discussed here. The first is an adult male of *Anaciaeshna isoceles* caught on breeding pond at village called Tachty at 4th June 2016. It is a rare species across the country, with a local occurrence in Slovakia. Species is becoming more frequent with the decreasing longitude. On the territory of PLA Karancs-Medves in Hungary it has more common occurrence.

The second species is *Aeshna grandis*, which hunting individuals were seen above forest clearings at rural area of village Hajnáčka on 17th August 2017. There are also first findings for the bilateral PLA Karancs-Medves territory. While the previous species occurs more in the southern parts of Europe and its occurrence is patchy, the range of *Aeshna grandis* is widespread and extend across northern Europe until Baikal region in Siberia. It means their zoogeographic borders just slightly touch these regions of Slovakia and Hungary. However, due to climatic changes this borderline is shaping, the *Anaciaesna isoceles* is extending his range to the north and could be more common (Dolný et al. 2016).

The last important species for the region of Cerová vrchovina Upland is the critically endangered *Libellula fulva* confirmed by two adult males recorded on 7th and 8th July 2016. It was seen at for this species unsuitable habitat, which was a drainage canal with cobblestone pavement almost without vegetation under water reservoir called Petrovce. Nevertheless, in the lower parts of this stream favourable microhabitats are known where larvaes would be able to finish the development process.

CONCLUSION

As exposed above, these regions still hold surprises even at the national scale. Relatively high proportions of rare species were found for Special Protection Area Poiplie and SPA Cerová vrchovina – Porimavie. In Slovakia, the six following, in this study confirmed species such as *Anaciaeshna isosceles*, *Anax imperator*, *Coenagrion scitulum*, *Epitheca bimaculata*, *Libellula fulva* and *Sympecma fusca* are protected by law. Currently, 43 species of dragonflies are described for Cerová vrchovina Upland which present 62% of overall species diversity of Slovakia. The results also could serve as a background for establishing desirable management measurement in these regions. Aim of these long-term researches is to support the biodiversity on a local scale and establish conservation attitude against pressure of negative anthropogenic activities.

ACKNOWLEDGEMENTS

This research was financially supported by the grant IGA FA MENDELU No. IP_60/2017 „Faunistic and ecological assesment of species of genus *Cordulegaster* and *Sympetrum* (Insecta: Odonata) in selected areas of Slovakia".

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THE METHODOLOGY OF BRYOZOA CULTIVATION IN THE LABORATORY CONDITIONS

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Abstract: The main aim of the study was to develop and verify a methodology for the cultivation of bryozoans in laboratory conditions. For the experiment, the bryozoan of the species *Plumatella emarginata* was used. The colonies came from the recirculating system of circular tanks located at Mendel University. The colonies of *P. emarginata* were placed into aquaria in adjusted Petri dishes and started to attach to the dish walls after 2–3 days. A total of 28 adjusted Petri dishes were used in 6 tanks. Majority of colonies (70%) were attached after 14 days. The colonies were not fed during the first trial while a mixture of green algae was offered to them during the second trial. Although the colonies were cultivated in optimised conditions, they did not grow any further and all colonies in both trials died after one month. We did not succeed in developing and verifying a methodology for the cultivation of bryozoans in laboratory conditions.

Key Words: Bryozoa, *Plumatella emarginata*, cultivation, laboratory condition

INTRODUCTION

Recently, there has been much advancement in the freshwater aquaculture in the Czech Republic. This mainly consists of the construction of specialized system (e.g., recirculating aquaculture systems) which are focused predominantly on salmonid fish farming (MZe ČR 2013). These systems are usually inhabited by other organisms, whose occurrence is mainly affected by local conditions. Aquatic invertebrates can be abundant, and their effects on the system differ among taxa. Some species have no effect on the recirculating aquaculture systems but there are also some species whose presence is undesirable. Bryozoans may cause serious problems in intensive fish farming both by blocking the piping with their growth and by being intermediate hosts to fish parasites. Laboratory breeding of bryozoans serves to define optimum conditions for their occurrence and development. Changes to these conditions might lead to their partial or total elimination.

Cultivation of bryozoans in laboratory conditions is generally very difficult. Bryozoans of the genus *Cristatella* and *Pectinatella* were kept alive only for several days (Wood 2005). On the other hand, Wayss (1968) was able to keep a living colony of *Plumatella repens* for 3 years. He used several different single-celled green algae in a mixture of Knop's nutrient solution and soil infusion at a ratio of 1:1. However, one of the most effective and least problematic cultivation systems that has been used for many years is using of fish-conditioned water. Fish are placed in large aquaria in water that never gets filtrated or changed. The aquaria are well lit and the fish are well fed. The water from these large aquaria is then used to rinse smaller, dark tanks where bryozoans grow (Wood 1996).

Morris et al. (2002) described another possibility of cultivating bryozoans in plastic aquaria with colonies of *P. repens* already attached on vertically positioned Petri dishes. The aquaria are filled with artificial freshwater of medium hardness (0.35 mM $CaSO_4$ ($2H_2O$), 0.5 mM KCl, 0.5 mM $MgSO_4$ ($7H_2O$), 0.1 mM $NaHCO_3$). They are intensively aerated and maintained at a constant temperature of 21 °C using water warmers. Water in the aquaria is changed weekly. Bryozoan colonies are fed daily with a mixture of grown algal cultures by suspension them directly in the tank. The mixture is composed of *Cryptomonas ovata* Pringsheim (300 ml), *Synechococcus leopoliensis* Komárek (150 ml), and *Pediastrum boryanum* Myen (50 ml). Cultures of *Chilomonas paramecium*

Ehrenberg and *Colpidium striatum* Stokes (10 ml each) are also added to the water in the first week to inoculate the aquaria with protozoans.

It is important to protect bryozoans from particles settling on their surface. The easiest way how to achieve that is to let the colonies grow on the bottom side of the artificial substrate. For example, individual plastic Petri dishes containing bryozoans may be inserted into glass tubes glued to the inner wall of the cultivation aquarium, or glass Petri dishes containing the colonies may be placed bottom up on stands in the aquaria. The third possibility – suitable for mass cultivation – is the colony growing on the bottom sides of glass sheets that are tilted and placed parallely to each other in a row, just as honeycombs in a beehive (Wood 2005).

The transfer of colonies of the genera *Lophopodella, Lophopus* and *Cristatella* is relatively easy. They are simply taken from the original substrate and placed directly onto the new substrate, which they attach to within 3–12 hours. Branching colonies attach to the new substrate only by the actively growing branch endings. Free branches of bryozoans of the genus *Fredericella* and some species of the genus *Plumatella* may be left undisturbed for a period of 1–2 days by leaving the branch endings in contact with the desired substrate. It is also possible to weigh the branches gently down with glass sticks until they hold onto the new substrate on their own. Generally, it holds true that those branches that are firmly attached to the substrate along their whole length, are seldom successfully transferred to another place. Where, however, it is possible to place a part of the old substrate with the colony onto a glass plate with wedges or rubber bands, the colony can grow over to the glass. Then the old substrate may be removed (Wood 2005).

Bryozoans can also be cultivated on artificial substrate *in situ*. Wöss (1996, 2000, 2002) transferred bryozoan colonies onto plates of wood and Perspex hanging vertically under a floating raft. Wood (1973) successfully used glass and grooved polyethylene substrate. Only very small colonies were found on vinyl or rubber foil.

MATERIAL AND METHODS

Four glass aquaria (A, B, C and D) of the dimensions of 44 × 31 × 25 cm and the volume of 31 litres were used for the trial. The water column height reached 20 cm. The aquaria were aerated using aerating rocks. The space of the aquaria was divided by a polystyrene partition in order to prevent excessive water swirling. To simulate the dark environment in which bryozoans are commonly found, the aquaria were shielded using cardboard boxes.

The aquaria were filled with water from the recirculating system of circular tanks located at Mendel University. The colonies of the bryozoan *P. emarginata* were also taken from the same system. The first sampling site was the filtration equipment; the second site was the piping at the outflow from the circular tanks. The colonies were taken using a scalpel. This method of sampling turned out to be the most gentle one and the least damaging to the structure of the colony.

The sampled colonies were divided into smaller parts of the size of approximately 2–3 cm^2. These parts were then installed on a specially adjusted Petri dish of 8.65 cm in diameter. A test tube filled with sand was glued to the centre of each dish. This caused the dishes to remain stabilized under water when submerged into the water bottom up. In order to keep the colony in the dish before it attached, each dish was equipped with a plastic grid.

This trial was conducted in two replicates. During the first trial, five Petri dishes were inserted into each aquarium with a part of the colony. The colonies received nourishment only from the organisms contained within the aquatic environment and were not let to feed otherwise. During the second replication, only two aquaria (A and B) with four Petri dishes were used for the cultivation of bryozoans. Again, food consisted of the organisms contained within the water, and furthermore, we added 10 ml of concentrated mixture of green algae (*Chlorella kessleri, Acutodesmus obliquus* and *Raphidocelis subcapitata*) every third day. The hydrochemical parameters of the water were continuously monitored and the collected water was supplemented with water from the system.

RESULTS AND DISCUSSION

Hydrochemical properties of water

Basic physical and hydrochemical measurements (water temperature, content of dissolved oxygen in water, oxygen saturation of water, and pH) were done using a multi-parameter probe HACH.

Table 1 Water temperature (°C)

Aquarium	Mean temperature (°C)	Lowest temperature (°C)	Highest temperature (°C)
A	21.8	21.2	22.9
B	21.5	20.5	22.6
C	21.4	20.4	22.4
D	21.4	20.4	22.2

Water temperature was relatively balanced in all the aquaria (Table 1). It follows from Table 1 that the water temperature was relatively balanced in all the aquaria. The differences in the lowest and highest measured values are due to the fact that water was added to the aquaria from the recirculating system, always slightly cooling the environment down.

The species *P. emarginata* is commonly found in standing waters in warm eutrophic areas within the depth of 1 m. It is mainly found in calm, stagnant waters of lakes and rivers where it has sufficient attaching material (plants, wood, rocks). For example, in the temperate areas of Northern America they occur to the largest extent at the end of spring when the water temperature rises above 20 °C (Wood 2005). In laboratory conditions, it has been proved successful placing of the colonies in an aquarium with medium hard, intensively aerated water of 21°C maintained by water warmers (Morris et al. 2002).

Table 2 Dissolved oxygen concentration (mg/l) and oxygen saturation (%)

Aquarium	Mean content of O_2 (mg/l)	Lowest content of O_2 (mg/l)	Highest content of O_2 (mg/l)	Mean saturation (%)	Lowest saturation (%)	Highest saturation (%)
A	8.4	8.2	8.6	96.8	95.5	97.8
B	8.3	8.1	8.6	95.6	94.5	96.6
C	8.3	8.1	8.5	95.5	94.1	96.7
D	8.4	8.1	8.6	95.5	94.3	96.6

No marked fluctuations in the oxygen content occurred during the trial (Table 2). Potential fluctuations might negatively affect the lifespan of the stocked bryozoan colonies.

Table 3 pH values

Aquarium	Mean pH	Lowest pH	Highest pH
A	7.65	7.48	7.93
B	7.68	7.50	8.05
C	7.69	7.48	8.07
D	7.80	7.63	8.06

The pH values recorded are very balanced in individual aquaria, pointing out to a slightly alkaline environment (Table 3). Wood (2005) described places where bryozoans in open waters are not commonly founded. They are low oxygen sites with pH values below 6, fast flowing rivers and streams where smooth and rounded stones did not allow the boulders to snugly adhere to the substrate and were carried away by the stream. Likewise, we would not find them on greasy, decayed or actively corroding substrates.

Results of cultivating Plumatella emarginata in laboratory conditions

Colonies of *P. emarginata* stocked in specially adjusted Petri dishes into the aquaria started to attach to the dish walls after 2–3 days. After 14 days, 70% of colonies were attached to the base. However, these colonies did not grow any further, and after one month, all colonies in both trials died.

Unsuitable temperature, dissolved oxygen content, and pH may be excluded as the cause of the die-off. The temperature was maintained around 21 °C which is in agreement with the study by Morris et al. (2002). The water was also sufficiently oxygenated, and pH did not decrease below the critical value of 6. One of the possible reasons for the failure was the possibility of damaging the colonies during sampling. Another problem may have been an insufficient amount of adequate food that the colonies need or also the instability of the surrounding environment (frequent lighting regime changes in the laboratory during the water measurements and manipulation with Petri dishes) as the colonies prefer shady places (Lukešová 2011). The presence of an aquarium with fish also needs to be taken into consideration as they may positively affect the growth and development of bryozoan colonies, as reported by Wood (1996).

CONCLUSION

Attempts to cultivate bryozoans of the species *P. emarginata* in laboratory conditions were not successful. Fourteen days after stocking the colonies into the aquaria, they became attached to the base. However, the colonies were unable to grow any further, and after one month, all colonies died. Besides the monitored water properties (temperature, dissolved oxygen content, pH), it is also necessary to monitor other variables that may have influenced the results of the trial; e.g., the contents of other substances in the water (such as phosphates, chlorides, nitrites, and nitrates), the amount and composition of food, light intensity, and possibly even including tanks with stocked fish.

ACKNOWLEDGEMENTS

The results and outputs were processed using the equipment financed by the project OP VaVpI CZ.1.05/4.1.00/04.0135 Teaching and research capacities for biotechnology disciplines and infrastructure expansion. The study was processed with the support of the project NAZV QJ1510077 Increasing and improving salmonid fish production in the Czech Republic using their genetic identification.

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INTRASPECIES VARIABILITY OF THE CHUB (*SQUALIUS CEPHALUS* L.) IN THE CZECH REPUBLIC AND POSSIBILITIES OF ITS MORPHOLOGICAL DETERMINATION

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Abstract: The aim of the study was to find whether it might be possible to discover individual lines of selected fish species, in our case the chub (*Squalius cephalus*), using meristic and plastic traits. The lines were detected using genetic methods; in particular, using analysis of mitochondrial (COI) as well as nuclear (RP1S7) markers. The studied fish originated from all river basins within the Czech Republic. A total of 30 plastic and 20 meristic traits were determined in 18 samples. Only in three traits, the level of significance was found to be lower than $P = 0.05$. In practice, however, not even these three traits can categorize particular individuals into the given lines. Therefore, no trait was found that would clearly define the morphological differences between the studied lines of the chub.

Key Words: chub, *Squalius cephalus*, morphometric characteristics, plastic traits, intraspecies variability

INTRODUCTION

With the advent of genetic methods and their increasing availability, molecular methods have collided with classical ichthyology based on morphological traits. For the needs of taxonomy, however, we need to find a common language for both these methods.

We are interested in understanding the intraspecies diversity mainly for the sake of a more efficient protection of the biodiversity of our ichthyofauna.

The genus Squalis is a relatively variable group of fish; its taxonomy and exact number of species in Europe have not yet been completely determined. This is supported by the fact that presently new species are being found, such as *Squalius janae* reported by Bogutskaya and Zupančič (2010) from Slovenia Bogutskaya and Zupančič (2010).

A number of authors have studied the genus Squalis; for example, Sanjur et al. (2003), and Doadria and Carmona (2006) on the Iberian Peninsula, Zupančič et al. (2010) in Croatia, Seifertová and Šimková (2010) in Europe, Lerch (2010, 2012) in the Balkans, and Saleh et al. (2017) in Iran.

The chub is an omnivorous fish species, commonly found in all types of running and sometimes even stagnant waters within the Czech Republic.

Historically, no lines/subspecies of chub have been reported on the territory of the Czech Republic or formerly, Czechoslovakia. Hrabě et al. (1973) who otherwise mention subspecies in fish, do not describe any subspecies in this particular species. Baruš and Oliva (1995) note minute differences in the branched rays in the dorsal and anal fins in individual fish from the Danube, Odra, and Elbe rivers, however, without defining specific subspecies.

Using genetic methods, the study *Molecular biodiversity inventory of the ichthyofauna of the Czech Republic* (Mendel et al. 2012) revealed that at least three chub lines inhabit the territory of the Czech Republic. My aim was to determine whether any differences can be found among these lines at the level of morphology, and thereby, whether the lines can be reliably distinguished without using DNA analysis.

MATERIAL AND METHODS

The material used came from samplings collected within the study Molecular biodiversity inventory of the ichthyofauna of the Czech Republic (Mendel et al. 2012). Captured individuals (n = 18) originated from all major river basins in the Czech Republic. The genetically-determined lines didn´t correlate with the origin of the fish. The sex of the fish wasn´t known. This material was fixed using formaldehyde and subsequently transferred into 70% alcohol. It was stored in the depository of the National Museum in Prague, from where it was loaned for the purpose of my study. The methods were based on the studies of Hrabě et al. (1973), Baruš and Oliva (1995), and Kottelat and Freyhof (2007). A set of a total of 15 meristic and 28 plastic traits was evaluated in the fixed fish. Subsequently, biometric coefficients were calculated from the plastic traits. Meristic values were evaluated as numbers of hard and soft rays on all fins, and as numbers of scales at several levels of the body. The number of gill rakers on the first branchial arch was not determined due to protection of the museum material from damage, and the number of vertebrae weren´t determined for technical reasons.

The size of studied fish ranged from 118 to 241 mm total length, or from 95 to 192 mm standard length. For biometric measurements, a digital calliper (Digital Calliper Powerfix Profi) with the accuracy to a tenth of a millimetre was used. In larger fish, some longitudinal dimensions (> 150 mm) were determined on the measuring board. Smaller fish were examined under a magnifying apparatus (Mantis Stereo Microscopes, Vision Engineering) at a ×2 or ×4 magnification. Measurements were done manually, and all horizontal dimensions were measured horizontally with the craniocaudal axis of the body. The determined values were processed statistically with the program Statistica 12 using the method of one-factor ANOVA and Canoco 5.04.

RESULTS AND DISCUSSION

Meristic traits

Of the total number of 15 studied traits, only one was found to be statistically significant. It was the number of soft rays in pectoral fins, where the *P* value = 0.036.

Practically speaking, however, even this trait is non-significant, as the minimum number of 16 soft rays occurred across all three lines, and therefore it cannot be used to distinguish among individual fish (Table 1).

Table 1 Meristic traits by three lines of chub

Feature		line 1 (n = 8)			line 2 (n = 7)			line 3 (n = 3)		
		Min	Max	Median	Min	Max	Median	Min	Max	Median
Scale counts above lat. line		7.0	8.5	8.0	6.5	8.0	8.0	7.5	8.0	8.0
Lateral line scales min		43.0	47.0	44.0	44.0	45.0	44.0	44.0	46.0	45.0
Lateral line scales max		44.0	48.0	45.5	44.0	45.0	45.0	45.0	46.0	46.0
Scale counts below lat. line		3.0	3.5	3.0	3.0	4.0	3.0	3.0	3.0	3.0
Circumpeduncular scales		14.0	15.0	14.0	14.0	15.0	14.0	14.0	15.0	14.0
Predorsal scales		18.0	24.0	20.0	19.0	22.0	21.0	19.0	20.0	19.0
Postdorsal scales		19.0	23.0	20.5	19.0	21.0	20.0	20.0	23.0	22.0
Preventral scales		26.0	31.0	28.0	23.0	29.0	28.0	26.5	27.0	27.0
Preanal scales		7.0	10.0	8.3	7.0	9.0	8.0	7.0	9.0	9.0
Scales A – C fin		9.0	13.0	11.0	11.0	11.5	11.0	10.0	11.0	11.0
D	hard	3.0	3.0	3.0	2.0	3.0	3.0	3.0	3.0	3.0
	soft	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0
C	{ }	19.0	20.0	19.0	19.0	21.0	19.0	19.0	19.0	19.0
A	hard	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
	soft	8.0	9.0	8.0	7.0	8.0	8.0	8.0	9.0	8.0
V	hard	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
	soft	8.0	8.5	8.0	8.0	8.0	8.0	7.5	8.0	8.0
P	hard	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
	soft	16.0	18.0	17.0	16.0	17.0	16.0	16.0	16.0	16.0

Legend: D – dorsal fin, C – caudal fin, A – anal fin, V – ventral (pelvic) fin, P – pectoral fin, lat. line – lateral line

Plastic traits

Were found to be similarly non-significant. Of the 36 relative indices (Table 2), only two were found to be significant; the **snout length in percentage of head length** $P = 0.002$. The smallest snout length relative to head length was found in line no. 1 (on average 26.95%), followed by line 2 (28.32%), and the longest snout length on average was found in line 3 (28.69%). The other trait was the **depth of caudal peduncle in percentage of body length**, where $P = 0.020$. On average, the first group had 11.00%, the second 11.71%, and the third group 11.42%.

Table 2 Plastic trait indexes by three lines of chub

Feature	line 1 (n = 8)			line 2 (n = 7)			line 3 (n = 3)		
%	Min	Max	Average	Min	Max	Average	Min	Max	Average
BD/SL	24.1	27.3	25.6	25.2	28.6	27.0	25.8	28.0	26.9
BD/BW	53.5	64.5	59.0	51.4	63.2	58.7	58.0	62.5	59.8
BW/SL	14.0	16.9	15.1	14.7	16.8	15.8	15.5	16.5	16.0
PD/ TL	42.8	46.5	44.4	43.2	46.7	44.6	43.3	44.5	44.0
PD/ SL	53.2	57.0	54.9	54.1	58.3	55.8	54.0	55.9	55.1
PD/PT	50.8	65.2	61.9	57.4	68.4	60.9	62.1	64.0	63.2
PT/TL	23.3	29.2	27.5	24.8	29.8	27.2	27.4	28.2	27.8
PT/SL	28.9	35.4	34.0	31.2	37.0	34.0	34.4	35.5	34.8
PVL/SL	48.7	52.0	50.6	49.8	51.7	50.9	50.0	51.9	51.2
PAL/SL	70.1	75.9	71.8	71.6	74.5	72.6	72.1	75.3	73.4
V-A/SL	19.9	23.8	21.7	20.6	22.9	22.3	21.8	23.8	23.1
LCP/SL	17.1	19.2	17.8	15.5	19.4	17.7	16.5	19.3	18.2
Von/SL	10.3	11.6	11.0	11.3	12.3	11.7	11.1	11.7	11.4
DCP/BD	41.3	44.3	43.0	39.5	45.4	43.4	41.8	43.1	42.6
WCP/BW	13.9	19.8	16.5	11.2	19.6	16.1	15.2	20.6	18.4
HL / TL	19.6	21.3	20.5	18.6	21.1	20.0	19.4	21.5	20.7
HL / SL	24.0	26.6	25.3	23.5	26.2	25.0	24.2	27.1	26.0
HL/BD	91.9	107.3	98.4	82.2	100.2	92.6	86.6	104.9	97.1
HL/PD	43.8	48.9	46.1	42.2	47.2	44.7	44.9	48.4	47.2
HW/BD	64.3	73.6	69.0	62.6	70.7	67.2	62.6	74.9	67.6
HW/BW	86.5	105.5	94.8	87.6	100.0	92.7	88.6	102.1	95.1
RL/HL	25.8	28.2	27.0	27.6	29.4	28.3	28.3	29.3	28.7
PL/HL	48.7	52.8	51.5	46.8	54.7	50.6	46.3	52.5	50.1
IW/HL	41.1	44.6	42.1	42.3	46.9	43.8	41.5	45.8	43.9
IW/HW	69.0	78.4	74.6	71.5	77.1	74.5	72.9	75.9	74.7
ED/SL	5.0	5.7	5.3	5.0	5.9	5.4	5.0	6.2	5.6
ED/HL	19.8	21.9	20.9	19.8	23.9	21.8	20.4	22.9	21.4
ED/RL	74.3	80.2	77.5	71.3	86.5	76.9	69.8	80.2	74.5
ED/IW	47.8	52.1	49.6	46.6	55.2	49.8	45.4	51.7	48.7
dD/SL	17.1	19.4	18.0	16.6	19.8	18.3	17.8	18.1	17.9
dC/SL	22.3	26.4	24.8	23.6	26.8	25.0	24.5	26.4	25.4
dA/SL	12.8	15.9	14.9	13.6	16.3	15.1	14.3	15.4	14.8
dV/SL	13.6	16.3	15.4	13.9	16.4	15.5	14.4	16.1	15.1
dP/SL	16.4	19.5	18.0	16.3	18.9	17.5	17.3	19.1	17.9
dD/BD	66.7	77.8	70.4	63.0	72.6	67.9	64.5	69.3	66.7
dA/BD	49.1	64.5	58.3	52.5	60.3	55.9	52.4	59.7	55.2

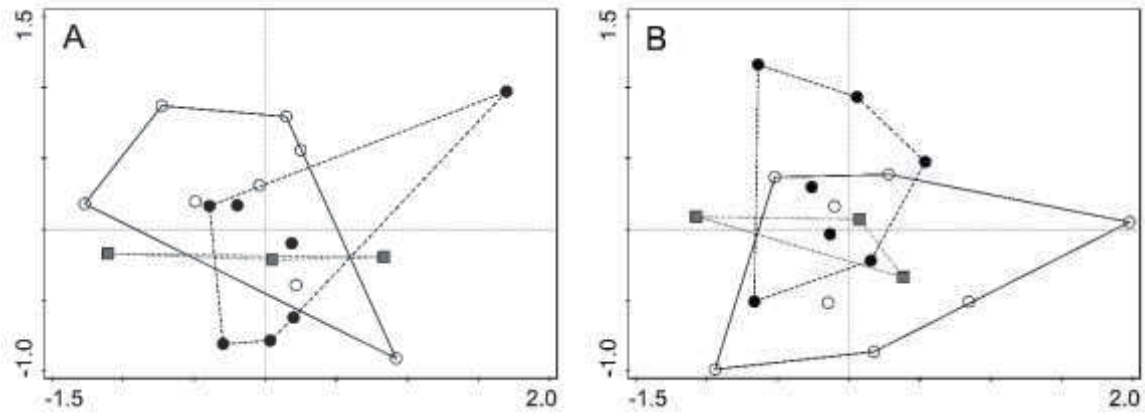
Legend: dD – depth of dorsal fin, dC – depth of caudal fin, dA – depth of anal fin, dV – depth of ventral (pelvic) fin, dP – depth of pectoral fin,dD – depth of dorsal fin, TL – total length, SL – standart length, BD – maximum body depth, BW – body width, HW – head width, PD – predorsal distance, PT – postdorsal distance, HL – head length, RL – snout length, PL – postorbital length, IW – interorbital width, ED – eye diameter, DCP – depth of caudal peduncle, WCP – width of caudal peduncle, LCP – legth of caudal peduncle, PVL – preventral length, PAL – preanal length, V-A – distance between the ventral and anal fin

All the other traits we statistically non-significant. For meristic traits, the value of $P = 0.124$ to 0.880; for plastic traits, the value of $P = 0.066$ to 0.994. It can therefore be stated that of the 55 plastic and meristic traits, only two statistically significant traits and one highly significant trait were found that might in theory help distinguish the three chub lines in the Czech Republic based on morphology. It must be added, however, that a relatively small number of fish were used for the analyses (n = 18), and that all lines slightly overlap in these traits. The results

of non-metric multidimensional scaling (NMDS) did not show significant differences among the population of Chub (Figure 1).

This issue is further complicated by the fact that the chub is the focus of sport fishermen and belongs among the rheophilic fish species which are the subject of fish stocking plans for running waters. In other words, with the transfer of stock material, mixing of the individual lines within the river basin and their mutual cross-breeding occur.

Figure 1 Non-metric multidimensional scaling of biometric (A) and meristical signs (B) of Squalius cephalus.



Legend: line 1 – empty circles, solid line; line 2 – full circles, dashed line; line 3 – shaded squares, dotted line

CONCLUSION

It may be concluded based on the study that for a reliable detection of individual chub lines in the Czech Republic, the use of genetic methods is necessary which, unfortunately, has so far been impractical for field ichthyology. Although some morphometric coefficients were found to be statistically significant, they cannot be used in practice as the values overlap within the lines. For further verification, a multiple number of chub samples need to be analysed.

ACKNOWLEDGEMENTS

The research was financially supported by the NAZV (QJ1620240).

The results and outputs of the study were processed using the equipment financed by the project OP VVpI CZ.1.05/4.1.00/04.0135 Výukové a výzkumné kapacity pro biotechnologické obory a rozšíření infrastruktury (Teaching and research capacities for biotechnology disciplines and infrastructure expansion).

This study was conducted in cooperation with ÚBO AV ČR Brno and the Department of Zoology of the National Museum in Prague.

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INFLUENCING THE PHOSPHORUS DIGESTIBILITY FROM FEED MIXTURES IN CARP BREEDING BY USING PHYTASE ENZYMES AND CITRIC ACID

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Abstract: The aim of the study was to verify the effect of phytase and citric acid addition on increased phosphorus digestibility from feed mixtures in carp breeding. Feed mixtures have many purposes in carp breeding, but can also be a potential source of water pollution by phosphorus. Five diets were used in this study. Their basis was a commonly used feed mixture KP1. The first mixture was also the control, and the subsequent ones were with the addition of 500 FTU (phytase unit), 1000 FTU, 500 FTU and 3% of citric acid and the last one was with the addition of 1000 FTU and 3% of citric acid. Six tanks, with seven pieces of common carp in each, were prepared for the study. The test was conducted in two repetitions. Excreta samples were collected during the test, and later analysed for phosphorus content. The obtained result have shown a significantly high influence of phytase and citric acid addition on phosphorus digestibility (factorial ANOVA: $F = 51.4$, d.f. = 2, $p < 0.001$), where digestibility of phosphorus increased by almost 30%. Furthermore, the amount of phytase in diets had no positive effect on the quantity of the digested phosphorus.

Key Words: phosphorus, phytase, citric acid

INTRODUCTION

Complete feed mixtures have many uses in carp breeding, especially in supplementary feeding of fry and stocked carp. Hůda (2009) reports that Rybářství Třeboň a.s uses for carp production almost 20% feed mixture. Schäperclaus and Lukowicz (1998) recommend the use of quality feed mixtures in the second phase of fry breeding, while in the first phase they recommend shredded glycid feeds. Feed mixtures are also used for winter breeding of carp fry in warming water using recirculation systems, or for market carp production in waste warming water (Filipiak et al. 1998, Guziur et al. 2003). In addition to the carp production, the mixtures are also used to improve their condition or health state. Mareš and Baranek (2006) recommend supplementary feeding of carp fry condition feed mixture (20–22% ML, 10 MJ/kg) at the end of the summer. Further possibilities for using complete feed mixtures in carp nutrition are in the period of possible occurrence of the disease, when drugs, probiotics and vitamins are added to the feed mixture. In that case the feeding of fry takes place within a short interval of several days or weeks (Hartman and Regenda 2014).

Complete feed mixtures are composed mainly of plant and animal sources, supplemented with substances for improving the feed stability, digestibility of individual components or reducing its impact on the environment. Due to the limited availability of fishmeal or the prohibition on the use of other animal components, the importance of the feed of a plant origin is increasing (Mareš et al. 2015). In addition to reducing the cost of feed production, a significant aspect when using the feed is environmental loading. Fish meals contain high amounts of organic phosphorus, but digestibility reaches only around 25% (Jirásek et al. 2005). The plant ingredients form the basic component of feed mixtures for omnivorous and herbivorous fish. Their importance is mainly found in the substitution of other nutrient sources (when the combination is correct), and also in the reduction of feed prices, as energy source and last, but not least, as a technological component (Mareš et al. 2015). However, phosphorus in plants is stored in the form of phytic acid or phytate,

which is, due to its complex structure very hard to digest for fish. Therefore, the phosphorus stored in it is unavailable to the fish organism (Kumar et al. 2011).

Digestibility of phytate can be influenced in several ways. One of the possibilities is the use of special cereal hybrid lines, with genetically reduced phytate content, but preserved with the same amount of total phosphorus. This fact was also confirmed by Malý et al. (2016). Furthermore, the content and phytate digestibility from the plant components, depending on the plant species, the conditions of cultivation of the individual components and above all, the adjustment of the individual commodities. In general, the digestibility of nutrients can be enhanced by technological modification of components (Mareš et al. 2015). Further possibility for affecting, i.e. increasing phytate digestibility is the use of phytate enzymes.

In nature, phytases are abundantly present in plants, as endogenous phytase, and also in microorganisms and through microorganisms, it is also present in animals, and animals. Especially in ruminants, the presence of microorganisms of the rumen microflora is very important in terms of enzyme production. Due to very low presence of microorganisms in the fish digestive tract, production of phytase enzymes is very limited; almost zero (Simmons et al. 1990). Nowadays, phytases are commonly added to fish feeds. These enzymes are industrially produced by genetically modified microorganisms, mainly yeasts and bacteria (Cao et al. 2007). The use of phytase enzymes by animals is affected by many factors, of which the ambient temperature and pH of the animal's digestive tract are the most important (Simmons et al. 1990). The majority of industrially produced enzymes show the highest activity in acidic environments (2.5–5.5). The use of phytase is not problematic in case of fish with a stomach. However, in the case of carp breeding, there is a problem, because of neutral pH values in the digestive tract and a zero use of enzymes (Ji 1999, Cao et al. 2007). Temperature is another limiting factor in the phytase utilization. The majority of microorganism-produced phytases tolerate maximum temperatures in the range of 40–60 °C. In the production of granulated feed mixtures by granulation or extrusion, the mixture is very often heated to more than 100 °C. At this point the denaturation of the enzyme proteins occurs, and the enzyme becomes unusable (Cain and Garling 1995, Lei and Stahl 2000, Cao et al. 2007). However, procedures which allow the use of phytases even in the feed mixtures treated in this way are known. For example, Vielma et al. (2004) has presented a method of spraying a liquid form of phytase on to the surface of the produced granules. Another option is to modify the feed mixture before pelleting, by adding some of the organic acids, most often citric. In that way, the pH value of the feed mixture decreases, and thereby the phytase activates in the neutral pH of the carp's digestive tract (Baruah et al. 2005).

In the presented experiment, the hypothesis of increasing the phosphorus retention from the feed mixture with the addition of phytase and citric acid in the carp breeding was confirmed. By adding the acid to the carp feed mixture, it activates the phytase added to the feed mixture, and thus releases the phosphate bounded to the phytic acid molecule. Increasing the digestibility of the phosphorus from the plant component of the feed mixture reduces the loading of the environment and the need for the addition of monocalcium phosphate to the feed.

MATERIAL AND METHODS

Characteristics of the experimental diets

Used enzymes

The digestibility of phosphorus from the feed mixture by using phytase enzyme and citric acid was monitored in this study. Phytase Phyzyme XP 10.000 TPT, (10.000 FTU/g) was chosen for this experiment. The phytase is produced by the Danisco Animal Nutrition company (Du Pont concern). It is produced using *E. coli* and it is available in liquid and powder form. Increased resistance to proteolytic enzymes, high relative activity over a wide pH range and thermostability (owing to Thermo Protective Technology) up to 95 °C are the most important properties of the chosen enzyme.

Base of feeding mixture

Granulated complete feed mixture for carp KP1 produced in the factory at Stříbrné Hory was used as a base for the feed mixture. KP1 was shredded and subsequently the mixture for the experiment was produced. The composition of KP1 is shown in the Table 1. Due to low protein content, the experimental mixture was enriched with 10% of extracted soy meal. The extracted meal was added to the mixture to increase its crude protein content.

Table 1 Basic composition of KP1

Composition	Analytical characteristics	%
wheat, wheat flour meal, rapeseed expeller, wheat bran, extracted soy meal, maize, $Ca(CO_3)^{2-}$, NaCl, soybean oil	moisture	14.00
	crude protein	17.89
	crude fibre	4.81
	total phosphorus	0.57

Experimental diets

The individual components (Table 2) were mixed for 2 hours using a food processor (KitchenAid Heavy Duty 5kpm5) in order to thoroughly homogenize the mixture. After mixing, water was added and dough was made. Granules were made from dough using the food processor. Granules were dried in the hot-air sterilizer STERICELL 111 (BMT Medical Technology s.r.o.) at 50 °C temperature, and placed in plastic boxes after drying.

Table 2 Composition of experimental diets

	Control	F500	F1000	F500C3	F1000C3
KP1	90%	90%	90%	87%	87%
extracted soy meal	10%				
pellet-dur	0.5%				
phytase		500 FTU	1000 FTU	500 FTU	1000 FTU
citric acid	-	-	-	3%	3%

Characteristics of the equipment

Six tanks of the 106 litres volume were used in this study. Tanks were divided in two unequal parts, where the bigger one was used for breeding. The smaller part was with a conical bottom and partially separated from the bigger one using plastic partition. This part was used for the sedimentation of excreta and samples collection. Adequately adjusted inflow and aeration provided the water circulation and discharging of excreta into the smaller sedimentation part of the tank. Tanks are connected to biological filter NEXUS 310, where water is purified.

Characteristics of the tests

A total of 42 pieces of common carp of the average weight of 97.95 ± 3.43 g were chosen for the feed tests. Fish were stocked in six tanks, each with seven pieces. Before initiating the test, fish were left for four weeks to acclimate to the environment and a further week to adapt to the new feed. Throughout the tests, fish were fed *ad libitum* amount of feed.

During the experiment, two tests lasting 14 days were carried out. After the termination of the first test, fish were left for one week to adapt to the new feed used in the following test. In the first test, mixtures with no citric acid, i.e. control mixture, mixture with 500 FTU and mixture with 1000 FTU were used. In the second test, fish were fed a control mixture, and mixture F500 and F1000 with addition of citric acid.

Basic physicochemical (water temperature, oxygen content (mg/l), oxygen saturation (%) and pH) were measured by multimeter HachLange HQ40d, Germany. Concentrations of ammonia ions, nitrites and chlorides were analysed using spectrophotometer WTW photoLab 6600 UV.VIS, Germany (according to Horáková 2007).

Characteristics of the sample collection and analyses

Analyses of the experimental diets

The samples of feeds were analysed for phytase activity. The analyses were done by the Central Institute for Supervising and Testing in Agriculture, according to EN ISO 30024.

Additionally, the content of phosphorus and fibres in feed was determined by the methods in Malý (2015).

Sample collection and analyses

Excreta samples were collected regularly every day, prior to the morning feeding. Samples were sucked using a pipette and filtrated through the filtration paper. Filtrated samples were deposited in sample containers, separately for each tank. After collecting a sufficient amount of excreta, the collected samples were homogenized and dried at 106 °C. Dried samples were grinded and sent for subsequent analyses. The phosphorus and fiber content were determined.

Phosphorus digestibility was determined using an indicator method (Malý 2016). Endogenous fiber naturally occurring in feed was chosen as an indicator.

RESULTS AND DISCUSSION

Phytase activity

In the control diet, phytase activity was detected at 257 FTU/kg. Phytase activity in feed mixture is due to a naturally occurring phytase in plant feed components. In mixtures with the addition of 500 FTU, an activity of 961 FTU/kg was determined on average. In the mixtures containing 1000 FTU, 1350 FTU/kg on average was documented. According to these results, we can assume there was a variance or error when weighing a very small amount of enzyme, which was subsequently mixed into the mixture. However, the trend of increase in the phytase content in feeds F500, when compared to the control and in F1000 when compared to F500 was attained.

The content of crude fiber and phosphorus

Table 3 Content of crude fiber and phosphorus in diets and excreta samples (%)

Feed			Test 1 without citric acid			Test 2 with citric acid		
	CF	P		CF	P		CF	P
control	5.60	0.6542	control[1]	24.36	1.0271	control[1]	25.94	1.0849
F500	5.08	0.6093	F500[1]	21.90	1.1033	F500C3[1]	24.94	0.3143
F1000	4.98	0.6086	F1000[1]	21.44	1.0977	F1000C3[1]	25.14	0.4196
F500C3	5.00	0.6143	control[2]	25.82	0.9718	control[2]	25.48	1.1482
F1000C3	5.19	0.5931	F500[2]	24.06	1.1482	F500C3[2]	27.78	0.3369
			F1000[2]	23.47	1.0909	F1000C3[2]	26.45	0.2555

Legend: CF – crude fiber, P – phosphorus, 1 – first repetition, 2 – second repetition

The fiber content in used diets is within the range of 4.98–5.6%. Producer of KP1 reports 4.81% of crude fiber content. An increased fiber content in the experimental diets was most likely due to the addition of 10% of extracted soy meal. The amount of fibres found in diets used corresponds to the research of Jirásek et al. (2005), who has reported the need for fiber in carp feeds to be maximally up to 6%. A higher fiber content negatively affects the digestibility of other nutrients.

Phosphorus content in diets used is within the range of 0.59–0.65%. Producer of feeds reports that 0.57% of phosphorus was registered in KP1. Therefore, the phosphorus content is consistent with the study by Jirásek et al. (2005), where the need for phosphorus in the feed for carp was reported to be ranging from 0.6–0.7%.

Digestibility of phosphorus

Phosphorus digestibility is significantly higher in diets with the addition of 3% of citric acid, which can be evidently seen when comparing Figure 1 and Figure 2.

Figure 1 digestibility of phosphorus (%)

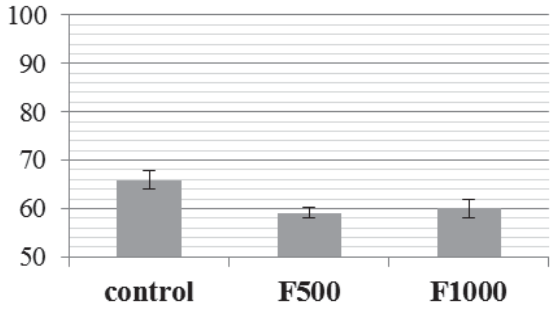
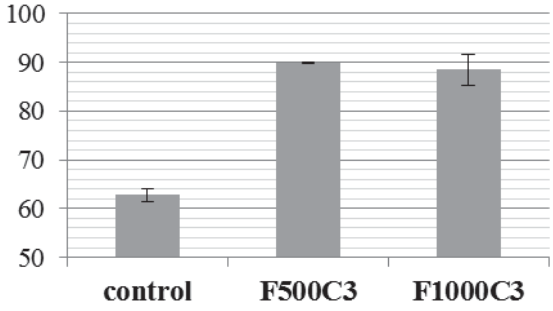


Figure 2 digestibility of phosphorus (%)



The digestibility of phosphorus for the control diet was at the level of 65.84% and 62.81%. A very similar result was attained by Malý (2015), who reported the digestibility of phosphorus of 63.89% in the control diet, composed only of KP1. In the same study, there was no increase in the phosphorus digestibility in diets with the addition of phytase enzyme Natuphos. A significant increase (27.13% and 25.66%) of phosphorus digestibility occurred in the case of two diets with the addition of citric acid. The digestibility of phosphorus was found to be positively influenced by an addition of 3% of citric acid and phytase (factorial ANOVA: $F = 51.4$, d.f = 2, $p < 0.001$). The same effect was recorded by Baruah et al. (2005), who established an increase in phosphorus digestibility in diets with an addition of 500 FTU and 3% of citric acid for *Labeo rohita* (Hamilton 1822). Phromkunthong et al. (2010) reported an increase in phosphorus digestibility in feed tests with common carp, when phytase in combination with citric acid and monosodium phosphate was used. Nwanna and Schwarz (2007) have reported a significantly higher digestibility of phosphorus in diets with 1000 and 4000 FTU. In the following year the same authors have again mentioned an increased digestibility of phosphorus when using phytase. In both studies, they reported positive results without the addition of citric acid.

CONCLUSION

Fish breeding in fishponds is certainly one of the potential sources of water pollution by excessive phosphorus. However, this is not the only problem in fishponds. The great part of fish production comes from recirculation systems, where fish are fed granulated feed mixtures. This way of breeding can partly extend into the carp breeding. In this study, the focus was on influencing phosphorus digestibility from feed mixtures designed for breeding of common carp. Five diets were evaluated: control, two diets with the addition of 500 FTU and 1000 FTU and two with the addition of 500 FTU and 1000 FTU and 3% of citric acid. After finishing the experiment, a positive effect ($p < 0.001$) of the addition of citric acid in combination with phytase on phosphorus digestibility was found. Digestibility increased by almost 30%, with no difference in the enzyme dose. The addition of just the enzyme had no effect on the phosphorus digestibility, when compared to the control. In conclusion, it can be said that the use of phytase enzymes with the addition of citric acid leads to a very significant increase in phosphorus digestibility. This data is important, not only from an economical point of view, but especially from an ecological point of view. The use of these products leads to a significant reduction in the amount of phosphorus released from fish breeding systems.

ACKNOWLEDGEMENTS

The research was financially supported by the IGA grant, no IP_12/2017.

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SECOND YEAR OF MONITORING OF AQUATIC INVERTEBRATES IN AN INTENSIVE FISH FARMING SYSTEM

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Abstract: The aim of the study was a year-on-year comparison of detailed research of aquatic invertebrates in intensive salmonid fish farming, determination of the taxonomic composition of the community, and specification of species that may be hosts to some parasitic disease agents. The monitoring was done on a Danish model recirculating system near the Pravíkov municipality in the Czech-Moravian Highlands. A total of 19 series of samples were taken in the vegetation periods of the years 2015 and 2016. In between the vegetation periods, the whole system was drained and disinfected which had a major impact on the aquatic invertebrates. A total of 51 taxa of aquatic invertebrates were recorded. In 2015, the mean abundance was 412 ind/m^2. In 2016 after disinfection, marked changes occurred in the taxonomic composition; the mean abundance here was 515 pcs/m^2.

Key Words: intensive fish farming, macroinvertebrates, fish parasites

INTRODUCTION

Aquaculture in the Czech Republic consists mainly of pond fish breeding; nowadays, however, there is an increasing number of specialized modern systems designed mainly for intensive salmonid fish farming (Ministry of Agriculture CZE, 2014). Such systems are often inhabited by various species of invertebrates through the water sources used, by the stocking of fish of various origins and also by natural pathways. The role of the invertebrates within the system and their possible effect on the facility is determined mainly by the ecological and biological properties of individual species.

Colonisation of the fish farming facility in Pravíkov by aquatic invertebrates is possible by several pathways. The premises are not roofed, therefore, the first possibility for some insect species is the immigration of adults from a nearby pond (approx. 20 m away). Invertebrates that are capable of colonising new habitats in this way include e.g. dragonflies, mayflies, dipterans, heteropterans, and aquatic beetles (Anderson et al. 2003). Another possibility of a colonisation is a nearby brook that serves as the water source for the system. Water is supplied to the system primarily from a bore hole; in the summer months, due to increased evaporation this source becomes insufficient and water is supplied from the brook. This water supply is not secured by a sufficiently dense mesh that would prevent invertebrates from infiltrating the system. The invertebrates can also enter the system alongside the fish stock.

Some species of aquatic invertebrates may be hosts to a wide range of fish parasites causing fish diseases. Parasites may cause serious problems in fish farming, especially if there are suitable conditions for development of their whole life cycle. Therefore, their presence in the system is undesirable (Palíková et al. 2014).

Proliferative kidney disease (PKD) is one of the most serious parasitic diseases. Their agents are connected with the invertebrates through their development cycle, and may occur in intensive salmonid fish farming systems. PKD is a threat to salmonid fish farming in Europe and North America (Okamura and Wood 2002). The agent is the myxozoan *Tetracapsuloides bryosalmonae*, its invertebrate hosts are bryozoans (Bryozoa) (Tops and Okamura 2005). Serious problems are also caused by the salmonid whirling disease (myxobolosis), the agent of which is the myxozoan *Myxobolus cerebralis*; with part of the development cycle occurring in blood worms (Tubificidae) (Svobodová

2007). We furthermore encounter the nematode *Raphidascaris acus* in intensive salmonid fish farming (Palíková et al. 2014) whose intermediate host may be the amphipod *Gammarus fosarum* (Moravec 2004a). Salmonid fish production is also affected by trematodes, predominantly *Diplostomum spathaceum* and *Crepidostomum* sp. (Svobodová 2007) whose development cycle is bound to freshwater snails (Brassard et al. 1982, Svobodová 2007). Isopod *Asellus aquaticus* is an intermediate host of thorny-headed worms (Acanthocephala); the rainbow trout is a host of the species *Acanthocephalus anguillae* or *A. lucii* (Moravec 2004b).

MATERIAL AND METHODS

Samples of water biota were taken in the recirculating facility for salmonid fish farming near the Pravíkov municipality in the Czech-Moravian Highlands. The premises are managed by the company Biofish s.r.o. Samples were taken at monthly intervals in the years 2015 and 2016. A total of 19 series of samples were taken. Three sampling sites we selected within the system with different conditions; namely, the bottom of the drainage canal from the breeding gutters (outflow), the bottom of the inflow canal into the breeding gutters (inflow), and the wall on the inflow to the system (wall).

Samples of aquatic invertebrates are most frequently taken using the benthic net (Niedobová and Řezníčková 2014). Considering the specific character of the recirculation system, the sampling device needed to be appropriately adjusted. A solid and sharp cutting edge was welded to the rim of the benthic net of the dimensions 23 × 30 cm which allowed for sampling from a solid surface. At the same time, a long handle was fastened to the net because of the approximately 2 m depth of water in the system. In cases of outflow and inflow, the sampling was done by traction along the bottom; the length of traction was 3 m in total. Samples from the wall were taken by scratching off the bryophyte growth. The substrate containing the animals was then caught into the net with a mesh size of 0.5 mm. Fixed samples were processed and evaluated during the year 2016 according to standard methodology (ČSN 757703).

The following physico-chemical properties of water were measured in the system: temperature, pH, conductivity, and dissolved oxygen concentration. Conductivity was measured using the conductometer HANNA Combo HI 98129, other parameters were measured using the multi-parameter probe HACH HQ40d.

Individual samples were evaluated based on the data on the taxonomic composition and abundance of macrozoobenthos. Due to the large number of samples and complex taxonomy, this study does not include the midges (Chironomidae); they do not play a role as intermediate hosts of fish parasites, either.

RESULTS AND DISCUSSION

The abundance and taxonomic composition of macrozoobenthos are affected predominantly by abiotic environmental conditions and by the life cycle of aquatic invertebrates. The abiotic conditions over the monitored period are summarized in the Table 1.

Table 1. Minimum, maximum, and the mean values of abiotic parameters of water in the system over the monitored period in the years 2015 and 2016.

	Inflow, wall			Outflow		
	min	max	mean	min	max	mean
Dissolved oxygen [mg/l]	7.8	12.2	9.5	6.5	12.0	8.7
pH	5.1	7.9	6.7	5.0	7.9	6.9
Temperature [°C]	3.2	18.1	11.9	3.3	18.3	12.5
Conductivity [µS/cm]	237	996	495	239	1029	453

Due to minimum differences of the measured values between localities, the system is evaluated as a whole. The oxygen concentration over the monitored period ranged within 6.5–12.2 mg/l, pH ranged from 5.0 to 7.9. Water temperature in the system decreased to 3.2 °C in the winter months; the highest temperature measured was 18.3 °C. Conductivity ranged within the values of 237–1029 µS/cm. Bregnballe (2015) reports the optimal values of hydrochemical properties

for the Danish model recirculating systems to be 70–250% for oxygen saturation of water and 6.5–7.5 for pH. Svobodová (2007) reports the optimal temperature for salmonid fish species to be 8–16 °C. All the measured values correspond with the recommended ranges, except for pH measured in September 2016 which decreased to 5.0; meanwhile it is recommended for pH not to drop below 6.2 (Brengballe, 2015).

Macrozoobenthos communities in recirculating systems are often also affected by technological interventions. Frequently, pH values are adjusted using sodium carbonate; as pH is adjusted to approach 7, such intervention does not interfere with the invertebrates in an important way. Therapeutic interventions may also be done within the system, having a major impact on the biofilter's function. For example, the use of antibiotics can completely kill off the biofilter bacteria (Palíková et al. 2015) causing major changes to the chemical status of water. Other frequently used therapeutic tools are formaldehyde and sodium chloride (Svobodová 2007). All therapeutic interventions have a major impact on the colonisation of the system by macrozoobenthos.

Composition of the macrozoobenthos community

In the two-year monitoring of the fish farming facility, permanent groups of aquatic invertebrates dominated unequivocally; i.e., such macrozoobenthos that are permanently bound to the aquatic environment. In the year 2015, the most abundant group was the subphylum Crustacea with the highest percentage in most samplings. Crustaceans were represented only by one species *Asellus aquaticus* which at the end of June and November represented up to 63% and 66%, respectively, of all the aquatic invertebrates. In the year 2016, *A. aquaticus* appeared in markedly lower abundances; with the lowest percentage of only 0.4% in September, and the highest percentage of 57% in March (Figure 1). This species is not sensitive to water pollution, feeding on organic remains, detritus, or fallen leaves, and due to the high fish breeding intensity, it has sufficient nourishment here. It may also become a problem when water lice enter the space of the biofilter. They feed on the bacterial growth, and may thus decrease its efficiency.

The efficiency of biological filtration of the bacterial growth may also be decreased by freshwater molluscs. In 2015 they occurred as the second most abundant group peaking at the beginning of June and in August with 53% and 32%, respectively, in the relative representation. The most abundant species was the mollusc *Lymnaea peregra*. In 2016, *L. peregra* occurred in the percentages of 0.4% in September up to 75% in April. The most abundant mollusc genus was *Pisidium* sp. Molluscs of the genus *Lymnaea* may be intermediate hosts of up to several species of trematodes. In the cyprinid fish breeding, it is predominantly *Posthodiplostomum cuticola* whose metacercariae parasitize on fish skin and hypodermis (Svobodová 2007). This trematode matures sexually in the intestines of waterfowl (Ondráčková et al. 2004) but those are prevented from entering the fish farming facility due to the use of netting. *L. peregra* may be a host of the trematodes *Crepidostomum metoecus* and *Diplostomum spathaceum*. Hosts of *Crepidostomum farionis* can be the pea clams *Pisidium* sp. (Olsen 1986), which were also noted in the system.

In 2015, an abundantly represented group was also the class Hirudinea; in October, leeches made up to 30%, and were thus the second most abundant group. In 2016 leeches occurred in a markedly lower percentage; only in February they made 36%, thus being the most abundant group. In the other months, the percentage ranged within 0–7%. In the leech digestive system, reproduction of monads of the genus *Trypanosoma* and *Trypanoplasma* occurs (Lom et al. 1986, Overath et al. 1999, Svobodová 2007). These monads live in the blood circulation of mainly cyprinid fish species, frequently causing massive infections (Svobodová 2007). At the monitored facility, only two leech species were found, and neither one of them is a fish ectoparasite and neither one serves as a host of blood monads. *Erpobdella octoculata* feeds on small aquatic invertebrates, and *Glossiphonia complanata* by sucking on the haemolymph of molluscs and annelids (Buchar et al. 1995).

An interesting group were the enchytraeid worms (Oligochaeta) which in the year 2015 occurred rather sporadically and made a maximum of 7% at the end of June. They were represented by only five taxa. In 2016, the group Oligochaeta was the most abundant group of invertebrates, their relative representation reached up to 97% in the month of September, and the species range extended to include 16 taxa.

Other groups of permanent fauna found in the recirculating system in both vegetation periods were Turbellaria, represented only by the genus *Polycelis* sp., and Cnidaria, also represented by one genus *Hydra* sp.

From among representatives of temporary fauna, i.e. the one that undergoes only a part of its life cycle in the aquatic environment, a very abundant group were the midges (Chironomidae) which commonly occur in all types of surface waters. However, they have not been evaluated in this study and they are not important as intermediate hosts of fish parasites, either. From among other representatives of temporary fauna, in both vegetation periods the following groups occurred: Ephemeroptera, especially *Baetis rhodani*; Coleoptera, especially *Brychius* sp. Diptera, most frequently *Limnophora* sp.; and furthermore, the groups Plecoptera and Trichoptera in negligible numbers. *Sialis fuliginosa* (Megaloptera) occurred in 2016.

Figure 1 Relative representation of groups in 2015 and 2016

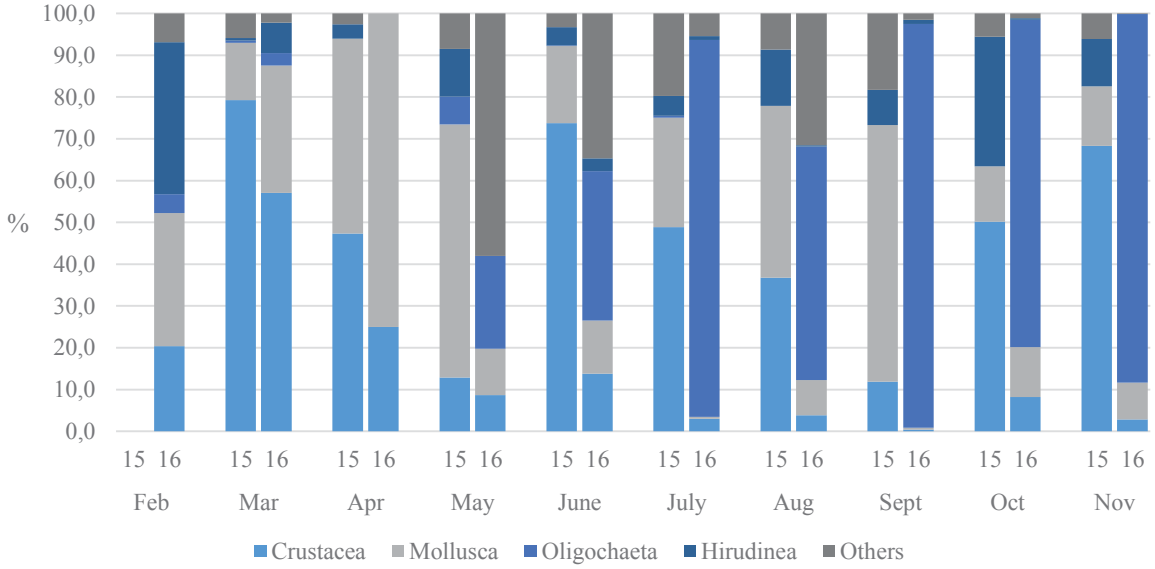


Figure 2 Abundance of aquatic biota in 2015 and 2016

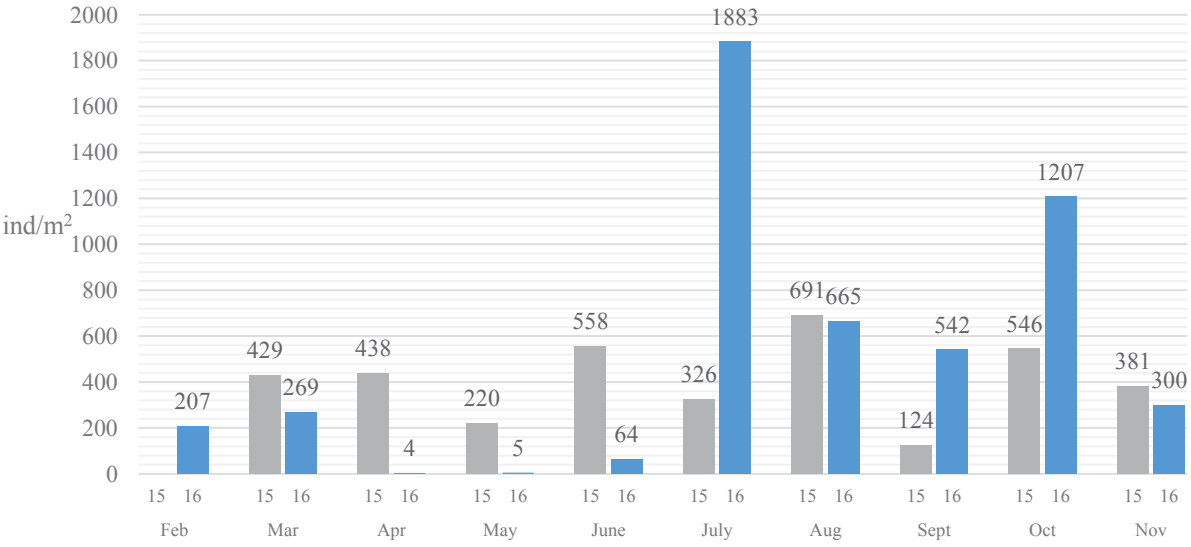
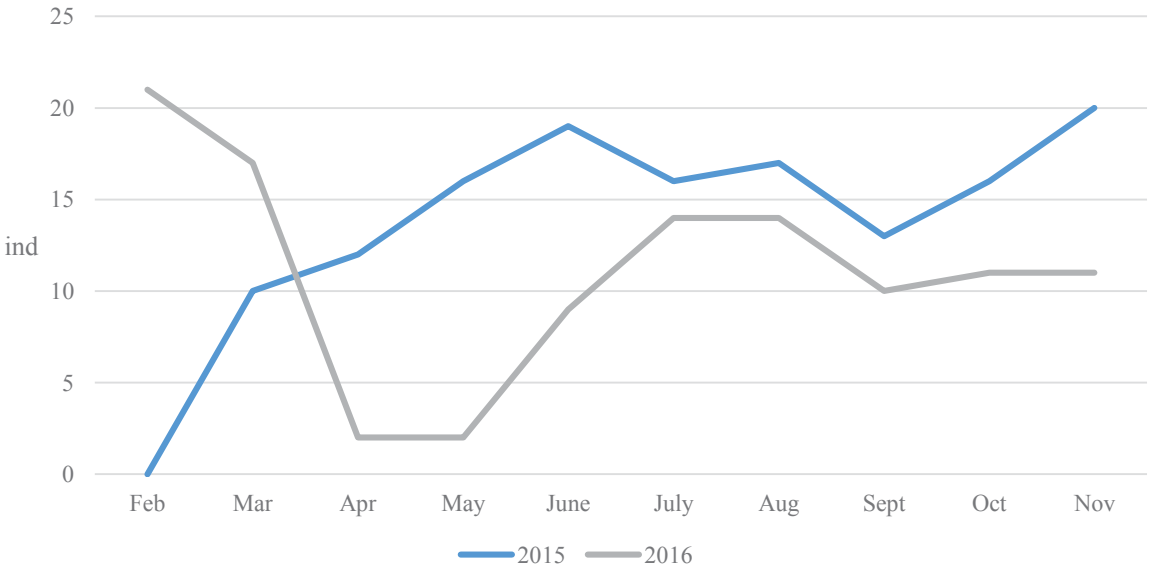


Figure 3 Number of taxa in 2015 and 2016



CONCLUSION

Colonization of Danish type recirculation facilities by aquatic invertebrates is not being studied at all in the Czech Republic at present. As presumed, marked differences were found in the incidence of invertebrates in the year-on-year comparison.

In between vegetation periods, the system was drained and the facility completely disinfected, having a major impact on the occurrence of invertebrates. In 2015, abundant bryophyte growth occurred here, providing the invertebrates with a suitable environment for shelter and a large amount of food. The growth was removed during the cleaning and was not restored during the following season.

Permanent fauna predominated at the facility over the monitored period; temporary fauna representatives made approximately a third of all the invertebrates. The most abundant groups were Crustacea, Mollusca, Oligochaeta, and Hirudinea. Chironomidae were not evaluated in this study and they do not have importance in terms of intermediate hosts of fish parasites. Furthermore, the groups Cnidaria, Turbellaria, Trichoptera, Ephemeroptera, Diptera, Coleoptera etc. were observed.

Upon evaluating the effect of the found taxa of invertebrates on the functionality of the system and the health condition of fish, several potentially dangerous species were determined. The crustacean *Asellus aquaticus* is an intermediate host of thorny-headed worms (Acanthocephala); furthermore, it devours bacterial growth and once it enters the biofilter space, it may decrease its efficacy similarly as the freshwater molluscs present in large numbers. The mollusc *Lymnaea peregra* is a host of the trematodes *Crepidostomum metoecus* and *Diplostomum spathaceum*; the genus *Pisidium* sp. is a host of *Crepidostomum farionis*. The other species do not pose a potential threat to the system as they are not hosts to fish parasites and occur in very small numbers.

ACKNOWLEDGEMENTS

The research was financially supported by the project NAZV "Increasing and improving the salmonid fish production in the Czech Republic using their genetic identification" [QJ1510077].

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OPTIMIZATION OF THE LYMPHOCYTE TRANSFORMATION TEST IN SALMONID FISH

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Abstract: The lymphocyte transformation test is a functional immunological assay commonly used in mammals, but in fish, there are some obstacles, making the optimization necessary (including determination of optimal samples, anticoagulants, mitogens and their concentration, serum type, temperature during incubation and its length). Samples obtained from rainbow trout (*Oncorhynchus mykiss*) included organs (head kidney and spleen) and blood collected using different anticoagulants (heparin, EDTA). The highest levels of cell proliferation were observed after 6–7 days of incubation. Peripheral blood lymphocytes showed the best results with pokeweed mitogen (50 µg/ml), while head kidney lymphocytes were most stimulated by phytohaemagglutinin (100 µg/ml). Compared with trout serum, FBS provided higher proliferation levels. Best results were obtained at 15 °C with peripheral blood lymphocytes, though only small differences were measured between different incubation temperatures with head kidney lymphocytes. Heparinized blood was proven to be an optimal sample, providing the best results. Optimization of this test improves possibilities for examining the health status of salmonid fish in trials as well as in practice.

Key Words: mitogen, LTT, thymidine, *Oncorhynchus mykiss*, lymphoblast, stimulation

INTRODUCTION

There are many factors (rearing conditions, stress, various pathogens and pollutants) with the ability to negatively affect health and meat quality of fish. Considering the worldwide growth of aquaculture and increasing demand for fish meat, requirements for disease diagnosis and prevention are also rising. As competence of the fish immune system is directly proportional to their general health status, immunological parameters can be used for its evaluation (Papežíková et al. 2016). There are many applied immunological methods; however, functional immunological assays are the most effective ones, telling us not only the current state of the fish immune system but also its ability to actively react to stimulants. The lymphocyte transformation test (LTT) is commonly used in mammals, but in fish, there are some differences, making the optimization necessary. Using mitogenic stimulation, lymphocytes are transformed into lymphoblasts (Scapigliati 2013). Mitogens selectively stimulate T cells (PHA, ConA), B cells (LPS) or both (PWM) (Kehrer et al. 1998). After several days of cultivation, labeled thymidine (radioactive ^{3}H) is added, incorporated into newly formed DNA and subsequently measured by a liquid scintillation counter in counts per minute (CPM). Overall, this test examines the ability of the immune system to respond to the above-mentioned negative factors (Ottinger et al. 2014) or to prophylactic and therapeutic interventions. Thus, reduced lymphocyte proliferation may be observed in case of immunosuppression. However, there are significant differences between fish species (and also

individuals) which should be taken into account. One of the facts we need to consider is a different temperature optimum (in salmonids 8–16 °C). Serum inactivation is also carried out at a lower temperature than normal in mammals and thermophilic fish (Sakai 1981). According to some authors (DeKoning et al. 1991), it is necessary to use homologous plasma in rainbow trout (*Oncorhynchus mykiss*) instead of FBS (fetal bovine serum, commonly used in mammals). The optimization comprises determination of optimal samples, anticoagulants, mitogens and their concentration, serum type, temperature during incubation and its length.

MATERIAL AND METHODS

Fish and rearing conditions

Rainbow trout (average weight 194.77 ± 68.44 g, total length 24.14 ± 2.64 cm) were kept in three breeding tanks with a volume of 1000 l and one tank with a volume of 1270 l (Mendel University in Brno, Czech Republic). The water was saturated with oxygen at an average value of 8.11 mg/l (i.e. 87.1%), while average temperature was maintained at 17,6 °C, pH at 7.10, $N\text{-}NH_4^+$ at 0.36 mg/l, $N\text{-}NO_2^-$ at 0.14 mg/l and Cl^- at 140.40 mg/l. A Nexus 310 biofilter was used to filter the water, while a UV-C lamp was used for disinfection. The diet used in this experiment was BioMar EFICO Enviro 920, a standard extruded feed for salmonid fish rearing. All fish were fed two times a day, feeding intensity was based on the supplier's recommendations.

Sample collection, isolation of mononuclear cells

Samples obtained from rainbow trout (*Oncorhynchus mykiss*) included organs (head kidney and spleen) and blood collected from the caudal vein using different anticoagulants (heparin, EDTA). The organs were cut, forced through a mesh and washed twice by centrifugation in phosphate-buffered saline – PBS (10 min, 450 g). Blood was diluted by RPMI-1640 medium (1 : 2). Subsequently, mononuclear cells were isolated by density gradient centrifugation (Histopaque, 1077 g/ml; 40 min, 800 g). After centrifugation, a layer of mononuclear cells (lymphocytes and a small amount of monocytes) was formed above the density medium. After collection, the cells were washed repeatedly in PBS (10 min, 450 g), counted (Mindray, China) and resuspended in L-15 medium (Leibovitz) to the required concentration (2×10^5 cells per well).

Lymphocyte transformation test

The cell suspension (180 µl) was dispensed in triplicates into wells of 96-well plates together with mitogens (20 µl) and heat inactivated trout serum or FBS (20 µl). The mitogens comprised plant mitogens (polyclonal activation): PWM – pokeweed mitogen (5, 10, 50 µg/ml), ConA – concanavalin A (1, 10, 20 µg/ml), PHA – phytohaemagglutinin (25, 50, 100 µg/ml) and LPS – lipopolysaccharide (1, 50, 100 µg/ml*; Actinobacillus pleuropneumoniae*). After the incubation period (2–8 days, 10–20 °C), labeled thymidine (radioactive ^3H) was added and the cells were harvested in the next 20 hours (Packard, USA). The amount of thymidine incorporated into DNA was measured by a liquid scintillation counter (Packard, USA) in counts per minute (CPM).

RESULTS AND DISCUSSION

The highest levels of cell proliferation were observed after 6 days of incubation with peripheral blood lymphocytes (= PBL) and 7 days with head kidney lymphocytes (= HKL, Figure 1). However, shorter incubation period is usually used in other studies (Harford et al. 2007, Müller et al. 2009). PBL showed the best results with PWM (50 µg/ml), while HKL were most stimulated by PHA (100 µg/ml). The lowest levels were recorded with ConA (Figure 2, 3). In the following experiment, however, PWM (10 µg/ml) showed best results with HKL (Figure 4). Due to insufficient amount of isolated cells, stimulation with LPS had to be examined separately (Figure 5, 6). Similarly to heparinized blood, EDTA was well stimulated by PWM (50 µg/ml), with no stimulation observed with PHA (100 µg/ml) (Figure 7). Different results were described by various authors (Chilmonczyk 1978, Tillitt et al. 1988, Agbede et al. 2005). In contrast with other findings (DeKoning et al. 1991), FBS provided higher proliferation levels than trout serum (Figure 8). Best results were obtained at 15 °C with PBL (Figure 9, 10), though only small differences were measured between different incubation temperatures with HKL. Heparinized blood (diluted by RPMI) was proven to be an optimal

sample, providing the best results (Figure 11). The spleen could not have been examined due to very low numbers of isolated cells.

Figure 1 Length of incubation (HKL/PBL-heparin, 15 °C, PWM10, FBS; n = 4/5)

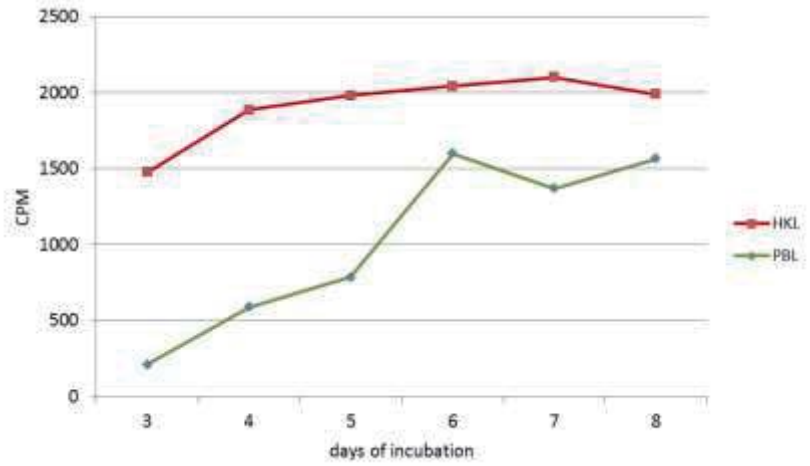
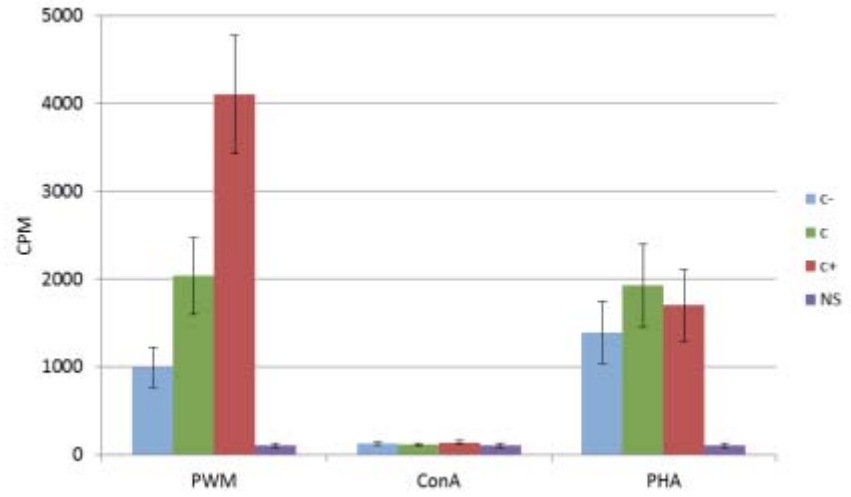


Figure 2 Mitogens – standard (c), lower (c-) and higher (c+) concentration (PBL-heparin, 7 days, 15 °C, FBS; n = 8)



Legend: NS – non-stimulated cells

Figure 3 Mitogens – standard (c), lower (c-) and higher (c+) concentration (HKL, 7 days, 15 °C, FBS; n = 6)

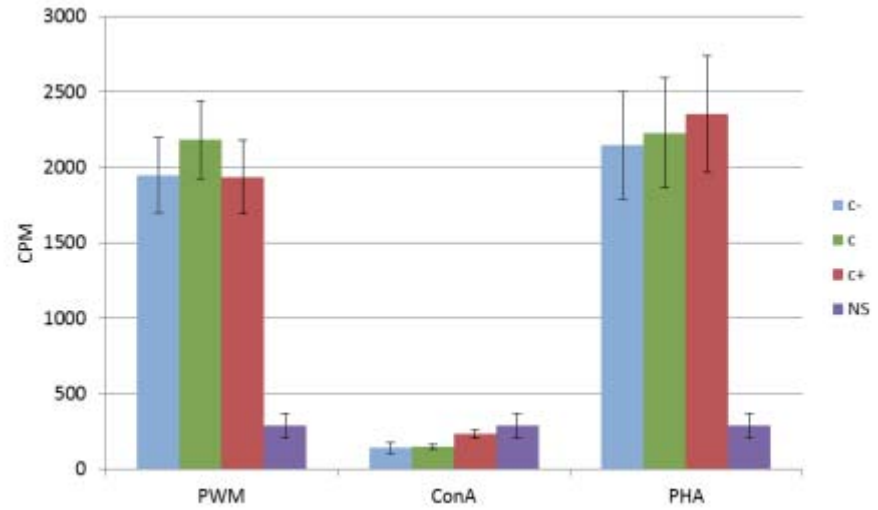


Figure 4 Mitogens – PWM, PHA (HKL, 6 days, 15 °C, FBS; n = 10)

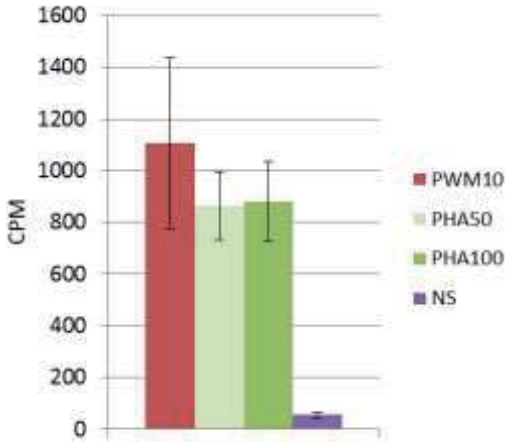


Figure 5 Mitogens – LPS (PBL-heparin, 7 days, 15 °C, FBS; n = 8)

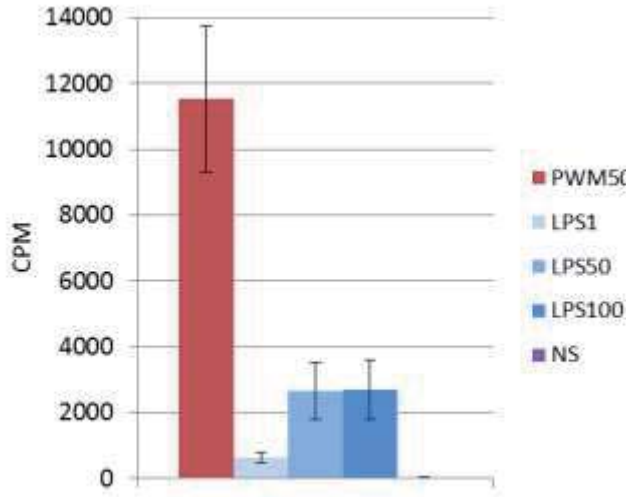


Figure 6 Mitogens – LPS (HKL, 7 days, 15 °C, FBS; n = 8)

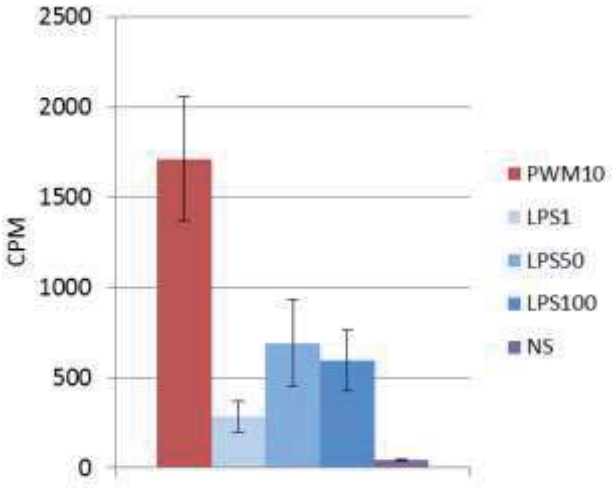


Figure 7 Mitogens – PWM, PHA (PBL-EDTA, 6 days, 15 °C, FBS; n = 10)

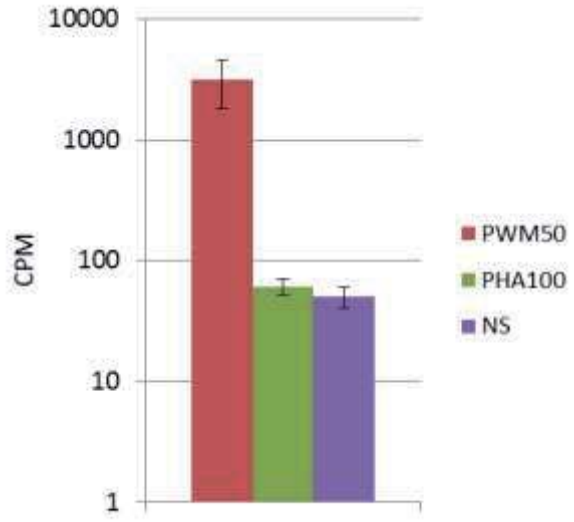


Figure 8 FBS, autologous and homologous trout serum (HKL, 7 days, PHA100, 15 °C; n = 8/HS 6)

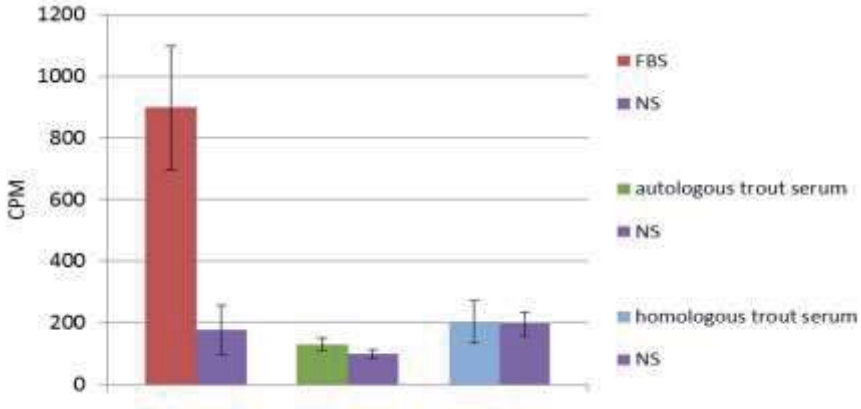


Figure 9 Incubation temperature (PBL-heparin, 7 days, PWM50, FBS; n = 8)

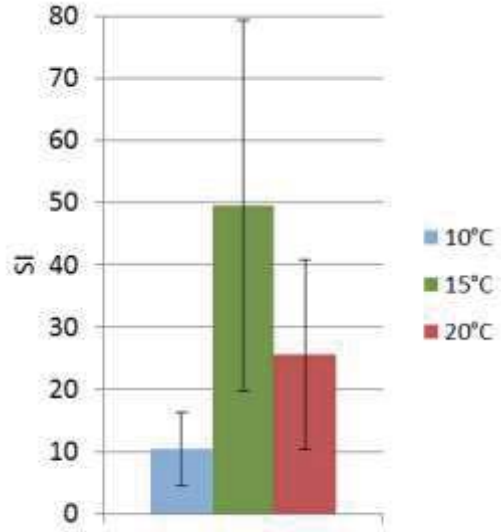
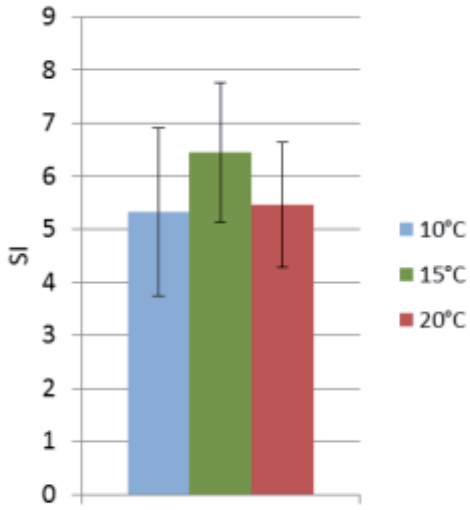
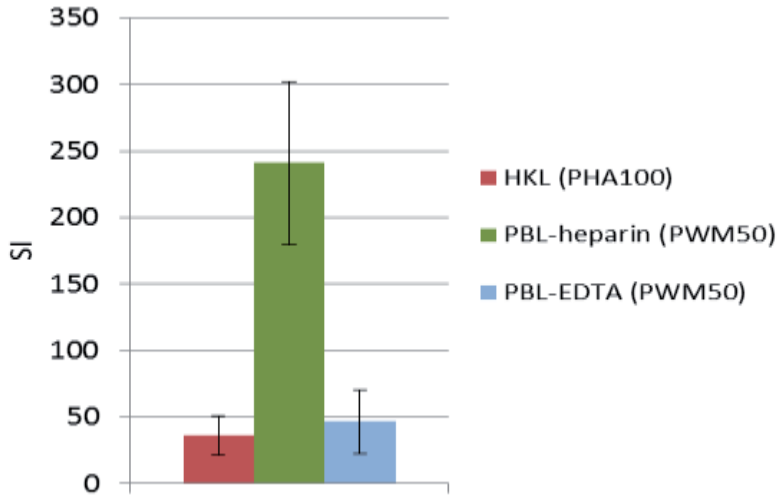


Figure 10 Incubation temperature (HKL, 7 days, PHA100, FBS; n = 8)



Legend: stimulation index (SI) = stimulated/non-stimulated cells

Figure 11 HKL, PBL-heparin and EDTA (7 days, 15 °C, FBS; n = 9)



CONCLUSION

There are many variables involved in the lymphocyte transformation test and studies show different findings. Heparinized blood was proven to be an optimal sample during this experiment. Maintaining the correct temperature when handling fish cells is essential (even during serum inactivation) and natural variability also needs to be taken into account. Optimization of this test improves possibilities for examining the health status of salmonid fish in trials as well as in practice.

ACKNOWLEDGEMENTS

This study was supported by the Ministry of Agriculture of the Czech Republic (MZe NAZV QJ 1510077) and IGA VFU Brno (216/2017/FVHE).

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USE OF BIO-ENZYMATIC PRODUCTS FOR THE REDUCTION AND MODIFICATION OF FISHPOND SEDIMENTS

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Abstract: Accumulation of sediments in fishponds is a big problem in the Czech Republic. Most owners of fishponds are not able to manage this problem financially, organizationally or professionally. A product that could degrade organic depositions at the bottom and in the water column using natural unmodified bacteria could be an excellent and innovative solution to the problem of silt removal in fishponds. The aim of the project was to examine the ability of bacterial-enzymatic mixture to decompose pond sediments and the effect of these products on the quality and composition of sediments. The experiment was conducted in laboratory conditions, so the results of this experiment do not match the results that could be achieved in ponds under natural conditions. The degradation of pond sediments was not confirmed during the course of the experiment. However, the influence of the product on the change in sediment composition and the increase in oxygen content in water was confirmed.

Key Words: sediment, water extraction, Mehlich extraction, bio-enzymatic preparations

INTRODUCTION

A significant change in strategy management of fishpond ecosystems occurred during the second half of the 20th century, especially in connection with the intensification of carp breeding and overall intensification of agriculture (Vrána 2002). Increase in sediment depositions at the bottom of ponds is occurring due to increased erosion in the catchment area, which is caused by an inadequate management practise of agricultural land. As a result, not only fish production, but also the function of fishponds in the process of pollution elimination is reduced (Kubík 2011, Plaster 2014).

Sediments from fishponds are often removed and deposited in piles, and recorded as pond mud (Havlíček 1969). Dredged sediments from fishponds, water reservoirs and water courses do not have to be regarded as waste, if the quality of sediment complies with the requirements listed in the Annex no. 9 of Act no. 185/2001 Coll. on waste, as amended (after adoption of amendment no. 9/2009 Coll) (Kubík 2011). Sediments from fishponds and reservoirs contain higher content of nutrients and organic material when compared to arable land. Considering the conditions of our soil and deposits of pond material, whether still in ponds or removed to fishpond dam, their use in agriculture is very small (Havlíček 1969). The amount of allochthonous material getting into the water increases risk of sediment contamination, which entails tightening of the legislative requirements for the use of sediments in agriculture. Nowadays, interest in the reuse of sediments is minimal and excavation from ponds and reservoirs is more expensive and more difficult.

A product, which should decompose organic parts of sediments in fishponds and reservoirs, can be purchased on the market. It is a bacterial-enzymatic mixture containing concentrate of spores and endospores of specially selected and purposefully cultivated strains of native soil bacteria. One of the specific properties of those bacteria is the ability to increase the production of the desired enzyme. All strains of bacteria should be non-pathogenic and naturally occurring in natural environment. These strains of soil bacteria were selected for specific efficiency and should not be genetically altered or modified. After introducing the mixture to water environment, spores

and endospores should revive in a short time, produce specific enzymes and consume the present organic sediment.

Regular use of PTP Plus should lead to the reproduction of bacteria, followed by continual cleaning of fishponds. Producers of the mixture further state that biological balance in fishponds occurs after couple of weeks of using this product. The amount of organic deposition, as well as turbidity at the bottom and in the water column, should be significantly reduced. The result should be a clear and transparent fishpond. Furthermore, a significant reduction in chlorophyll a, which is a measure of algal biomass, should be achieved, as well as an increase in the oxygen content in the water. The product should be safe for animals and cannot be overdosed (Baktoma 2017).

MICROBE-LIFT/Sludge-Away from USA company Ecological Laboratories, Inc is a similar product available on the market. This product has organic and microbial base and it is formulated specifically for the removal of organic bottom solids that are slow to degrade. It works faster at warm temperatures; however, it may be used effectively at any temperature year-round. The product may slightly discolour pond water for a short while. Company also states that product precipitates phosphorous, improves transparency of fishpond and it is safe for fish, plants and environment. It will accelerate the solubilisation and biological digestion of organic solids in pond. As a result of this increased oxygen demand, the oxygen uptake rate in the pond will increase in the process, along with the aquatic life's need for oxygen. The fishpond must be sufficiently oxygenated (> 4.0 mg/l of dissolved oxygen) during the time of use of this product (MIKROBELIFT 2017).

MATERIAL AND METHODS

Sediments from eutrophic fishponds Bohuslavický I and Bohuslavický III, situated in cadastral area of municipality Bohuslavice u Konice in Olomouc Region, were used in this study. Sediments were collected during the vegetative period on 9. 5. 2017 from the surface layer (0–15 cm).

Sediments were deposited in six graduated cylinders. Height of sediments layer was 20 cm, and 30 cm of tap water was added. All cylinders were placed in a room with a constant temperature (12 ºC) and limited light access. Bio-enzymatic mixture PTP Plus was applied in two of graduated cylinders with the mixture of sediment and water. In the first one the applied concentration was according to the instructions of the producer and in the second one 100 times greater. The third cylinder was without any product, and it was used as a control. The dosing according to the instructions recalculated on the water surface of the cylinders was following:

1st week – application of 9.5 mg

2nd and 3rd week – application of 4.8 mg

4th and 5th week – application of 3.2 mg

6th and 7th week – application of 1.6 mg

Other product (MICROBE-LIFT/Sludge-Away from USA company Ecological Laboratories, Inc) available on the market has been tested for comparison with the effect of PTP Plus. MICROBE-LIFT was applied in two graduated cylinders (fourth and fifth) with prepared water and sediment. One was with concentration recommended by producer and the other with hundred times higher concentration. The last cylinder was used as a control, so it was without application. The product was applied every week, for five weeks, according to the instructions, recalculated to the volume of the cylinder, i.e. 0.12 ml of product every week.

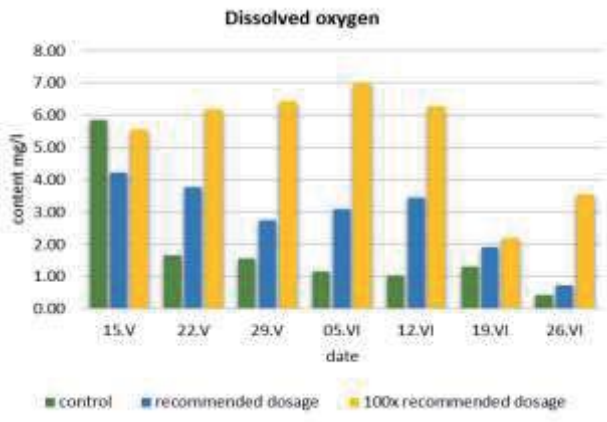
Basic hydrochemical parameters: dissolved oxygen, water temperature, pH (Hach HQ40d) and water conductivity (Hanna combo) were measured in cylinders every week during the product's application. PTP Plus and MICROBE-LIFT were applied for the first time on 15 May 2017 according to the above-mentioned instructions. Eleven weeks (31. 7. 2017) after the first application, all sediments were analysed. Dry matter of the sample was determined and water extraction according to standard ČSN EN 12457-4 and according to Mehlich III was done. The content of available nutrients (N, P) in sediments was determined in both extracts. At the same time, the loss of sediment in cylinders was monitored and the share of organic matter before and after the application of products was measured. Chemical parameters from the extract were analysed using standard methods according

to Horáková (2007) and Zbíral (2016). The results are expressed in dry weight units of the used sediments.

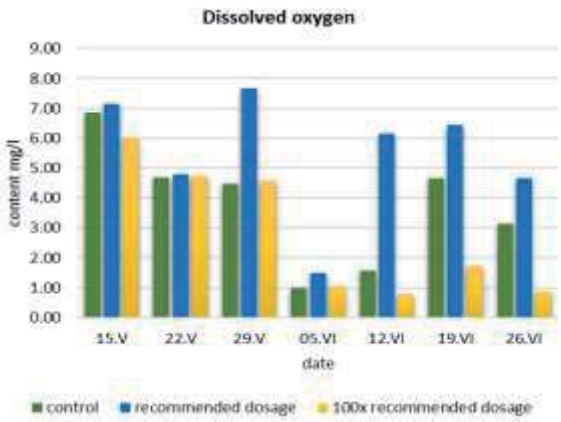
RESULTS AND DISCUSSION

Figure 1 Dissolved oxygen content

A) PTP plus application



B) Microbe-lift application

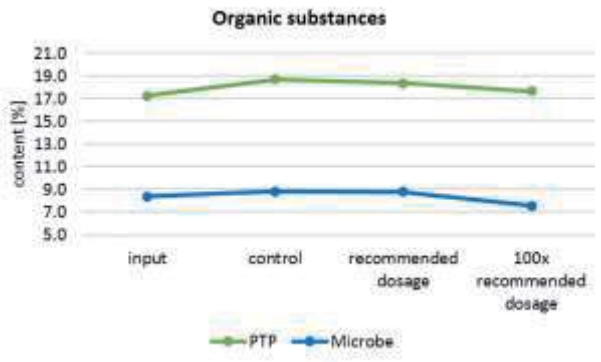


Dissolved oxygen content was higher in cylinders where PTP plus was applied than in cylinder without mixture, with the exception of the first application (15. 5. 2017). During the almost entire period of PTP plus application, the highest oxygen content was measured in cylinder where a hundredfold dose of the product was applied, and the lowest in the cylinder with control, i.e. without application (Figure 1A). On the other hand, in case of Microbe-lift, the lowest values of dissolved oxygen were in the cylinder with a hundredfold dose. The highest values were documented in the cylinder where the product was applied in recommended dose (Figure 1B).

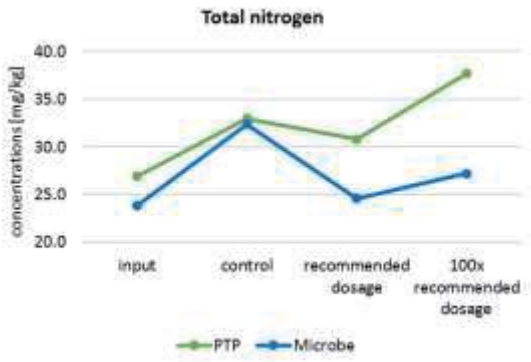
The amount of dissolved oxygen actually increases during the application of both products, when compared to the control. In the case of Microbe-lift, where an oxygen content of more than 4 mg/l is necessary for the functioning of the product, it is important to keep accurate dosing. Other measured hydrochemical parameters (temperature, pH, conductivity) were relatively balanced in the cylinders with PTP plus product. Significantly increased values of conductivity were detected in the cylinder with hundredfold dosage of Microbe-lift. The other measured hydrochemical parameters were again relatively balanced.

Figure 2 A) Content of organic matter and B) concentration of total nitrogen in water extraction

A)



B)



Content of organic matter in sediments from fishponds Bohuslavický I and Bohuslavický III is presented in Figure 2A. Values "input" are values of sediment, which was analysed immediately after the sampling, i.e. 9. 5. 2017. Other values are from analyses conducted after eleven weeks (31. 7. 2017). PTP plus was applied to the sediments from the Bohuslavický I pond from 15. 5. 2017

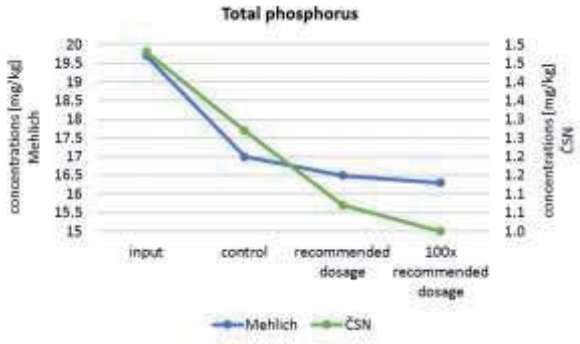
to 26. 6. 2017 and Microbe-lift/sludge-away was applied to the sediment from the Bohuslavický III pond from 15. 5. 2017 to 12. 6. 2017. The values in graduated cylinders with recommended dosage and hundredfold higher dosage than recommendation of producers were recorded. "Control" represents the values of sediments taken from cylinders without any application (only sediment and tap water), eleven weeks after the beginning of the test.

The highest amount of organic matter in sediments from Bohuslavický I fishpond was in the control (Figure 2A). A slight decrease in the amount of organic substances occurred in sediments treated with PTP plus; but the lowest values were in the sediment analysed on the day of the sampling (input). A similar trend is also shown in the second curve presenting the effect of Microbe-lift. The lowest value was documented in the sediment where a hundredfold higher dosage of the product was applied. However, the values do not differ by more than 2%, so in this case, neither one product is evidently functional.

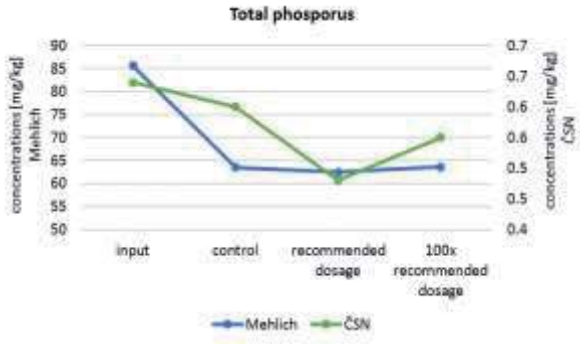
Concentration of the total nitrogen in sediments decreased in cylinders with recommended dosage of both products, when compared to the control. However, an increase in values was detected in the sediment treated with the hundredfold dosage; values of the sediment treated with PTP plus exceeded and the control values. It is clear from Figure 2B that the lowest total nitrogen concentration was measured in fresh sediments, i.e. those analyzed on the day of the collection.

Figure 3 Concentration of total phosphorous in sediments determined in extract according to standard ČSN EN 12457-4 and according to Mehlich III

A) PTP plus application



B) Microbe-lift application



The amount of total phosphorus declined with an increased concentration of the PTP plus product (Figure 3A). The value of total phosphorous also decreased in control, when compared to the input. In this case, we can evaluate that the PTP plus slightly decreases the phosphorus concentration in sediments. Concentration of the total phosphorus also decreased significantly in the control sample when compared to the input sample, in sediment from Bohuslavice III treated with Microbe-lift, as can be seen in Figure 3B. In this Figure, the differences in values obtained from different methods of extraction were recorded. According to Mehlich extraction, the product has no effect on the change in phosphorus concentration. Values obtained by standard ČSN EN 12457-4 show a decreased concentration of phosphorous in sediments treated with recommended dosage. However, increase occurs at higher dosage. In this case, the effect of the product on the sediment cannot be evaluated.

Amount of calcium in water extract increased after PTP plus application; the value in control also was slightly higher, when compared to the input analyses (Figure 4A). Therefore, in this treatment, the product had influence on the increase in the amount of available calcium in the sediments. In case of Microbe-lift (Figure 4B), more distinctive increase was documented in control when compared to the input value. Values obtained from different methods of extraction have differed. According to Mehlich analyses values increased and according to the standard ČSN values are more balanced, with a slight decline. The influence of Microbe-lift product is minimal.

Figure 4 Concentration of calcium in sediments determined in extract according to standard ČSN EN 12457-4 and according to Mehlich III

A) PTP plus application *B) Microbe-lift application*

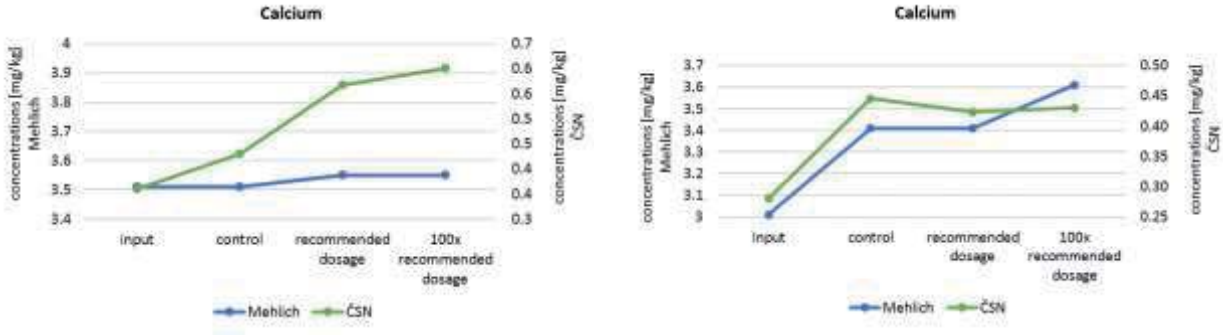
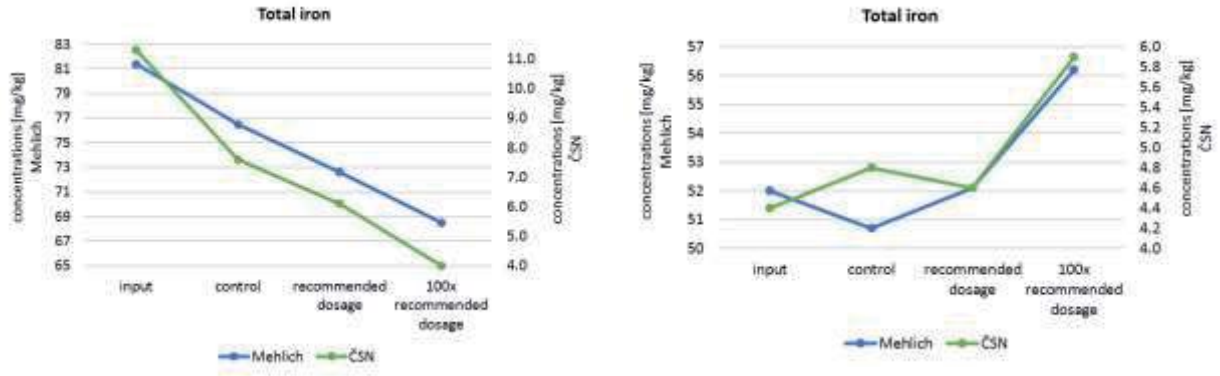


Figure 5 Concentration of iron in sediments determined in extract according to standard ČSN EN 12457-4 and according to Mehlich III

A) PTP plus application *B) Microbe-lift application*



Concentration of total iron declined in sediments from Bohuslavice I with a higher concentration of PTP plus (Figure 5A). In that case, there was a slight decline in concentration of iron in sediments. The value of total iron was lower in control, when compared to the input. In Microbe-lift treatment, values of total iron vary, depending on the type of the extraction method used. According to Mehlich, there is an increase in the values of the treated sediments, and only in control the value is lower than in the analysed fresh sample (Figure 5B). Values in the water extract prepared according to the standard ČSN EN 12457-4 are more balanced. A slight decline of iron concentration occurred in the graduated cylinder with recommended dosage, but the concentration rises again at a hundredfold higher dose. In this case, the effect of the product on the sediment again cannot be clearly assessed.

CONCLUSION

PTP plus producer states that this mixture should decrease the amount of organic sediment, and thereby also the turbidity at the bottom and in the water column. Furthermore, a decrease of phosphates and increase in oxygen content in the water should occur. The product does not decline the amount of organic sediment according to the conducted laboratory experiment. The height of sediment layer was the same in graduated cylinders during the entire period of application and four weeks after the application. Values of organic matter slightly decreased during the product's application, but only in a negligible amount. The content of dissolved oxygen in water actually increases with an increasing concentration of the product. Concentration of phosphorus in the sediments slightly decreased with the higher concentration of the product. The amount of available calcium in sediments is influenced by the PTP plus treatment; higher values in cylinders with higher dosage. Concentration of total nitrogen in water extraction was reduced when the product

was applied at the recommended dose. Total iron in extractions was also analysed and as with phosphorus, its concentration decreased with an increasing concentration of the product.

Concurrent product Microbe-lift should also decrease the amount of organic sediments and precipitate phosphorous. A condition for the functioning of this product is sufficiently oxygenated water (> 4.0 mg/l of dissolved oxygen), but this condition has not always been met. Values of dissolved oxygen increased in cylinder with the recommended dosage of the product, but decreased in sediment treated with a hundredfold concentration. The amount of organic matter slightly decreased during the application of the product, but also at a negligible quantity. The amount of total nitrogen also slightly decreased in cylinder with a recommended dosage. Other parameters cannot be clearly evaluated. Used extraction methods (according to Mehlich and standard ČSN EN 12457-4) show different values in order of magnitude in both products. In sediments treated with Microbe-lift, different tendency of increasing and decreasing was noticed in each of the methods. In PTP plus the treatment values are different, but the curves in figures are with the same or similar tendency of ascending and descending.

The results of our experiment show that none of the products fulfilled all of the producers claims. However, the experiment conducted under laboratory conditions does not fully correspond with natural conditions in fishponds. For the best possible functioning of the products, sufficient oxygenation and mixing of water in the cylinders should be provided. In fishponds, weather conditions and fish stocks contribute to water and sediment movements, and in addition, there is a number of other factors, which can have influence on the efficiency of the products.

ACKNOWLEDGEMENTS

The research was financially supported by the Internal Grant Agency project no. IP 070/2017.

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PHYTOPLANKTON DYNAMIC OF SMALL FISHPONDS DURING THE APPLICATION OF BACTERIAL PRODUCT

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Abstract: Qualitative and quantitative analyses of phytoplankton community were carried out in fishponds Bohuslavice 1, Bohuslavice 2 and Bohuslavice 3 situated in the District Prostějov, during the period from April to August 2017. In Bohuslavice 2 and 3 bacterial product PTP PLUS was applied in several repetitions in April and May with the aim of decomposing the organic sediments at the bottom and improving the oxygen regime. At the same time, selected hydrochemical parameters were measured. The highest values of oxygen saturation were documented at the beginning of the season, after which they continuously decreased. Taxa from eight divisions were recorded in the phytoplankton community. In terms of diversity, Chlorophyta was the most diverse group, followed by Bacillariophyta and Euglenophyta in each of the studied fishponds. The highest abundance in Bohuslavice 1 and 2 was recorded in the beginning of the research period, and this was the only time when cyanobacteria were present in higher numbers. In Bohuslavice 2 the highest value of abundance was in July, with *Tetrastrum triangulare* as the most dominant species (48.87% of total amount of cells); and this was the highest abundance registered in all three ponds during the entire research period. The most dominant group during greater part of the season was Chlorophyta with common representatives from genera *Monoraphidium, Desmodesmus, Planktosphaeria, Tetrastrum*. Very numerous members of phytoplankton community were also genera *Trachelomonas*, from the group Euglenophyta and colonial *Fragilaria* from Bacillariophyta. The oxygen content, diversity and abundance of phytoplankton were probably influenced by the cover of free floating plants *Lemna minor* and *Spirodela polyrhiza*, which were present in different scales in all three ponds.

Key Words: green algae, composition, physicochemical parameters, taxon, duckweed

INTRODUCTION

Fishponds are generally man–made shallow water bodies and their functioning is conditioned by human activity. Czech Republic is one of the European countries with the highest density of fishponds (Wezel et al. 2013). Function of fishponds could be different, e.g. provision of habitat to support freshwater biodiversity (De Bie et al. 2008), ornamental, recreational, but in Czech Republic most of them are used for fish production.

Phytoplankton, as the photoautotrophic part of the plankton, is a major primary producer of organic carbon in the pelagic of the seas and of inland waters (Reynolds 2006) and forms the base of the aquatic food webs, which supports the zooplankton and fish (Graham et al. 2009).

Continual succession of dominant species of phytoplankton communities occurs due to dynamic changes of growth factors such as light, temperature and nutrient concentration in an aquatic environment (Chan 1980), as well as the presence of zooplankton, fish stock density (Azim et al. 2003) and presence of submerged and floating plants (Bicudo 2007).

Intensive fish production has an important influence on both the structure and dynamics of an aquatic ecosystem. According to the seasonal means in concentrations of total phosphorus at the end of 20th century, about 80% of Czech fishponds were described as eutrophic (Přikryl 1996). However, the fishery management is not the only source of nutrients, but also run-off from surrounding land and in present time internal loadings area a very important factor. It is known that most of the ponds have a nutrient pool in sediments.

Phosphorus precipitation, aeration, sediment treatment, sediment removal, biomanipulation and different chemical and bacterial products have all been used for restoration of water systems and reducing of the internal load of nutrients.

Phytoplankton analysis gives an overall idea of the environmental condition of the water body. Both quality and quantity abundance of plankton communities in fishponds are of great importance for successful aquaculture management.

MATERIAL AND METHODS

The studied fishponds Bohuslavice 1 (B1) (1 ha), Bohuslavice 2 (B2) (0.8 ha), Bohuslavice 3 (B3) (0.6 ha) are situated along the flow of the Šumický stream in municipality Bohuslavice in the District Prostějov, within the Olomouc Region (Czech Republic). In two of the ponds (Bohuslavice 2 and 3) bacterial product PTP PLUS (Baktoma) was repeatedly applied in May and June 2017 with the goals to decompose the organic sediments at the bottom and in the water column. One of the benefits of this treatment, according to the producer, should also be increased oxygen content (Baktoma 2017).

A cover of free floating duckweed plants (*Lemna minor* and *Spirodela polyrhiza*) was noticed at the monitored ponds in beginning of April. In August, these plants have completely overgrown B3, while 80–90% surface of B1 was covered, and B2 was covered to a small extent during July and August. From June to August the submerged plants (mainly genera *Ceratophyllum*) were documented in B1 and B3.

Temperature, dissolved oxygen, pH, transparency and conductivity were measured immediately on locality using mobile instruments (Hach Lange, Hanna instruments, USA and Secchi disk) always in the same place (outflow) at the same time (in the morning).

The samplings of phytoplankton were conducted once a month in the period from April to August 2017, except in the period of applying product PTP PLUS, when samples were collected bi-weekly. Samples were taken using 20 µm planktonic net. Phytoplankton species and genera were identified in the live material under a light microscope Olympus BX51 using standard keys. Determined taxa were classified into eight divisions: Cyanobacteria, Dinophyta, Cryptophyta, Chrysophyta, Xantophyta, Bacillariophyta, Euglenophyta and Chlorophyta (Reynolds 2006).

Quantitative phytoplankton samples, 50 ml each, were taken with plastic bottles from the surface water layer and preserved in Lugol's solution. Samples were concentrated using filtration equipment by Marvan (Marvan 1957), after which the abundance of algae and cyanobacteria was calculated by counting cells in a Bűrker chamber. The data were expressed as a number of cells per millilitre.

RESULTS AND DISCUSSION

The values of measured physicochemical parameters in all three fishponds are summarized in Table 1.

Table 1 Minimum, maximum and mean values of the measured abiotic parameters in monitored fishponds

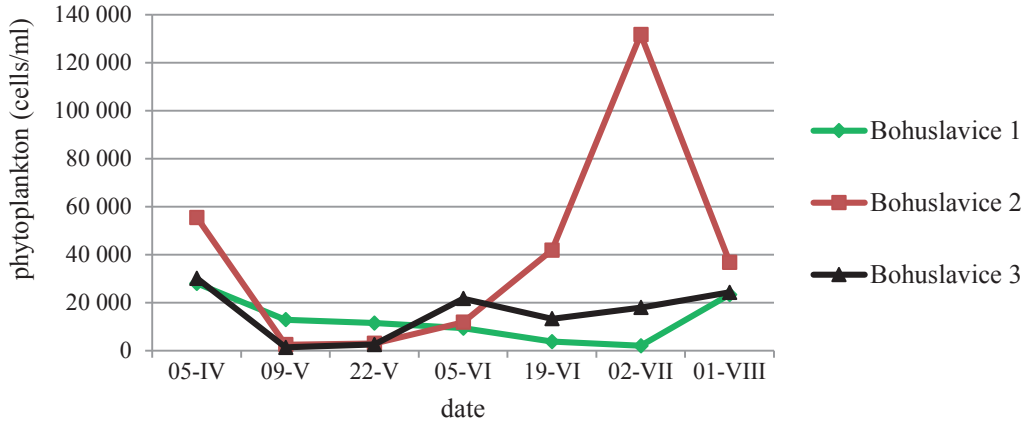
Area	Bohuslavice I			Bohuslavice II			Bohuslavice III		
	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min
Temperature (°C)	21.9	17.9	13.9	21.4	17.7	12.8	20.1	17.1	12.9
Dissolved oxygen (%)	122.4	57.5	3.2	131.9	44.7	5.5	142.5	61.5	5.2
pH	8.25	7.21	6.35	8.69	7.42	6.48	8.85	7.75	6.78
Conductivity (mS/m)	50.8	43.0	34.6	45.8	43.8	42.7	42.2	39.2	35.4
Transparency (cm)	110	95	50	100	63	25	125	98	50

The oxygen content was high in the spring, while in the following months it decreased, reaching a minimum during the summer months in every pond. The cover of free floating plants, which was very developed throughout most of the sampling period, could be the cause of oxygen depletion in the water (Pasztaleniec and Poniewozik 2013, Parr et al. 2002). During the course of this study, the positive effect of the applied bacterial product PTP PLUS on oxygen content was not documented.

A total of 116 taxa from eight divisions were documented in Bohuslavice 1 fishpond, 88 in Bohuslavice 2 and 92 in Bohuslavice 3. The most diverse group was Chlorophyta with 43.10% in B1, 48.86% in B2 and 39.13% in B3, followed by Euglenophyta 17.24% (B1), 19.32% (B2) and 19.57% (B3) and Bacillariophyta 19.83% (B1), 17% (B2) and 19.57% (B3). Cyanobacteria were registered in all three ponds, with 7 taxa (6.03%) in B1, 3 (3.41%) in B2 and 5 (5.43%) in B3, but majority of them was documented in April and July. Chlorophyta was the most diverse division during the entire sampling period, with exception of May in B1 and B3, when Bacillariophyta was the most common, and August, when Euglenophyta was the most diverse group in B3.

The greatest phytoplankton abundance was observed in July in Bohuslavice 2, while in Bohuslavice 1 and 3 the most abundant phytoplankton was documented in April, but with lower values than in Bohuslavice 2. The lowest abundance was in the first half of May in Bohuslavice 2 and 3 and in Bohuslavice 1 in July (Figure 1).

Figure 1 Change in phytoplankton abundance in studied fishponds



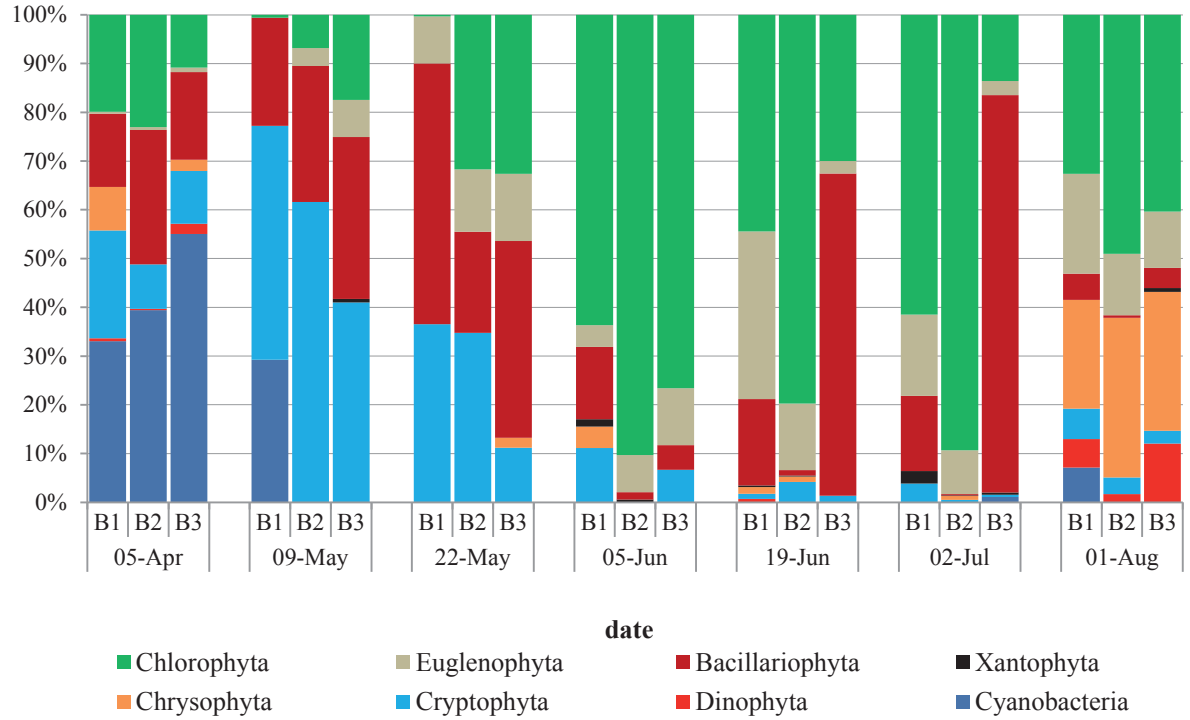
The most abundant group in April was Cyanobacteria (Figure 2), represented by *Aphanizomenon* and *Pseudanabaena*. *Aphanizomenon* was the most abundant taxon in B2 (28.69%) and B3 (51.38%), while in B1 *Cryptomonas* (22.12%) was the most numerous. In May, *Cryptomonas* and colonial diatom *Fragilaria* were the most dominant taxa in all ponds. Green algae represented mainly by *Planktosphaeria gelatinosa* and different species of Euglenophyta were also present in a significant percent in B2 and B3 during the second half of May (Figure 2).

The dominant phytoplankton organisms in all monitored fishponds from June to August were the representatives of green algae, with the exception of second half of June and July, when Bacillariophyta was the most abundant division in Bohuslavice 3 (Figure 2).

In the first half of June, *Planktosphaeria gelatinosa* was the most numerous in B1 and B2, while the representatives of genus *Monoraphidium* were the most dominant in B3. In the second half of June and in July, when in Bohuslavice 1 the lowest abundance was documented, the most dominant were genus *Trachelomonas* and common genera of green algae (*Scenedesmus, Desmodesmus*). The same organisms were also the most dominant in Bohuslavice 2 at the end of June, while in July when the abundance reached a maximum in value (Figure 1), *Tetrastrum triangulare* was the most numerous species (48.87% of the total amount of cells) in this pond. At the same period, colonial diatom *Fragilaria* was the most dominant taxon in Bohuslavice 3.

In August the green algae were still the most abundant group in each of the ponds. However, Chrysophyta and Euglenophyta also comprised a significant part of the phytoplankton community (Figure 2), with *Mallomonas* and *Trachelomonas* in B1 and B2, and *Synura, Dinobryon* and *Trachelomonas* in B3 as the main representatives.

Figure 2 Abundance of cyanobacteria and algae divisions documented in Bohuslavice 1 (B1), Bohuslavice (B2) and Bohuslavice 3 (B3) during the season from April to August 2017



In this study, the influence of bacterial product on phytoplankton community in ponds was not registered. Abundance of phytoplankton in B1 (without application) and B3 (application) over entire season was similar; while in B2 (application) was considerably higher only in July. Likewise, in some months, dominant species were same in different fishponds.

Although the highest richness of species was documented in B1, majority of them were common to all three ponds. The majority of dominant representatives tolerate a lower nutrient content, but with different light requirements (Kruk et al. 2012). Persistence of a floating cover can be the cause of low phytoplankton abundance, due to a low light intensity, which impairs photosynthesis (O'Farrell et al. 2009, Pasztaleniec and Poniewozik 2013). The abundance of phytoplankton in B1 and B3 was low during entire season. On the other side, in B2, it fluctuated throughout the season, while reaching its highest peak in July, when the duckweed cover, which was highly developed in June, was present only near the bank. *Ceratophyllum*, which was registered from June to August in B1 and B3, could also be the reason for lower abundance in these two fish ponds. It is well known that species of the genus *Ceratophyllum* can suppress the phytoplankton community through different mechanisms (van Donk 2002).

CONCLUSION

The phytoplanktonic community of monitored fishponds during most of the study period was dominated by the representatives of green algae. Cyanobacteria were the dominant division only at the beginning of season, while during the rest of the season they had been observed in a negligible number. Cryptophyta were documented during the entire period, but only in April and May in high proportions. Bacillariophyta and Euglenophyta were important members of the phytoplankton community throughout the study period. During the course of this study, the effect of the applied bacterial product PTP PLUS on phytoplankton was not recorded. The free floating plant cover impaired the development of phytoplankton community, resulting in the low abundance throughout the greater part of the season. In Bohuslavice 1 and Bohuslavice 3, the lower abundance values then in Bohuslavice 2 were additionally influenced by the presence of a submerged plant *Ceratophyllum*. However, for a more detailed analysis of the fishpond phytoplankton, data on the fishpond management, which is currently unattainable, are also required.

ACKNOWLEDGEMENTS
This study was supported by the project of Internal Grant Agency of the Faculty of AgriSciences No. IP_2017/070.

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ECOLOGICAL QUALITY OF POOLS, RIVER BRANCHES AND OXBOW LAKES OF THE MIDDLE ELBE REGION

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Abstract: Middle Elbe region is well known for its vast network of pools, river branches and oxbow lakes with preserved stands of water macrophytes. These sites are determined by richness of stands and their morphology to evaluate its ecological potential. Ecological potential of water systems of the Middle Elbe region has been monitored in seven sites, located in the Labe floodplain area between Týnec nad Labem and Káraný. The combination of Ecological quality ratio and Trophic state index was used to evaluate this potential. The monitored sites are significantly affected by the eutrophication process. This methodology has been verified to be applicable for evaluation of ecological potential of similar sites. It has been verified that this methodology is applicable to evaluation of ecological potential of similar sites. It has been found that ecological potential of examined localities displays status influenced by eutrophy. The monitored sites are characterized by the presence of valuable water and wetland plant communities. Some species of aquatic plants (like *Nymphaea alba, Potamogeton lucens* or *Scutellaria hastifolia*) have disappeared as a result of increasing trophy, advancing ecological succession and the destruction of stands.

Key Words: overgrown localities, trophic state index, ecological quality ratio, macrophytes

INTRODUCTION

Distribution of pools, river branches and oxbow lakes in the landscape is the result of modelling, geomorphological, hydraulic and hydronamic processes (Giusti and Schneider 1965). The original lotic system by the separation from the main flow becomes a closed lentic biotope with its own dynamics (Moss 2010).

This type of system becomes a specific refuge for a variety of animals and plants and create specific and unique biocenological communities. Moreover, these sites are very important in terms of preserving the diversity of species. It is therefore necessary to contribute on preserving their most natural environment and reducing or eliminating most of the anthropic impacts (Moss 2010).

The aim of this study is to find out the ecological status and quality of pools river branches and oxbow lakes in the Middle Elbe region. These habitats belong to the Czech Republic well-preserved nature areas with significant species diversity. Aquatic and wetland stand are among important components of the environment. The anthropogenic catchment is mostly minimal; the localities are loaded by the activities of anglers – these biotopes are in general managed by the Czech Anglers Union. This ultimately means the stocking of cyprinids to the assessed overgrown localities and affecting their overall ecological status. In some cases, the stocking of herbivorous fish results in a top-down effect and in the negative impact on aquatic plants (Jeppesen et al. 1997).

Another objective was to verify the available methodologies for assessing such water bodies and to set the concept that suits best the above-mentioned requirements and which yields comparable results with already published outputs.

MATERIAL AND METHODS

Characterization of localities, monitoring and methods of assessment

Examined localities are situated in the Central Bohemian region between cities Týnec nad Labem and Káraný (see Table 1). The average depth of the sites ranges between 0.7 and 2.6 meters. River branches Bajkal and Grádo are donated by water directly from River Elbe, while other sites are predominantly dependent on the amount of precipitation.

Trophic state Index (TSI) was evaluated using Carlson´s method (Carlson 1977, Walker et al. 2007). The TSI ratings are based on SD and TP equations. Transparency and total phosphorus were measured 10 times during the period 2016 – 2017.

Ecological quality ratio (EQR) was evaluated using two methods (Borovec 2013, Bund and Solimini 2007).

Coefficient of determination was calculated using maximum depth colonisation (MDC) method (Canfield et al. 1985).

Chemical data (NO_3^-, $P\text{-}PO_4$) were classified into three groups A – low quality, B – medium quality and C– high quality (Maggioni et al. 2009). These parameters were measured twice times during the period 2016– 2017.

Determination of vascular plants was based on the Key to the Flora of the Czech Republic (Kubát et al. 2002).

The nomenclature of aquatic and wetland plants was united based on the *List of vascular vegetation of the Czech Republic* (Danihelka et al. 2012).

Endangered taxons were filed based on the *Red List of vascular plants of the Czech Republic* (Grulich 2012). C1 – critically endangered species, C2 – strongly endangered species, C3 – endangered species, C4a – rarer taxons requiring further attention – known status, C4b – rarer taxon requiring further attention – unknown status. Critically endangered species (C1) and strongly endangered species (C2) are also listed for the reason of endangerment.

For classification of vegetation units the third volume of Vegetation of the Czech Republic (Chytrý et al. 2011) was used.

Table 1 Overview of localities

Locality	Altitude and Latitude	Site characteristics
Bejkovna	50° 2' 4.9404279" N, 15° 19' 44.9269867" E	Pool, 0.5 ha
Bezedná	50° 4' 54.947121" N, 15° 10' 18.0067062" E	Oxbow lake, 0.8 ha
Okrouhlík	50° 5' 25.6276941" N, 15° 10' 24.7272491" E	Oxbow lake, 0.4 ha
Grádo	50° 10' 15.608924" N, 14° 45' 8.4723473" E	River branch, 14.0 ha
Bajkal	50° 7' 3.4320522" N, 15° 9' 38.6876678" E	River branch, 2.8 ha
Václavka	50° 10' 48.5327034" N, 14° 46' 20.8340549" E	Oxbow lake, 1.7 ha
Homolka	50° 10' 47.0981396" N, 14° 46' 44.182148" E	Pool, 1.5 ha

RESULTS AND DISCUSSION

Trophic state index

Values of TSI (dimensionless value) in examined localities were situated in the range 54.3–59.9; mean ± SD; 56.9 ± 2.0 (see Figure 1).

Suggested TSI range 50–70 classifies eutrophy with decreased transparency, anoxic hypolimnia during summer, macrophyte problems evident and dominance of blue-green algae (Walker et al. 2007). Decreasing of cyprinids stocking and reduction of eutrophic load are recommended.

Ecological quality assessment

Values indicating the ecological status of the sites (see Table 2) indicate that these are nutritionally loaded systems with a middle degree trophy. The assessment of the number of macrophyte

species according to (Borovec 2013) proved the same result – to be ineffective. Therefore, this metric, which can only be implemented on lake category reservoirs, cannot be applied in this case. The indicator maximum depth colonization cd (MDC) is rather indicative classification. On the contrary, the best ranking is based on (Maggioni et al. 2009) expressed as chemical data classes (CDC), adapted to our conditions, where five sites are listed as less eutrophied and two sites as moderately eutrophied. In the summary, therefore, all of the water courses below can be identified as eutrophied (Brown and Simpson 2001).

Figure 1 Trophic state indices of examined localities (dimensionless value), 2017

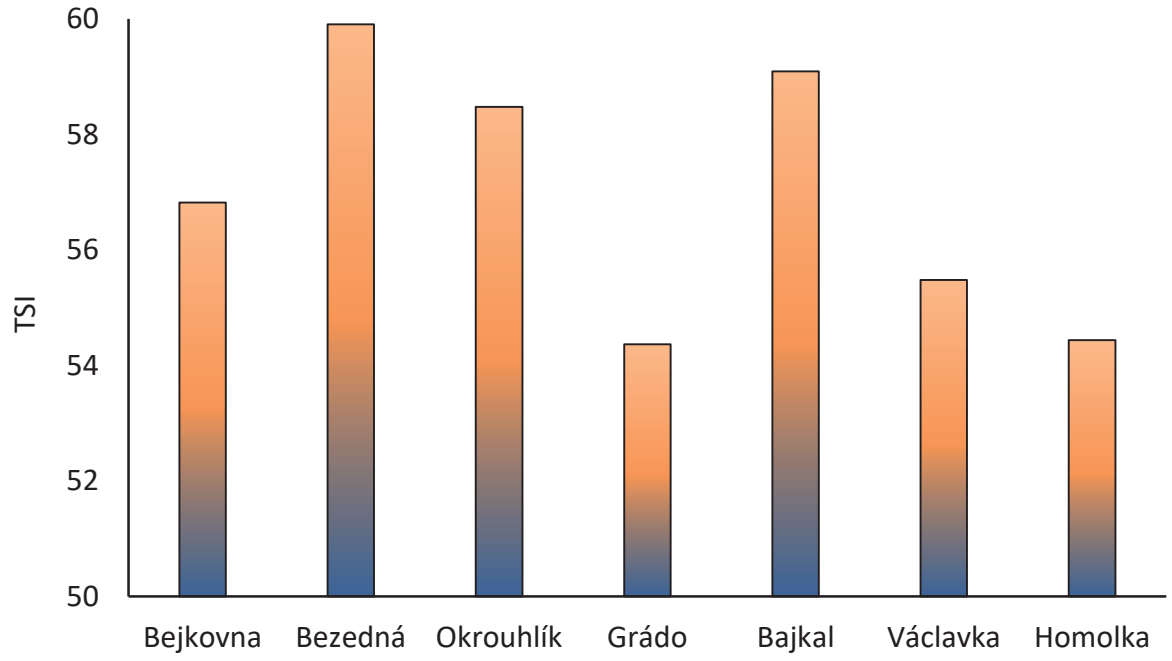

Table 2 Ecological quality assessment of localities – Ecological quality ratio; number of macrophytes species, maximum depth colonisation – coeff. of determination and chemical data classes (A – low eutrophy, B – medium eutrophy and C – high eutrophy)

Locality	EQR-NS	cd(MDC)	CDC
Bejkovna	1	0.37	A low eutrophy
Bezedná	1	0.26	B medium eutrophy
Okrouhlík	1	0.31	A low eutrophy
Grádo	1	0.46	A low eutrophy
Bajkal	1	0.29	B medium eutrophy
Václavka	1	0.42	A low eutrophy
Homolka	1	0.43	A low eutrophy

Macrophytes assessment

The Bejkovna pool is the last largest pool in the Middle Elbe region with species-rich stands of water macrophytes (see Table 3). Between years 2000 and 2002 there were recorded 23 associations of water macrophytes. During the year the level of the water column fluctuates strongly, in dry years there is only a small area with sufficient depth for fish survival. This type of vegetation was destroyed by intensive breeding of fish and water poultry in the Middle Elbe in most areas (Rydlo 2002). The locality is currently part of SCI (Site of Community Importance) Lžovické tůně. At the sampling point, rich stands of associations of *Hottonietum palustris*, *Utricularietum australis*

and *Nymphaeo albae – Nupharetum luteae*. The *Scirpetum lacustris* association with the dominant *Schoenoplectus lacustris* is also represented. In the loose reed beds critically threatened *Ranunculus lingua* (C1r) is represented with high coverage, there is also an endangered *Sium latifolium* (C2b).

Table 3 Aquatic and wetland communities at the sampling points (+ plant community is present, - plant community is absent), A – Hydrocharitetum morsus – ranae, B – Ceratophylletum demersi, C – Hottonietum palustris, D – Utricularietum australis, E – Nymphaeo albae-Nupharetum luteae, F – Nymphaeetum candidae, G – Lemnion minoris, H – Phragmitetum communis, I – Glycerietum fluitantis

Locality/Taxon	A	B	C	D	E	F	G	H	I	number of species
Bejkovna	-	-	+	+	+	-	-	-	-	46
Bezedná	-	-	-	-	+	-	+	+	-	35
Okrouhlík	+	-	-	-	+	-	-	+	-	32
Grádo	-	-	-	-	+	-	-	+	-	42
Bajkal	+	-	-	-	+	+	-	+	+	40
Václavka	-	-	-	-	+	-	-	+	-	39
Homolka	-	+	-	-	-	-	+	+	-	28

The association of *Hydrocharitetum morsus-ranae* in the Czech Republic is vegetation strongly decaying from our wetlands, mainly due to the destruction of alluvial waters, eutrophication and the exposure of habitats to severe disturbances, eg. by waterbirds (Chytrý et al. 2011). The Okrouhlík locality with vegetation of the Common Frogbit is part of the Tonice and Bezedná nature reserve. Rydlo in 1989 reported rich occurrence of *Nymphaea alba* (Rydlo 1991). The subject of protection is, among other things, the protection of the pool system with the occurrence of White Water-lily. To the SCI, together with Bezedná pool and Bajkal branch, also belong Libické luhy where one of the subjects of protection is Natural eutrophic lakes habitat with *Magnopotamion* or *Hydrocharition* - vegetation type. At the sampling point, the association of *Hydrocharitetum morsus-ranae* is present. Unfortunately, this valuable vegetation is threatened by the increase of the trophy, the related process of succession and the breeding of semi-wild ducks.

Growths of critically endangered White Water-lily (*Nymphaea alba*) have grown on the site of Bezedna in the past, at present this vegetation has not been found in the pool, *Nuphar lutea* vegetation prevails here nowadays. The white water lily vegetation in this complex of pools was strongly retreating already in the nineties (Rydlo 1991). Rare species of *Apiaceae* such as *Sium latifolium* and *Berula erecta* grow in locally loose reed beds, with vulnerable *Silene baccifera* growing on the edges of the stands.

The Bajkal river branch in the Libický luh, which flows into the River Elbe, originated in the regulation of the flow. Due to the connection with Elbe the dispersion of diaspores of water and aquatic plants is facilliated. In the 1970s, for example, *Nymphaea candida* appeared (Rydlo 2008), stands of this species are still locally present. In the history of botanical research 45 species of water macrophytes and species of exposed bottoms were recorded there (Rydlo 2008). At present, the edges of the pool are overgrown with representative vegetation with the Common Frogbit, which is an endangered species in the Czech Republic (C2b), there is also endangered *Cicuta virosa* (C2b), vulnerable *Lemna trisulca* (C3) or *Leersia oryzoides* (C3). The connection with Elbe has positive influence on the development of aquatic and wetland vegetation and the pool is still one of the species-rich localities in the Middle Elbe.

The Grádo river branch at the sampling site is overgrown by association of *Nymphaeo albae – Nupharetum lutae* with dominant *Nuphar lutea*, the association of the *Phragmition communis* is directly associated with this vegetation. In loose reed beds grow endangered *Cicuta virosa* (C2b), endangered *Sium latifolium* (C2b), with vulnerable *Silene baccifera* (C3) on edges of stands. In the sand grasslands there is critically threatened *Hierochloë odorata* (C1b) and endangered species of *Plantago arenaria* (C2b). The locality is partially a part of SCI Káraný - Hrbáčkovy tůně where one of the subjects

of protection is a habitat Natural eutrophic lakes with *Magnopotamion* or *Hydrocharition* – vegetation type. In this part of the nature reserve grows vulnerable Common Bogbean (*Menyanthes trifoliata*).

The location of Václavka is a sunny oxbow lake, dominated by vegetation of *Nymphaeo albae – Nupharetum luteae* association with *Nuphar lutea* and association of *Phragmition communis*. At the sampling site there also occurs vulnerable *Lemna trisulca* (C3) there. An endangered species of *Sium latifolium* (C2), near-threatened *Schoenoplectus lacustris* (C4a) was found in the reeds, poplars, several individuals of endangered *Epipactis albensis* (C2b) orchid were recorded. The site is a part of SCI Káraný – Hrbáčkovy tůně.

The Homolka site is a shaded pool where stands of *Ceratophyllum demersi* association dominate the site, there are also scattered lemnids, such as *Lemna trisulca* (C3) and *Lemna minor*. More valuable aquatic macrophytes are no longer present in the pool.

CONCLUSION

The ecological quality of the studied localities was slightly variable due to the eutrophication load found there. Although the localities were elected so that the anthropogenic effect was as low as possible, some significance of this element could not be excluded. When evaluating individual indicators, it emerged that in the Czech Republic there is no compact methodology that would be able to comprehensively evaluate similar types of sites and would be simply feasible in practice. For this reason, foreign methods were tested in their combination. Recommendations to maintain at least the current state of the shoulders and pools are to change the management of the sites and bring them back to their early stages of succession. Management of well-developed communities of *Hydrocharitetum morsus-ranae*, *Hottonietum palustris*, *Utricularietum australis* is not needed, it´s important to consistently protect habitats from destruction, sometimes is necessary to gently remove a part of the sediment to maintain the seed bank at the bottom of the water habitats. This conservation management should be carried out over a period of several decades. Associations of *Nymphaeo albae – Nupharetum lutae* and *Nymphaeetum candidae* require restoration of the flow of river branches and pools. From the point of view of the development of aquatic macrophyte communities we can state that the monitored sites are still valuable places with many rare species of aquatic and wetland plants. Some species of aquatic plants like *Nymphaea alba*, *Potamogeton lucens* or *Scutellaria hastifolia* have disappeared as a result of increasing trophies, advancing ecological succession and the destruction of stands.

ACKNOWLEDGEMENTS

The research was financially supported by the Specific Research Projects No. 2116/2016 and 2117/2016 from the University of Hradec Králové (PrFUHK). We would like to thank Romana Prausová and Kateřina Zelenková for methodological help and Michaela Luňáková for English language correction.

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AGROECOLOGY AND RURAL DEVELOPMENT

URBAN AND PERI-URBAN FORESTRY IN THE FACE OF CLIMATE CHANGE IN CAMEROON: CHALLENGES AND NEW PERSPECTIVES FOR SUSTAINABILITY

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Abstract: This study assessed the status of urban and peri-urban forestry, evaluated the effect of population growth on urban forests landscape and designed innovative strategies that will ensure sustainability and improvement of urban living environment. It was investigated through secondary data, field investigation, enquiries to relevant stakeholders, direct assessment and observations of urban and peri-urban forest landscapes in Cameroon. These analyses highlight the extreme diversity of environmental resources from urban forests in Cameroon. Rapid urban population growth, limited land area, and poor implementation of government policies are some factors affecting urban forests development and are responsible for vicious cycle of environmental degradation in urban areas in Cameroon. The results contribute elements for strategic and operational planning. Urban forestry management is an important strategy to improve urban living and working environments. There is a need to highlight successful strategies and actions concerning the management of urban and peri-urban forests. Involvement of all stakeholders and users in reflections and in the implementation of policies concerning the management of the urban and peri-urban forests should be adopted and advocated, to ensure sustainable development and to ensure that cities, trees and forests grow together to meet the needs of urbanized societies.

Key Words: urban forestry, sustainable development, urbanization, environmental degradation, management strategies, Cameroon

INTRODUCTION

Urban forestry is a relatively new, multidisciplinary approach in international forest research. Rapid urbanization and climate change raise several issues for those who are responsible for developing policies and making decisions at local, national and international levels. Urban trees provide a significant contribution to building resilient cities and improving health and well-being, such as through mitigating natural disasters, providing ecosystem services, reducing energy costs or increasing property values. However, urban and peri-urban forest ecosystems in Cameroon are steadily increasingly under pressure from their city populations.

Urban forests can be defined as networks or systems comprising all woodlands, groups of trees, and individual trees located in urban and peri-urban areas; they include, therefore, forests, street trees, trees in parks and gardens, and trees in derelict corners. Urban forests are the backbone of the green infrastructure, bridging rural and urban areas and ameliorating a city's environmental footprint (Salbitano et al. 2016).

Global urbanization and climate change raise several issues for those who are responsible for developing policies and making decisions at local, national and international levels. At the beginning of the twenty-first century, the city/forest relationship is a major issue for sustainable land management in many countries, particularly in Cameroon. Urban and peri-urban forests provide a range of products and services both locally and globally. There is increasingly strong evidence and a growing consensus that trees, woods and forests have a key role to play in climate change mitigation and adaptation, and in delivery of ecosystem services in both rural and urban areas (Atkinson and Townsend 2011). Urban and peri-urban trees play a key role and provide a significant

contribution to building resilient cities and improving health and well-being, such as through mitigating natural disasters, providing ecosystem services, reducing energy costs or increasing property values. Soil protection and management, erosion control or siltation control, space structuring, recreation and/or leisure, water quality, treatment of wastewaters and bioremediation, biodiversity and ecosystem conservation, agriculture and livestock production, non-wood forest products are some examples of environmental goods and services (FAO 2012). At the same time forests, woods and trees face unprecedented challenges. Climate change, pollution, globalization, increasing competition for natural resources and pressure on land-use, financial and economic crises and constraints all have serious implications (Atkinson and Townsend 2011). The environmental impacts of forest degradation and deforestation, can become critical and effect climate change, biodiversity, desertification and/or carbon-related processes. These impacts are found at all levels of spatial integration from the single plot to the global arena.

Cameroon's forests, which cover about 60 percent of the country, play a vital role for people and the economy. According to the FAO, forest cover in Cameroon is around 20 million hectares. They provide services and sustenance directly and indirectly to local communities and city dwellers alike. Yet, until recently, Cameroon lacked a comprehensive information system to actually monitor and manage its forests. There was no integrated system or entity tracking the various forest uses, like logging concessions, community forests, hunting zones, and more. The information that was available was scattered amongst different institutions, wasn't publicly accessible, or was of a quality insufficient to support legality claims and effective land use decisions. This lack of information exacerbated the unsustainable use of forest resources and sparked conflicts between competing forest stakeholders, such as loggers and community groups (Tessa 2002).

The dynamics of growth, availability and management of the timber resource are often unknown; especially since suburban areas are frequently without the most basic management tools. In many cases, the management of timber resources is only one aspect of land management and therefore the management of wood as it relates to energy is often disrupted by other external considerations (Marien 2008). Nowadays, many governmental efforts have mainly focused on forest management in concessions and protected areas. Urban forestry is one of the promising strategies to address the multifaceted problems associated with urbanization. Urban forestry addresses "*the land in and around areas of intensive human influence, ranging from small communities to dense urban centers, that is occupied or potentially occupied by trees and associated natural resources*" (Strom 2000). Urban forestry is a relatively new, multidisciplinary approach in international forest research. It has been defined as the art, science, and technology of managing trees and forest resources in and around urban community ecosystems for the physiological, sociological, economic and aesthetic benefits trees provide society (Helms 1998). The basic challenge for urban forestry is to develop and maintain a sustainable urban forest resource that meets multiple societal and personal needs (Fuwape and Onyekwelu 2010).

The objective of this paper is to provide an overriding policy advice and some good practices in order to improve policy development and decision-making to optimize the contributions of trees and forests to cities.

MATERIAL AND METHODS

The development of urban forestry in Cameroon was investigated through the analysis of secondary data derived from literature, field investigation (enquiries to relevant stakeholders, on-the spot assessment), and inventory of urban and peri-urban facilities in the country. A combination of qualitative and quantitative research methods was then adopted. Qualitative research technique employed was content analysis of relevant official and policy documents, added to in-depth interviews, which let respondents to express themselves openly. This brought out of a comprehensive view and insights into the formulation of a problem. The results of the analysis were used to develop protocols for interview and questionnaire surveys, conducted in order to determine the benefit and problem of urban forestry. The influence of population growth and urbanization on urban forests were also assessed.

RESULTS AND DISCUSSION

The major types of urban forestry in Cameroon include:

(i) semi-private space like green space in residential and industrial areas
(ii) designated parks, street trees and roadside plantations
(iii) public green areas like green parks, botanical gardens, recreational gardens
(iv) public and private tree plantations on vacant lots, green belts, woodlands and peri urban tree plantations
(v) rangeland, and forests close to urban areas
(vi) natural forest under urban influence, such as nature reserves, national parks, forests for eco-tourism
(vii) trees planted for environmental protection such as wind break, watersheds protection, etc.

Urban forest trees species and their characteristics

The types of major tree species planted in urban centers for landscape enhancement, environmental protection, provision of timber and non-timber forest products and other benefit varied with ecological zones and cultural values of the people: *Adansonia digitata, Azadirachta indica, Terminalia catapa, Gmelina arborea, Tectona grandis, Delonix regia, Khaya senegalensis, Cassia siamea, Terminalia mantaly, Balanites aegyptiaca, Ziziphus spinachrisiti, Ficus gnaphalocarpa, Eucalyptus* species *(Eucalyptus camaldulensis), Acacia* species, *Rhizophora* species, *Avicennia* species, different species of palm, ornamental palms and coconut trees, etc.

Benefits

Urban forest resources in Cameroon can play active roles in providing goods and services to alleviate poverty, improve livelihoods, and enhance the wellbeing of inhabitants. Urban forestry practices such as gardens and parks, peri-urban agroforests, botanical gardens and protected zones play vital role in nature conservation. Incorporating trees in urban landscape improves biological conservation and biodiversity. The benefits of the different elements of urban forestry is enhanced by policies and legislation, strategic planning and management, urban forestry practice, financial mechanisms and operational maintenance, ownership and access, key actors and stakeholders, education and capacity (Åkerlund et al. 2006).

In addition to providing essential goods and services, current urban forestry practices are services and amenity oriented. Urban green spaces with trees as major component play important roles for healthy, liveable and sustainable cities. Trees and green spaces help keep cities cool, act as natural filters and noise absorbers, improve microclimates, conserve biodiversity, protect and improve the quality of natural resources, including soil, water, vegetation and wildlife. Trees contribute significantly to the aesthetic appeal of cities, thereby helping to maintain the psychological health of their inhabitants. Consequently, urban forestry management is an important strategy to improve urban living and working environments (Fuwape and Onyekwelu 2010).

Factors affecting urban forests development in Cameroon

According to the FAO (2006), the annual average total deforestation rate in Cameroon, including urban and peri-urban deforestation for the 1980–1995 period was 0.6%. The rate reportedly rose to 0.9% for the 1990–2000 period and reached 1% between 2000 and 2005 of the total forest cover estimated at 22.5 million ha, or 48% of the national territory (de Wasseige et al. 2009). There is deforestation for pressure on forest resources because of high demand for usable wood, energy and other non-timber forest products.

The following direct and indirect causes of deforestation and forest degradation, in order of importance are underlying as: agriculture, illegal timber offtake, firewood, industrial logging, mining, population growth, construction of roads, bushfires, an inheritance system that can lead to land fragmentation, and forest cover degradation (MINEP 1998). Wood as a source of energy is listed amongst the direct causes of deforestation. Peri-urban forests play a key role in providing

firewood and charcoal. Fuelwood and charcoal cover the largest market for forest products, especially in terms of volume of felled trees (Essama-Nssah and Gockowski 2000). The extent of forest degradation is important near peri-urban areas as a result of wood offtake for energy (Ndoye and Kaimowitz 2000), as each urban household spends an average US$ 55–59 per year on fuelwood, which means that cumulatively, the 1.3 million urban households in Cameroon spend US$ 65–70 million on wood each year (Topa et al. 2009). This type of wood consumption is pinpointing the link between population growth (particularly in urban areas) and deforestation. A recent global study found that forest losses are greatest in areas with accelerated urbanisation, where the per capita trade in agricultural products is the highest (DeFries et al. 2010).

The Rapid urbanization and population growth

Uncontrolled and rapid urbanization on a limited land area has impacted the country's urban and peri-urban forest to varying degrees and has radically transformed the social context for urban people. Rapid urbanization (occasioned by demographic switch from rural to urban society and/or growing urban population) is outstripping the planning and carrying capacity of municipal authorities in Cameroon. All urban and peri-urban forest ecosystems are-to varying but steadily increasing degrees-under pressure from their city populations. According to the Central Intelligence Agency–CIA (2014), the urban population rate is 52.1% of total population (2011) and the rate of urbanization is 3.23% annual rate of change (2010–15 est.). Over the past five decades, population growth in Cameroon has more than quadrupled. Population growth rate is 2.6% (2014 est.) (IndexMundi 2014). Population growth is much higher in urban settlements than in rural ones. The rural exodus and a largely uncontrolled demographic situation, together with often inadequate governance, are leading to the chaotic development of sprawling towns in which urban poverty is rife and getting worse. There has been no adaptation in terms of infrastructure and energy consumption patterns to cope with this sharp rise in urban population. The current trend of urbanization in Cameroon, coupled with the growing urban population, is redefining urban forestry practices in the country and has presented new challenges and opportunities.

Other challenges of urbanization include: shortage of land, provision of food, energy and wood for construction, deteriorating air quality, higher air temperatures, increased noise levels, greater psychological stress, etc. (Fuwape and Onyekwelu 2010).

Land degradation

Peri-forest degradation affects not only the largest cities (e.g., Yaounde, Douala) but also all major cities and towns in areas dominated by savannah. Small cities and village populations in forest areas are barely affected. Uncontrolled collection of resources often exceeds the recovery potential of forest stands especially given the short distance from peri-urban forests to urban markets. In the absence of strategies and management plans, these facts translate into the degradation of natural woodlands, forests, parks, trees and shrub savannahs. This degradation of wooded ecosystems can lead to deforestation, with well-documented and disastrous ecological, economic and social consequences.

Poor implementation of government policies

Most cities have master plans to govern and cope with heavy population pressure, their implementations have been poor (poor governance, inadequate governance, absence of appropriate strategies and management plans).

Often, little is known about the growth dynamics, availability and management of wood resources, especially inasmuch as the peri-urban zones concerned are often left out of official forest policies and strategies, and tend to lack even the most cursory management tools.

The new strategies for spatial development planning adopted by the government are characterized by several weaknesses, such as spontaneous land privatization and inconsistent land reform, insufficient information on land-use and ownership. Strategies also reflected imperfect and incomprehensive legislative frameworks regulating spatial development planning, undivided competencies in matters of land regulation, weak coordination of activities and lack of cooperation among agencies.

Some generic recommendations also applicable to sustainable management of peri-urban woodland and natural forests in Cameroon: (FAO 2012):

- definition of appropriate criteria and indicators to analyse the evolution of urban and peri-urban forestry with regard to the main causes of the degradation of wooded ecosystems (urbanization, energy, agriculture, livestock, infrastructure etc.);
- practical, transparent application of national laws and regulations, if need be helping to boost existing arrangements and make them more appropriate to the current situation;
- improvement in the knowledge of forest dynamics, particularly in the case of natural stands suffering urban pressure, and promotion of appropriate management plans;
- involvement of all stakeholders and users in reflections and in the implementation of policies concerning the management of the urban and peri-urban forests;
- promotion of private plantations dedicated to supplying wood fuel on the outskirts of towns, by securing land tenure, clarifying the taxation system and strengthening infrastructures;
- boosting of and support for local strategies for sustainable management of the resource, formalization of value chains and improvement in the energy performance of local equipment and techniques;
- carrying out of life-cycle assessments of the various urban energy value chains in order to compare the impacts of the various energy sources, especially the position of wood vis-à-vis alternative fossil fuels;
- highlighting of successful strategies and actions concerning the management of urban and peri-urban forests, and improving the policy development and decision-making to optimize the contributions of trees and forests to cities;
- development of systems of payment for the environmental services of urban and peri-urban forests, especially global services (carbon, biodiversity etc.), ensuring that local people are involved in this dynamic;
- the planting of trees, the use of multi-purpose tree species;
- Associate the management of peri-urban wood resources with local communities;
- Community and traditional management approaches.

CONCLUSION

Clearly understood national and local policies, or even well-organized profitable private initiatives, can act as a catalyst for the development of active peri-urban forestry. Urban forests are essential for healthy environment.

Urban and peri-urban forestry should be highly promoted for (eco)tourism. The Ministry of Forestry and Wildlife is supporting the reforestation campaign with councils and groups empowered to plant trees all over the country. Councils have been challenged to implement the concept of urban forestry and enhance the sustainability vision of the Ministry of Forestry and Wildlife with the aim to reduce by 32 per cent greenhouse gas emissions on the strength of the commitment by Heads of State at the COP 21 Summit in Paris, France in 2015.

This research provided policy advice and highlighted some good practices in order to improve policy development and decision-making to optimize the contributions of trees and forests to cities.

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IMPORTANCE OF ISOLATED FOREST COMPLEXES FOR STABLE POPULATIONS OF SMALL TERRESTRIAL MAMMALS IN LOWLANDS OF SOUTH MORAVIA (THE CZECH REPUBLIC)

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Abstract: The relative abundance and diversity of small terrestrial mammals (Rodentia, Soricidae) were evaluated in lowlands of south Moravia. Three larger isolated forest complexes (> 50 ha) in intensively managed landscape were assessed: seminatural forest, production forest and pheasantry. Animals were captured using snap-traps. A total of 5426 individuals belonging to 12 species were trapped. The most abundant and most dominant species were *Apodemus flavicollis*, *A. sylvaticus* and *Clethrionomys glareolus*, while other species were present in much lower numbers. The highest relative abundance (n = 2696, rA = 11.102), diversity (H' = 1.21) and number of species (n = 12) were found in pheasantry. The lowest abundance (n = 1202, rA = 7.705) and diversity (H' = 1.04) were in seminatural forest. The lowest number of species occurred in production forest (n = 6). The differences between localities were not statistically significant ($p > 0.05$). Larger isolated forest complexes are suitable for long-term stable populations of dominant species only (*Apodemus* spp., *Clethrionomys glareolus*).

Key Words: shrews, Soricidae, rodents, Rodentia, small mammal community, agricultural landscape

INTRODUCTION

Isolated forest complexes are very significant refuges for communities of small terrestrial mammals in intensively managed landscape. A number of authors underline the importance of forests for maintenance of species richness in agricultural landscape (Dudich and Štollmann 1983, Pelikán 1989, Trnka et al. 1990, Ylonen et al. 1991, Suchomel and Heroldová 2004, Suchomel et al. 2012). They mostly evaluated a small fragments of forest (biocorridors, windbreaks, shelterbelts, woodlots etc.) but some of the contributions are concerned on the large lowland forests (Zejda and Pelikán 1969, Zejda 1976, Suchomel and Heroldová 2004, Suchomel et al. 2012).

This study concentrates only on large isolated complexes of forests. It is compared abundance and diversity of small terrestrial mammals (Rodentia, Soricidae) inhabiting three isolated forest complexes in lowlands of south Moravia. These localities differ their area, variability of biotope, tree composition and forest management.

MATERIAL AND METHODS

The study used data from surveys of small terrestrial mammal communities from the years between 2002 and 2012. This material was obtained from three larger forest complexes, isolated within the intensively managed landscape of South Moravia (The Czech Republic). The study sites are defined groups of forest types and characterized by different degrees of intensity to which they are exploited by people (Suchomel and Heroldová 2004).

The study sites belong to the climatic region T4 (W4), according to E. Quitt. The climatic region is characterized by a very long, a very warm and a very dry summer, the transition period is very short with a warm spring and a warm autumn, the winter is short, warm, dry to very dry, with a very short duration of snow cover (40–50 days). The average air temperature in January is from -2 to -3 °C,

in July 19–20 °C and in April and October 9–10 °C. The total precipitation in the growing season reaches 300–350 mm and in the winter period 200–300 mm. (Květoň and Voženílek 2011)

„Seminatural forest" (120 ha; Horní les; GPS: 48°48'40.904"N, 16°47'16.066"E)

First locality is situated near the town of Lednice na Moravě. This is a seminatural forest cover without forestry intervention, characterized by a group of forest types *Ulmeto-Fraxinetum carpineum.* The dominant species are common ash (*Fraxinus excelsior*), English oak (*Quercus robur*), black poplar (*Populus nigra*), *large-leaved linden* (*Tilia platyphyllos*), and common maple (*Acer campestre*).

„Production forest" (60 ha; Hájek; GPS: 48°57'28.004"N, 16°35'36.323"E)

Second locality is situated near the village of Vranovice. This is a typical production forest, characterized by a group of forest types *Carpineto-Quercetum acerosum.* The dominant woody species are English oak (*Quercus robur*), durmast oak (*Q. petraea*), and black locust (*Robinia pseudoacacia*).

„Pheasantry" (280 ha; Rumunská bažantnice; GPS: 49°2'9.528"N, 16°42'5.675"E)

Thirds locality is situated near the town of Židlochovice. This is made use of as an intensive pheasantry. These location is the most variable area of the three, with regard to microhabitats. It includes a number of miscellaneous woody species of various age categories as well as small open areas, such as small fields, meadows and wetlands. The dominant woody species in this location are English oak (*Quercus robur*), durmast oak (*Q. petraea*), Scotch pine (*Pinus sylvestris*), common spruce (*Picea abies*), and black poplar (*Populus nigra*). The following groups of forest types were identified here: *Ulmeto-Fraxinetum carpineum, Saliceto-Alnetum* a *Carpineto-Quercetum acerosum.*

In all trial plots, trapping of small mammals was realized at even intervals, five times a year in the years between 2002 and 2012. The animals were catched by snap traps or combination of snap traps and pitfall traps that were arranged in the shape of the letter Y (Pelikán 1967, Suchomel and Heroldová 2004). The traps were laid in lines of twenty and were about 5 metres apart. The trap systems in the shape of the letter Y included 10 pitfall traps witch were placed in the ground approximately 5 m apart, always three in each arm and one where the arms converge. Usual 2-litre plastic bottles with cut-off bottlenecks were applied for the pitfall traps and a snap trap was laid along each of these traps. Solid plastic foil was stretched along the traps for tend the small mammals right into the traps. Oil lamp wicks were used as baits. They were fried in pork fat or smeared with peanut butter. Traps were left in place for four days (i.e. 3 nights) and checked each morning (Pelikán 1967). Captured individuals were dissected in the lab. They were determined according to the species, body length, body weight, sex, and sex condition (Suchomel and Heroldová 2004). 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), species diversity (H') and equitability (E) (Magurran 1988).

Due to different trapping methodologies (different numbers of traps) at individual locations, it was necessary to determine the relative abundance of small mammal species (rA). This is expressed by the equation: rA = 100*n/P (n – number of individuals trapped; P – number of trap nights). Table 1 shows the total number of trap-nights in individual location. The abundance obtained in this way reflects the absolute abundance (Pelikán 1967).

Relative abundance and diversity among habitats were statistically evaluated using one-way ANOVA. These calculations were performed using Statistica 12 CZ.

RESULTS AND DISCUSSION

A total of 5426 individuals of twelve small terrestrial mammal species were trapped in all study sites within the period of ten years. The most abundant species was *Apodemus flavicollis* (n = 2956, rA = 5.218, D = 54.37%), followed by *Clethrionomys glareolus* (n = 1342; rA = 2.420, D = 26.67%) and *Apodemus sylvaticus* (n = 810, rA = 1.362, D = 13.57%), all of these three species were eudominant (D > 10%). *Microtus arvalis* (n = 274; rA = 0.462; D = 4.64%) was subdominant (D = 2–5%). *Apodemus microps* (n = 12, rA = 0.017, D = 0.16%), *Micromys minutus* (n = 3, rA = 0.006, D = 0.07%), *Microtus subterraneus* (n = 8, rA = 0.013, D = 0.13%), *Mus musculus* (n = 2, rA = 0.004, D = 0.04%), *Crocidura leucodon* (n = 4, rA = 0.005, D = 0.05%), *Crocidura suaveolens* (n = 3, rA = 0.004,

D = 0.04%), *Sorex araneus* (n = 10, rA = 0.019, D = 0.23%) and *Sorex minutus* (n = 2, rA = 0.004, D = 0.04%) were the least abundant species and all were subrecedent (D < 1%). Table 1 shows the values of dominance and relative abundance at each of the locations.

Table 1 Values of dominance (D), relative abundance (rA), diversity (H') and evenness (E) of individual mammals species determined in trial plots

Species	„Seminatural forest“		„Production forest“		„Pheasantry“	
	D[%]	rA [%]	D [%]	rA [%]	D [%]	rA [%]
Apodemus flavicollis	48.75	3.756	61.06	5.981	53.30	5.917
Apodemus microps	-	-	0.07	0.006	0.41	0.045
Apodemus sylvaticus	6.41	0.494	16.43	1.609	17.88	1.985
Clethrionomys glareolus	41.26	3.179	17.02	1.667	21.74	2.413
Micromys minutus	0.17	0.013	-	-	0.04	0.004
Microtus arvalis	2.66	0.205	5.24	0.513	6.01	0.667
Microtus subterraneus	-	-	0.20	0.019	0.19	0.021
Mus musculus	0.08	0.006	-	-	0.04	0.004
Crocidura leucodon	-	-	-	-	0.15	0.016
Crocidura suaveolens	-	-	-	-	0.11	0.012
Sorex araneus	0.58	0.045	-	-	0.11	0.012
Sorex minutus	0.08	0.006	-	-	0.04	0.004
Number of individuals	1202		1528		2696	
Number of species	8		6		12	
Number of trap-nights (NTP)	15600		15600		24285	
H'	1.04		1.07		1.21	
E	0.50		0.60		0.49	

Apodemus flavicollis was the most abundant species in all monitored habitats. The highest number of captured individuals was in pheasantry (n = 1437, rA = 5.917), but the highest number of relative abundance achieved in production forest (n = 933, rA = 5.981). It was least common in seminatural forest (n = 586, rA = 3.756). *Clethrionomys glareolus* was eudominant species together with *Apodemus flavicollis* in all sites. These species seemed to be the most adaptable. The most individuals of *Clethrionomys glareolus* (n = 583, rA = 2.413) were captured in pheasantry and the most relative abundance (n = 496, rA = 3.179) achieved in seminatural forest. It was least common in production forest (n = 260, rA = 1.667). Important species was also *Apodemus sylvaticus*, but only a dominant species (6.41% of occurrence) in the location seminatural forest. It was most common in pheasantry (n = 482, rA = 1.985) and least common in seminatural forest (n = 77, rA = 0.494). Table 1 indicates that other species were significantly less common.

Populations of shrew in all sites (p = 0.000), were significantly less common, and in comparison with some species of rodents occurred only marginally or occasionally. In production forest the shrews were not found at all. The most dominant species of shrews was *Sorex araneus* (D = 0.23%). Even though shrews, mainly *Sorex araneus*, may in some forest habitats achieve dominant values in the communities of small mammals (Suchomel at al. 2014), their abundance in forest, in comparison with 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). Despite the low proportion of shrews 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 point of view, mountain forests are then even more important refuges, especially in terms of their abundance (Suchomel at al. 2014, Dokulilová and Suchomel 2016, 2017).

Comparison of the relative abundance of the individual species within a growing season shows that the most individuals were trapped in summer and in autumn (Table 2).

Table 2 Comparison of relative abundance values (rA) of small mammals on particular plots at different stages of vegetation season

Species	„Seminatural forest“			„Production forest“			„Pheasantry“		
rA [%]	spring	summer	autumn	spring	summer	autumn	spring	summer	autumn
Apodemus flavicollis	1.006	6.172	4.654	3.223	8.152	7.358	3.104	8.024	7.315
Apodemus microps	-	-	-	0.016	-	-	0.031	0.055	0.054
Apodemus sylvaticus	0.220	0.578	0.881	1.195	1.914	1.855	1.313	1.839	3.387
Clethrionomys glareolus	1.226	5.165	3.302	0.660	2.492	2.107	0.719	3.864	2.955
Micromys minutus	-	0.017	0.031	-	-	-	0.010	-	-
Microtus arvalis	0.079	0.347	0.189	0.079	0.776	0.881	0.156	1.226	0.631
Microtus subterraneus	-	-	-	-	0.050	-	-	0.044	0.018
Mus musculus	-	0.017	-	-	-	-	-	-	0.018
Crocidura leucodon	-	-	-	-	-	-	0.010	-	0.054
Crocidura suaveolens	-	-	-	-	-	-	-	0.022	0.018
Sorex araneus	0.016	0.050	0.094	-	-	-	-	0.033	-
Sorex minutus	-	-	0.031	-	-	-	-	-	0.018

The richest habitat in terms of species was pheasantry with 12 species, while the poorest habitat for species was production forest with 6 species. The highest total diversity of small mammals was identified in pheasantry (H' = 1.21), followed the location production forest (H' = 1.07). The lowest diversity was determined in location seminatural forest (H' = 1.04). The most balanced community was identified in production forest (E = 0.60), followed the location seminatural forest (E = 0.50) and the lowest value was detected in pheasantry (E = 0.49). Overall, in terms of diversity and abundance of population, pheasantry and production forest appear richer than seminatural forest (Table 1). The location pheasantry (the largest study area 280 ha) is made use of as an intensive pheasantry. It is the most variable area of the three. It is mosaic of mostly forest biotopes, but also small open areas, such as small fields, meadows and wetlands while location seminatural forest (120 ha) is seminatural forest cover without forestry intervention. The location production forest (the least study area 60 ha) is a typical production forest. It seems, the area of study sites has not influence on abundance of small mammals.

The comparison of relative abundances ($p = 0.428$) and diversity ($p = 0.964$) among forest complexes (seminatural forest, production forest, pheasantry) were not significantly different ($p > 0.05$). It seems the methods of management are not so significant for existence of small mammals in lowland forest, if these methods offer a suitability conditions for them. In spite of the localities pheasantry and production forest seems to be preferable (the higher number of abundance and diversity were founded here), maybe because of the much more variability due to intensively forest management. The locality seminatural forest, where the forest management is not here, it seems to be not so preferable (lower number of abundance and diversity were founded here).

The larger isolated forest complexes are important refuges in the agricultural landscape of South Moravia, in spite of the comparing with small and fragment forest, which are of ecotone type, they achieve less diversity (Pelikán 1986, Trnka et al. 1990, Suchomel and Heroldová 2004). On other hand, stable populations of dominant species (*Apodemus flavicollis, Clethrionomys glareolus*) in isolated forests are in conflict with forest management (Heroldová et al. 2012).

CONCLUSION

This study highlights the importance of isolated lowland forest complexes for small terrestrial mammal communities in south Moravia. The highest abundance and diversity were found in pheasantry but the comparison of relative abundances and diversity among study sites were not significantly different ($p > 0.05$). It seems the methods of management are not so significant for existence of small

mammals in lowland forest, if these methods offer a suitability conditions for them. In spite of, the results of abundance and diversity shows that localities of pheasantry and production forest appear richer than seminatural forest. It can be due to intensively forest management in these localities, thanks to they are more variable. Larger isolated forest complexes are suitable for long-term stable populations of dominant species only (*Apodemus flavicollis, A. sylvaticus, Clethrionomys glareolus*).

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PERCEPTION SOCIAL FARMING IN CZECH REPUBLIC AND GREAT BRITAIN

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Abstract: This work deals with the current state of a social farming in the Czech Republic. It explains and defines this concept, theoretical bases, specifications and sources of funding, moreover, it ponders over the benefits for rural development and local communities. The work includes an analysis of the current state of social farming in the Czech Republic and Great Britain and, a SWOT analysis of the Czech and British care farms. The subsequent comparison results in many similarities such as the perceived strength, the benefit to society, or the common feature of the lack of salary valuation of workers who work with people with a health or social disadvantage.

Key Words: Social farming, care farming, green care, Czech Republic, United Kingdom

INTRODUCTION

Social farming (SoFar) creates a new outlook on agriculture and agricultural activity. In the Czech Republic, the term *sociální zemědělství* has started to be used for these activities. It is the equivalent for English terms *Social Farming, Green Care Farming, Farming for health, Social Agriculture and Farming Therapy.* This is one of the possibilities how to integrate health and socially disadvantaged people into society. These people can be employed in the agricultural environment, furthermore, it can also help reduce their health or social problems. Simultaneously, there is also an opportunity of diversifying the income of farmers who make affords to create conditions that enable disadvantaged people to engage in normal farm activities for the purpose of securing their development, support and improvement of the physical and mental state (Hromadová 2016).

The exact definition of SoFar has not yet been established as there is not any legal definition or basic text that would determine what can still be included in social farming and vice versa. The basis for defining social farming is the Own-initiative Statement of the European Economic and Social Committee (EESC) published in 2012 based on the COST Action 866 *Health Benefits of Green Care report* and can be expressed as a sum of agricultural sources, both livestock and plants, in order to create social services in rural or suburban areas. The relatively new concept of social farming has a close connection to multifunctional agriculture and fully fits into the concept of rural development as it enables farmers to diversify their income. Moreover, it brings contribution to society since it provides social services and improves current services for rural residents by using agricultural and rural resources (NAT/539 2012).

Multifunctional agriculture links the production of commodities, non-commodities and pluriactivity of agricultural farms. We understand the production of commodities as services and goods (e.g. animal products or cereals) for which we have a market. On the other hand, for non-commodities, such as landscape or biodiversity, the market does not exist. They also include "damage" such as soil degradation or water pollution. Pluriactivity of the farm can be understood as an offer of another services and products of the company and diversification of the income portfolio of the farm (Ratinger 2011).

Social farming belongs to the activities called *green care*. This term involves all activities that share the experience of natural elements that improve or maintain the mental and physical state of humans (Chovanec 2015). All the subsequently listed activities such as social farming, social and therapeutic horticulture, animal assisted interventions, movement – physical exercise for therapeutic purposes, ecotherapy, wilderness therapy and nature therapy are the part of the green care (Sempik 2015).

Social farming has a wide range of target groups. It might be people who are disadvantaged on the labour market, as defined by Act No. 435/2004 Coll., on employment. However, the largest group involves people with physical, mental, psychic, sensory or combined disabilities, and persons at risk of social exclusion, as defined by Act No. 106/2008 Coll., on social services. The social farms also target on the general public (children, youth, adults and seniors) and serve for pedagogical purposes or become a meeting place for the local community (Moudrý et al. 2015).

There are many social farms abroad especially in Western European countries. These farms on the high-level can be found in countries such as the Netherlands, Finland, Norway, Belgium, Great Britain, France, Italy and Germany (Hine et al. 2008).

The aim of social farming is to create new jobs, services, educational activities, and to implement different types of therapies for a wide range of people with a health or social disadvantage by using available agricultural resources at the particular site. In addition, social farming creates and offers commodities same as other agricultural companies.

The farm environment has a beneficial effect on human psyche due to the structured activity with regular daily/seasonal rhythms and a rapidly visible result of work. It mediates contact with nature which can be a source of stimuli, interest and joy for individuals. People also learn responsibility through plant or animal care. There are the following studies that looked at the positive effects of a social farm on individual target groups such as people with mental disorders, drug addicts or addicted in another way, people with mental, health or combined disabilities.

For example, according to the study by Jan Hassin conducted in the Netherlands to evaluate the benefits of a social farm to young people with raising problems, it has become clear that thanks to the *work and stay* program the parents' relationship with these young people improves as well as spending of their leisure time, moreover, their self-confidence and self-acceptance increased (Elings 2012). The book *Pillen, praten en bewegen* describes that physical activity assists to improve mental health. Fresh air exercise helps people with mental disorders (Van der Stel, 2005 by Elings 2012). According to the study by the University of Essex in the UK, the time spent engaging in various farming activities across different target groups significantly reduces feelings of anger, confusion, depression, tension and fatigue while it helps them to feel better (Hine 2008). In the Netherlands, the quality of care and life of people with dementia were in the study compared to traditional nursing homes, small-scale living facilities and green care farms. It has been proved that a social farm is a valuable alternative to traditional facilities (Boer et al. 2017).

From a general point of view, we can say that social farming benefits society and fits into the concept of sustainable development. It can complement well with ecological farming. As demonstrated by Hassink (2006), European farmers dealing with social farming are also farmers who are involved in the environmental protection, leisure and educational events.

In the Czech Republic, there are 27 farms and therapeutic gardens, which claim to use the concept of social farming. Among them is a farm Biostatek whose leadership through participation in the international project M.A.I.E. brought the concept of social farming into the Czech Republic. Other farm operators, who are social farms today, were doing same work without realizing that it is a concept of social farming and are now discovering the possibilities that can bring them to be in this category. With the intention of starting a social farm, the Sociální farma, s.r.o. and farm Kálal, z.s. have been established.

Most of these farms in the Czech Republic, in addition to social farming, are also involved in other activities such as ecological or biodynamic agriculture (Malonty, a. s., Svobodný statek na soutoku etc.) or environmental education, upbringing and raising (e.g. Toulcův dvůr, Biostatek). Some of them do not exactly match the definition of social farming. The social farm is to be located in the countryside, however, Toulcův dvůr or Lipka – school facility for environmental education,

are located in the cities. Zahrady u splavu, Terapeutická zeleninová zahrada, Prevalco, o.s. and Lipka are therapeutic gardens. Svobodný statek na soutoku, o.p.s. is very specific mainly as it follows Camphill Česke Kopisty which is part of the Camphill community. Usedlost Nad Prameny, Farma Wenet broumov, z.s., Farma Kálal, z.s. also run agrotourism. The common feature of social farms in the Czech Republic is the fact that the operator is a non-governmental non-profit organization. Another common feature is participation in the environmental education, upbringing and raising of public awareness.

In the Czech Republic, people with health or social disadvantage are entitled to get a protected job. The employer receives a contribution for the protected job from the Labour Office which also contributes 75% of the actual expenditure on wages and salaries including social security contributions and state employment policy and public health insurance, but not more than 8 800 CZK. However, this contribution will be received only by an employer who employs more than 50% of the total number of people with health or social disadvantage.

Care Farming UK is a professional charitable company accountable to its members, and is led by care farmers and care farming supporters. There are approximately 250 care farms in the UK and another 35 in the Republic of Ireland at this moment. Majority of care farms, counting also prospective care farms, are usually commercial farm businesses, charities, Community Interest Companies (CICs) (all at 24%), or charitable companies limited by guarantee (22%). Subsequently, 13% are Social Enterprises (SE), 9% private limited companies and 18% are oriented towards other organisational arrangements, namely: sole trading, education centres, therapeutic communities, community groups, independent provident societies etc. The farms count, although it varies, 35 attending clients per week on an average. Services for the clients, provided by most of the UK Care Farms, include people with learning difficulties (93% of care farms), autism spectrum disorders (86%), adults with mental illhealth (70%), people with physical disabilities (53%) and young people excluded from school or with behavioural issues (50%). Care farms in the UK also prepare day sessions for their clients which usually cost around £52. Sessions happen regularly between 1-3 times a week, for 8 months to a year (Care Farming in the UK and Ireland 2017).

MATERIAL AND METHODS

Description social farming in Czech Republic and Great Britain

We used the own field survey in this study to describe the current state of social farming in the Czech Republic. The main source of information about the Czech social farms was acquired via the excursion survey which was part of the accredited course of the Ministry of Education, Youth and Sports - Introduction to Social Farming, and a three-day educational stay in POMOC Týn nad Vltavou z.s. with a visit to the apple orchard in Temelín and the composting plant JAROŠOVICE, s.r.o., operated by SoFar. We also visited Farma Ledce, which employs mentally disabled people, and Toulcův Dvůr, oriented towards people with disabilities.

There have been many interviews with job assistants, social workers, workers in social services, managers of the organization and the users of social services and social farming. We used semi-standardized interviews questioning the strengths and weaknesses of social agriculture, moreover, the strengths and weaknesses of their farms and perceived opportunities and threats.

British farms were selected by comparison with the Czech farm. The reason for this choice was the long tradition, the large representation of social farms in the UK and their precise mapping by Care Farming UK. About two hundred farms were approached by e-mail. Everyone was asked the same questions about the strengths and weaknesses of their farm and perceived opportunities and threats. Of the aforementioned number of farms, eleven respondents replied, the remaining respondents either did not respond, referred to the Care Farming UK website, or did not want to answer as they were no longer concerned with social farming. We made a comprehensive view of social farms and SoFar in the UK from the data obtained.

A SWOT analysis has been used to evaluate data from the semi-standardized interviews and internet questionnaire.

RESULTS AND DISCUSSION

On the Czech side were mentioned as strengths benefits for society as a whole (prevention of criminal phenomena etc.), followed by improving the quality of life of disabled persons or socially disadvantaged, since it improves the mental and physical health of disadvantaged people. Also, thanks to SoFar, health and socially disadvantaged people can get new experience and skills which they could not obtain anywhere else Furthermore, diversification of farms income, although this diversification is currently not entirely clear, positive contribution to the local community in the form of employment of disabled or socially disadvantaged people from the surrounding area, positive effects on the quality of life of the disabled person's whole family as it offers services directly at the place where the person resides, farm products etc.

Weaknesses are scattered activities, an insufficient salary, burnout syndrome and psychological difficulty in the work of social workers, workers in social services and work assistants, furthermore, the reduced work performance of employees (clients) as a result of their disability and a risk of an accident at work and indifference towards the farming.

Opportunities include the social farming support by the Ministry of Agriculture of the Czech Republic, forthcoming social enterprise act, social farming program PGRLF, as, Rural Development Program 2014–2020, the Employment Operational Program, the Integrated Regional Operational Programme (IROP), substitute payments.

Threats can be seen as a lack of employees (clients), adverse weather, government's unwillingness to help develop social farming, lack of product and service as well as a trust in solidarity and appreciation of the role of the entity and its contribution to society.

Table 1 SWOT analysis social farming in Czech Republic

STRENGTHS	WEAKNESSES	OPPORTUNITIES	THREATS
Diversification of farm activities	Scattered activities	Ministry of Agriculture of the CZ	Lack of employees (clients)
Contribution to the society	Burnout syndrome Psychological difficulty	Social farming program PGRLF, a.s.	Adverse weather
Quality of client's life improvement	Insufficient salary		Lack of product and service
Positive contribution to the local community	Risk of an accident	Rural Development Program 2014-2020	Government's unwillingness to help develop social farming
The quality of life of the disabled person's whole family	Indifference towards a farming	Sale of farm products	
	Complicated administration		
		Employment Operational Program	Lack of appreciation of the SoFar role
		Integrated Regional Operational Programme	Adverse weather
		Substitute payments	

Many factors in the SWOT analyses of the British side did not differ from those of Czech. This can be clearly seen in strengths and weaknesses. The opportunities and some of the threats were more pronounced. Among the opportunities, we can name project grants as well as volunteers willing to work on a farm. The threats mentioned in the questionnaire were the lack of clients, inadequate financial support from the state, constantly changing legislation, UK's quitting the membership of European Union, as a leaving from the EU may mean the absence of some grants, complicated

negotiations with the authorities and a lack of appreciation of the role of the entity and its contribution for the society.

The mentioned weaknesses were the weather dependence, the possibility of unsuitable work on the farm for every person, the low salary evaluation of employees who work with people with health and social disadvantage and the cost of providing security aids for them.

Table 2 SWOT analysis social farming in Great Britain

STRENGTHS	WEAKNESSES	OPPORTUNITIES	THREATS
Diversification of farm activities	Burnout syndrome Psychological difficulty	Support for mental health that appears to be worsening in the UK	Lack of employees (clients)
Contribution to the society	Scattered activities	Project grants	Adverse weather
Quality of client's life improvement	Risk of an accident	Volunteers	Lack of product and service
Positive contribution to the local community	Indifference towards a farming		Inadequate financial support from the state
Membership in Care Farming UK organization			Changing legislation
Farm products			Leaving UK from European Union
			Lack of appreciation of the role SoFar

CONCLUSION

SWOT analyses of the Czech and British social farms results in more similarities than differences. Both SWOT analyses show that a strength is a contribution to the society, but at the same time it can be said that the Czech and British farmers feel a lack of appreciation of their work by the wider public. Among the weaknesses on both the British and the Czech side, there is an insufficient salary assessment of workers who help people with a health or social disadvantage. Logically resulting is a uniformly perceived threat which is the dependence on the weather.

For a comparison with the Czech farm, a British farm was chosen which picture is drawn from a questionnaire survey. It is clear from the fact that farmers find the benefits for society since it improves the mental and physical health of disadvantaged people. SoFar positively affects the quality of life of the whole family of a disabled person as it offers services directly at the place where the person resides. Also, thanks to SoFar, health and socially disadvantaged people can get new experience and skills which they could not obtain anywhere else.

Social farms in the UK have the advantage of an established Care Farming UK organization and a greater awareness of social farming, although there is a lack of appreciation of the farms activities. For social farming in the Czech Republic, it might be an inspiration to found an organization to help individual social farms in their development or to give instructions for those interested in social farming. It could simplify the administration regarding the acceptance of persons with health or social disadvantage in social farms, which is today a significant weakness of Czech social farming, and to increase overall awareness of the Czech society about the social aspect that agriculture can have. Moreover, volunteering should not be neglected as the source of farm development in the Czech Republic, which is positively reported by British farms.

ACKNOWLEDGEMENTS

The research was financially supported by the project IGA_FA_ IP_2017/016 Social farming and its future in the Czech Republic.

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EVALUATION OF LAND USE TRENDS IN THE VINEYARD VILLAGE OF ČAJKOV (SLOVAKIA)

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Abstract: In this contribution we focus on evaluating the land use trends in the village of Čajkov (Southwest of Slovakia). Čajkov represents a village with a long tradition of vineyard cultivation. The aim of our study was to identify and evaluate the land use development trends in the village of Čajkov over two time periods (1949–1986 and 1986–2016), based on a comparison of secondary landscape structure changes. We discovered that Čajkov is one of the few villages in Slovakia where traditional vineyards have not disappeared over the last 70 years, but on the contrary have increased.

Key words: Čajkov village, land use trends, traditional agricultural landscape, vineyard landscape.

INTRODUCTION

Traditional agricultural landscape (TAL) can be defined as a mosaic of small-scale arable fields and permanent agricultural cultivations such as grasslands, vineyards and high-trunk orchards (Špulerová et al. 2016). It represents areas, where the same system of land use management has been maintained for centuries. From the 1950s to 1980s, more than half of TAL was destroyed in Slovakia as a result of agricultural intensification and collectivisation (Lieskovský et al. 2014). The actual trend in Slovakia is that traditional agricultural landscape is decreasing due to abandonment (Lieskovský et al. 2013, Mojses and Petrovič 2013, Izsóff et al. 2016). According to Supuka et al. (2011), the greatest area of vineyards in Slovak territory was in 1720, when they occupied an area of about 57 000 ha. Since 1720, the area of vineyards has reduced to 17 598 ha (ÚKSUP Bratislava 2016), out of which about 14 000 ha are productive.

In this contribution we focus on the analysis of development trends in land use changes. We chose the years 1949 and 1986 for the analysis of historical landscape structure, and for the current landscape structure the year 2016. The aim of this paper is to identify and evaluate trends of land use changes in the vineyard village of Čajkov over the last 70 years.

MATERIAL AND METHODS

In 2016, we undertook field research to create a map of the current landscape structure (CLS), which was necessary for updating the ortho photo images from 2007. Maps of historical landscape structures (HLS) were created using the panchromatic aerial photos from 1949 and 1986. The visualization of all maps was realised in the ArcGIS 10.2 geographic information systems. For mapping landscape features we used a modified legend according to Petrovič et al. (2009), because it is applicable to the historical landscape structure and current landscape structure of Slovakia as well. Instead of the original four-digit coding, we used two-digit coding for mapping landscape features in this study. Therefore, we identified 16 classes of secondary landscape structure features on the second level (Table 1).

Table 1 Secondary landscape structure legend

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 amenities
		62	Residence and technical vegetation
		63	Sports, cultural, recreational objects and grounds
		64	Production, technical objects and grounds
		65	Transport objects and grounds

After comparing the changes in the area of secondary landscape structure features, we analysed changes in land use trends using the ArcGIS 10.2 program tools. Using the classification scheme according to Feranec et al. (2002) and Cebecauerová (2007), we identified 18 land use trends. The code mark is stated in Table 2 and shown in Figures 2 and 3.

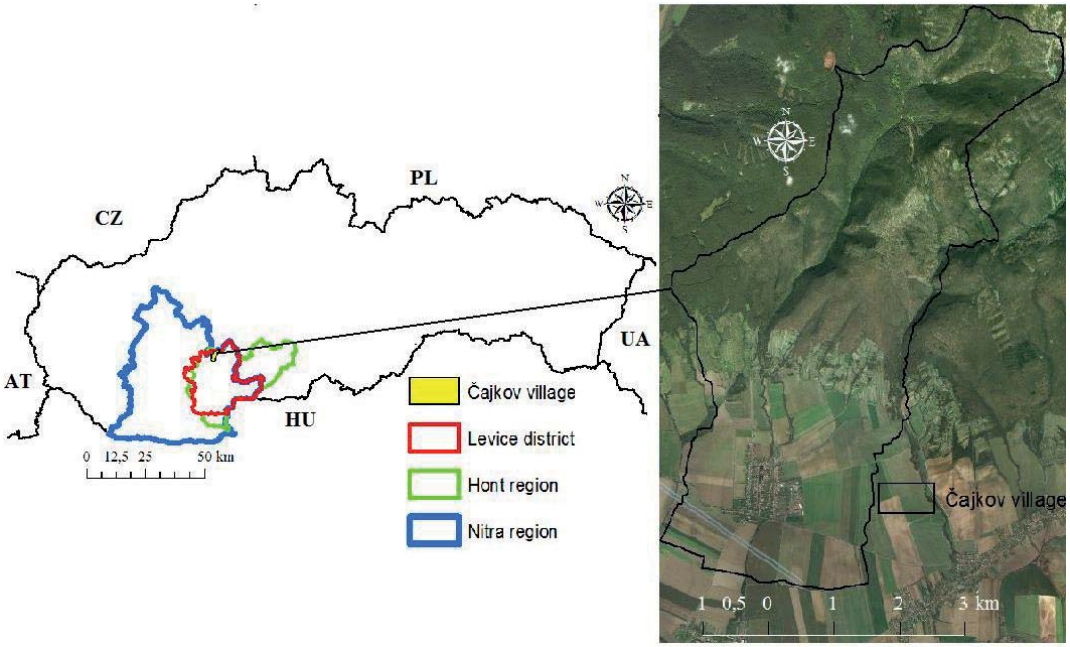
Table 2 Land use trends legend

1	Unchanged	10	Industrialization
2	Urbanisation	11	Exploitation of mineral resources
3	Deurbanisation	12	Intensification of urbanisation
4	Afforestation	13	Extensification of urbanisation
5	Deforestation	14	Agricultural intensification
6	Forestry intensification	15	Agricultural extensification
7	Forestry extensification	16	Greening
8	Draining	17	Deindustrialisation
9	Flooding	18	Overgrowing and succession of abandonment areas

Localization of the study area

The village of Čajkov is located in the Middle-Hron river region (Southwest of Slovakia). A large part of the territory enters into the forest, which belongs to the Štiavnica Mountains Protected Landscape Area (CHKO Štiavnické vrchy). The southern part of the village is situated on the Danubian upland, which is mostly covered by agricultural fields. The area of the village is 2389.01 ha. Čajkov belongs to the Levice district (Nitra region). The Čajkov vineyards are situated along the northern boundary of winegrowing in the Slovak Republic. According to the historical structures of agricultural landscape division (Špulerová et al. 2011), the Čajkov vineyards belong to the Hont region (see Figure 1).

Figure 1 Study area location in Slovakia



RESULTS AND DISCUSSION

The current landscape is the result of various development factors – urbanisation, economic activities, land management etc. Land use trends are the responses of landscape to the pressure of human activities. In this contribution, we present the most important results, which significantly affected land use and landscape perception.

Historical landscape structure of Čajkov in 1949

In 1949, forests occupied the largest part of Čajkov. In total, they represented an area of 1281.34 hectares (53.63%). The second largest area was occupied by small-block fields (31.85%). The area of the vineyards reached 59.78 ha (Table 3). The group of large-block vineyards, mining areas and raw soils and production, technical objects and grounds was not recorded.

Table 3 Comparison of area of SLS features in Čajkov 1949–2016

Year	1949		1986		2016	
SLS	Area (ha)	Area (%)	Area (ha)	Area (%)	Area (ha)	Area (%)
11	1281.34	53.63	1305.24	54.64	1334.45	55.86
12	46.49	1.95	35.42	1.48	74.49	3.12
21	144.04	6.03	175.6	7.35	113.89	4.77
31	41.94	1.76	657.53	27.52	642.17	26.88
32	761	31.85	14.55	0.61	8.39	0.35
33	29.17	1.22	39.07	1.64	29.35	1.23
34	0	0	41.48	1.74	35.35	1.48
35	59.78	2.5	67.95	2.84	80.99	3.39
36	2.54	0.11	0.65	0.03	1.7	0.07
41	0	0	1.12	0.05	0	0
51	3.19	0.13	3.97	0.17	4.04	0.17
61	8.82	0.37	17.21	0.72	24.76	1.04
62	3.05	0.13	3.94	0.16	15.84	0.66
63	1.01	0.04	2.58	0.11	2.68	0.11
64	0	0	12.13	0.51	9.9	0.41
65	6.64	0.28	10.57	0.44	11.01	0.46
Total	2389.01	100	2389.01	100	2389.01	100

Historical landscape structure of Čajkov in 1986

During 1986, forests still occupied the largest area (54.64%). Like most villages in Slovakia, Čajkov did not avoid merging small-block fields into large-block fields. Agricultural intensification caused the area of large-block fields to increase by 615.59 ha. Despite the agricultural intensification process, the area of meadows, pastures and small-block vineyards did not decrease as in other villages in Slovakia. On the contrary, the area of small-block and large-block vineyards has significantly increased. The riverbed´s adjustment in built-up areas caused an increase of water features from 0.13% (1949) to 0.17% (1986). All groups of features from houses and built-up areas increased.

Current landscape structure of Čajkov in 2016

The prevailing groups of features were groups of forest (55.86%) and large-block fields (26.88%), covering 82.74% of the research area. In 2016, there was an extinction of mining areas and raw soils. Compared to 1949, traditional agriculture features – small-block vineyards and orchards increased. An area of non-forest woody vegetation also increased by more than 50% as a result of new trends in landscaping. Garden areas decreased at the expense of the construction of houses and amenities.

On the basis of comparison of changes in the area of secondary landscape structure, we identified 18 development trends in land use changes (Table 4) in the Čajkov village.

Table 4 Land use trends of Čajkov in 1949–1986 and 1986–2016

Year	1949–1986		1986–2016	
Trends	Area (ha)	Area (%)	Area (ha)	Area (%)
Unchanged	1199.56	50.21	2127.04	89.03
Urbanisation	25.01	1.05	6.34	0.27
Deurbanisation	3.12	0.13	4.44	0.19
Afforestation	57.3	2.4	68.82	2.88
Deforestation	57.79	2.42	28.66	1.2
Forestry intensification	0	0	0.94	0.04
Forestry extensification	209.99	8.79	4.47	0.19
Draining	0.78	0.03	0.51	0.02
Flooding	2.47	0.1	2.57	0.11
Industrialization	19.61	0.82	12.13	0.51
Exploitation of mineral resources	1.25	0.05	0	0
Intensification of urbanisation	0	0	1.28	0.05
Extensification of urbanisation	2.46	0.1	11.5	0.48
Agricultural intensification	671.98	28.13	10.99	0.46
Agricultural extensification	126.62	5.3	75.48	3.16
Greening	0.97	0.04	3.16	0.13
Deindustrialisation	0	0	1.94	0.08
Overgrowing and succession of abandonment areas	10.1	0.42	28.74	1.2
Total	2389.01	100	2389.01	100

The most important land use changes in Čajkov started in the second half of the 20th century. Figure 2 demonstrates that there were significant changes in a large part of the village. Between 1949 and 1986, almost 50% of the area was changed as a result of various land use trends. As we can see, the process of agricultural intensification was the most significant (28.13%). Collectivisation caused a merger of the mosaic of small-block fields into large-block fields (Figure 2), which led to the destruction of traditional extensive farming. The merger of small-block fields into large-block fields has been reflected not only in the economy (higher yields), but also in the formation of natural hazards (decrease of biodiversity, wind and water erosion etc.). Trends of afforestation and deforestation were balanced. A significant trend was forestry extensification (8.79%). In 1979, the protected landscape area of Štiavnické Mountains was created, so this trend was caused by the slowing down of logging. That is the reason why forestry intensification could not exist. During this period, trends of urbanisation (1.05%) and industrialization (0.82%) were visible. Development of technical infrastructure brought new landscape features to the TAL and it has

continued also after 1986. These features have negatively affected the aesthetics and permeability of the TAL.

Figure 2 Land use trends 1949–1986

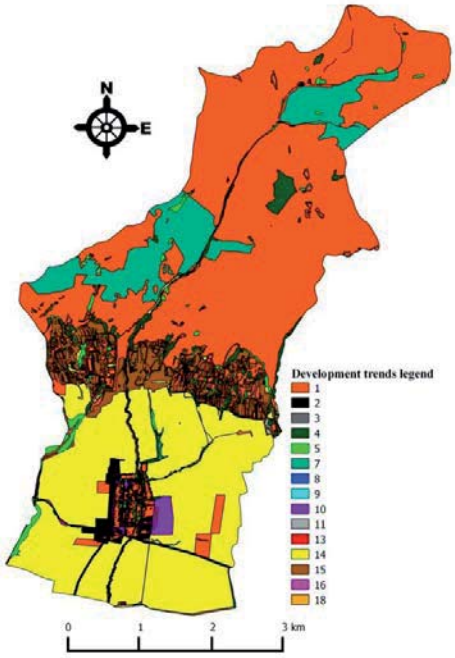
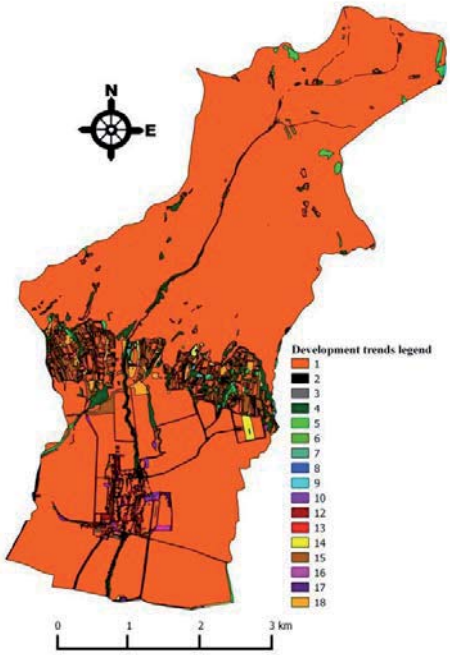


Figure 3 Land use trends 1986–2016



During 1986–2016 the village was more stable in terms of land use. 89.03% of the study area was unchanged. The number of houses in the village is gradually increasing because of the affordable price of building land. Over the last 2 years, we have noticed the trend of suburbanization as well. Suburbanization poses a higher threat to traditional vineyard landscape. The traditional architecture of rural and vineyard landscape is replaced by modern features. Typical examples are high grey fences, which completely disrupt the specific character of traditional agricultural landscape. TAL is losing much of its aesthetical value. The trend of industrialization in Čajkov and in the town of Levice (only 9 km from Čajkov) is increasing. Over the next 10 years we expect rapid growth in the number of new houses in vineyards as a response to industrialization and the gradual increase in real estate prices. The processes of flooding (0.11%) and draining (0.02%) were associated with the regulation of the river bed. The parts of the forest that underwent logging in the past have overgrown gradually. Afforestation trend was recorded on 68.22 ha (2.8%) of land. Overgrowing and succession of abandonment areas were recorded on 28.74 ha (1.2%) of study area. Abandonment of meadows and pastures is the result of people lifestyle change. They are discontinuing with tradition of breeding and grazing cattle.

The abandonment of vineyards related to imported wines, increased production costs (e.g. pest sprays) and insufficient agricultural subsidies. This made vine growing unprofitable (Lieskovský et al. 2013). Although the abandonment trend of the traditional small-block vineyards is noticeable in neighbouring villages (Izsóff et al. 2016) and overall in Slovakia (Lieskovský et al. 2013, Mojses and Petrovič 2013), inhabitants in Čajkov are strongly linked to traditional land use. Therefore, the area of vineyards has increased over the last 70 years.

CONCLUSION

Over the last 70 years, Čajkov has changed significantly. In Slovakia, traditional agricultural landscape has been preserved, especially in less accessible and less fertile localities (Špulerová et al. 2010). This village is an exception. We can designate it as a forest-rural landscape with a significant representation of vineyards, whose area has grown over the last 70 years. The most significant trends in land use changes that affected the village were intensification and collectivisation of agriculture from 1949 to 1986. It was caused by the change to the political system after the year 1948. As an important feature of TAL, the growth of vineyards is positively perceived, because

extensively managed vineyards contribute not only to ecological, environmental and cultural diversity, but also to positive landscape perception. The trend of industrialization poses a risk. If it grows significantly, suburbanization will increase. These processes could be disastrous for the TAL. Local people have a very important role to play. They are an irrecoverable source of knowledge for future generations. It is important to support the younger generation to ensure traditions of TAL management. Otherwise, we are risking a loss of our national wealth. Villages such as Čajkov are now becoming rare and thus highly valuable in Slovakia with irrecoverable historical and biocultural values.

ACKNOWLEDGEMENT

The contribution was prepared within the grant project of the Ministry of Education of the Slovak Republic and the Slovak Academy of Science VEGA no. 2/0171/16 "Changes in Slovak Landscape Driven by European Union Agricultural Policy."

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SAMPLING AND ANALISYS OF SEDIMENTS FROM SMOLENSKÁ WATER RESERVOIR BASIN

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Abstract: The aim of this paper is to evaluate the ecotoxicity of sediment from Malonínský brook, sedimentation reservoir and Smolenská water reservoir, which are located on this creek. The second objective is to compare the concentration of the risk elements contained in the sediment from the Smolensk water reservoir with the limit values set out in the Decree No. 257/2009 Coll., as amended. A methodology for the collection and evaluation of sediment samples, which is based on valid legislation and valid CSS standards, was developed. The area of interest was divided into 4 parts. For each part of the territory, one single mixed sample was created. In the laboratory of the Department of Applied and Landscape Ecology, an ecotoxicity test was carried out by the method of Inhibition of Growth of Root of Higher Plants according to CSS EN ISO 11269-1. The chemical analysis of the risk elements was processed only for sediment from the Smolenská water reservoir. After comparing the measured concentrations of the individual indicators (mg/kg dry matter) for the mixed sample from the Smolenská water reservoir, it is clear that the limit values given in Annex 1 to Decree No. 257/2009 Coll. are not exceeded. From the results of the ecotoxicity test, it can be seen that the mixed sample of sediment collected from the Malonínský brook in part 4 does not reach the required values according to Decree No. 257/2009 Coll. and is ecotoxic. Mixed sediment samples from part 2 and part 3 are not ecotoxic, but the test results are closer to the limit. The sediment from part 1 – above the Smolenská reservoir is not ecotoxic. It is not advisable to build the pond sediment as a waste, but after meeting the requirements for the concentration values of the risk elements as for the fertilizer. It is necessary to mix sediment before placed on farmland. The results show that for the sediment from the site, the ratio of substrate mixing to the sample is 3:1, i.e. 75% of the substrate and 25% of the sediment volume.

Key Words: mixed sample, sampling site, ecotoxicity, inhibition of root growth, homogenization

INTRODUCTION

Smith (1978) defines the term sediment as follows: "The sediment is the product of the accumulation of material originating from weathered and eroded rocks and brought to the place of storage either as a solid particle or in solution. The material is mostly stored in layers, by physical, chemical or biological processes." Sediment is a material that results from erosion. Erosion processes are natural and landscape-shaping agents that have been acting on it for a long time. From the point of view of the problem of alleviation of water reservoirs, it is mainly water erosion. Water takes material from the area into watercourses. The soil particles moving in the flows can be divided into suspended matter that are scattered throughout the flow profile and the bed load that are sun-drenched by sliding, rolling or jumping (Krešl 2001). The sediment travels to water reservoirs, where water drift decreases and sedimentation increases. This phenomenon cannot be completely avoided, but it needs to be minimized (Tlapák and Herynek 2002). The effect of this phenomenon is to reduce the capacity of retained water in the reservoirs and at the same time to worsen water quality as a result of its eutrophication. One of the most important symptoms of eutrophication is the excess growth of planktonic algae and cyanobacteria. Algal blooms lead to a decline in water quality with multiple ecological and socio-economic consequences (Smol 2010).

In the future, there is a shortage of phosphate ores, and therefore phosphorus, which is used for the production of fertilizers. The Czech Republic has a huge number of ponds, in which there

is naturally a large potential for the retention of phosphorus and other nutrients. The ability of ponds is the containment of phosphorus, which can then be recycled back into farmland via pond sediment (Potužák 2015). This is a progressive alternative to conventional soil fertilizer delivery.

Unloading takes place to increase the water capacity in the reservoirs and to reduce the negative effects of the sediment on the quality of the water in the reservoirs. After dredging, the sediment must be disposed of in accordance with current legislation. In practice Kotrba (2016) offers several ways to dispose of extracted sediment: utilization for agricultural land fund according to Decree No. 257/2009 Coll.; landscaping, loading of underground spaces according to Decree No. 294/2005 Coll.; storage in another facility pursuant to paragraph 14, Section 2 of Act No. 185/2001 Coll.; use as building material in accordance with paragraph 14(2) of Act No. 185/2001 Coll.; landfill pursuant to paragraph 14, Section 1 of Act No. 185/2001 Coll.; by-product according to § 3, Sect. 5 of Act No. 185/2001 Coll.

MATERIAL AND METHODS

Methodics

The aim of this paper is to evaluate the ecotoxicity of sediment from Malonínský brook, sedimentation reservoir and Smolenská water reservoir, which are located on this creek. The second objective is to compare the concentration of the risk elements contained in the sediment from the Smolensk water reservoir with the limit values set out in the Decree No. 257/2009 Coll., as amended.

First, a methodology for the collection and evaluation of sediment samples was developed, which is based on valid legislation and valid CSS standards. The area of interest was divided into 4 parts: part 1. – Malonínský brook from Bělá u Jevíčka to sedimentation reservoir, part 2 – sedimentation reservoir, part 3. – Smolenská water reservoir and part 4. – Malonínský stream from Smolenská water reservoir to body of unfinished highway Wroclaw – Vienna.

In the preparation for fieldwork, sampling points of partial samples were selected in selected parts of the territory. The exact location of the supply points has been adapted to local conditions for fieldwork. In Parts 1, 2 and 4, there were four sampling points for the partial samples, and in Part 3 there were six sampling points. After sampling (Figure 2 on the left and in the middle) samples were homogenized on site (Figure 2 on the right) and quadrupled. For each part of the territory, one single mixed sample was created. In total, four mixed samples were generated. Mixed samples were placed in sample boxes (Figure 3 on the left), which were transported to the Department of Applied and Landscape Ecology. The sampling and transport of the sub-samples took place according to valid standards.

In the laboratory of the Department of Applied and Landscape Ecology, an ecotoxicity test was carried out by the method of Inhibition of Growth of Higher Plants Root according to CSS EN ISO 11269-1. A full test was performed using seed of barley (*Hordeum vulgare*). A series of sample concentrations with substrate and sediment were generated for the test and according to the CSS methodology a procedure for the processing and evaluation of the results was chosen. Two replicates were performed in the test. Subsequently, the results were also compared with Table 1 of Annex No. 3 to Decree No. 257/2009 Coll., as amended. The process of the test is illustrated in Figure 3 on the right, Figure 4 on the left, in the middle and on the right.

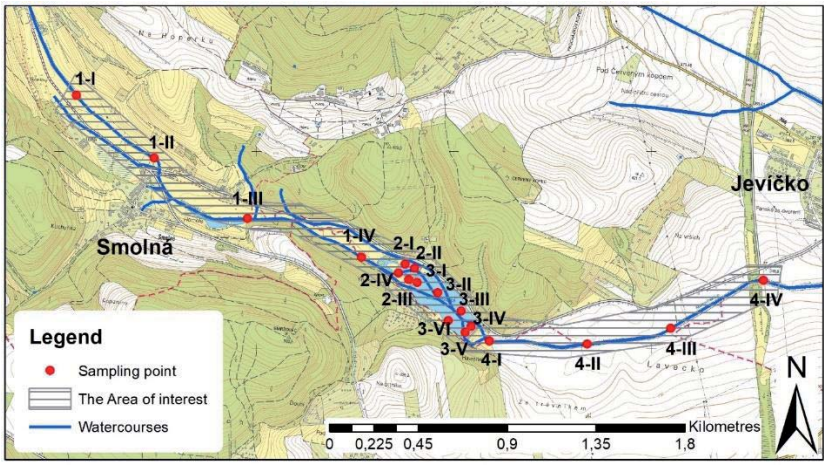
Chemical analysis of risk elements was processed only for sediment from the Smolenská water reservoir (Figure 3 in the middle). This analysis was carried out by a specialized laboratory of ALS Czech Republic, Ltd. The following risk elements were analysed: As, Be, Co, Cr, Cu, Ni, Pb, V and Zn. The resulting concentrations of the risk factors in the dry matter for the individual indicators (mg/kg of dry matter) in the mixed sample were compared with Annex No. 1 to the Decree No. 257/2009 Coll., as amended.

Definition the interest area

The area of interest is located west of Jevíčko in the Pardubice Region. The area of interest is defined by the water courses of the Malonínský brook, the Smolenská water reservoir and the sedimentation reservoir, which is located at the entrance of the Malonínský brook into

the Smolenská water reservoir. The area of interest begins under the bridge of the unfinished Wroclaw – Vienna highway and ends at a road bridge at the edge of the Bělá u Jevíčka (see Figure 1). The Arabic numerals represent the part number of the territory. The Roman numerals represent the numerical designation of the sampling points.

Figure 1 The interest area with sampling points.



Legend: Basic map: Czech Office for Surveying, Mapping and Cadastre. Modified by author.

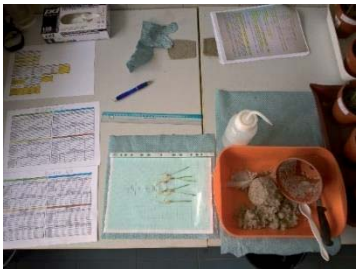
Figure 2 Sediment extraction from the Smolenská reservoir (on the left), Sediment output from sedimentation reservoir (in the middle), Homogenization of partial samples (on the right).



Figure 3 Mixed sample prepared for transport (on the left), Sediment output from the Smolenská reservoir for chemical analysis (in the middle), Jars with substrate and sediment mixes immediately after foundation (on the right).



Figure 4 Jars with substrate and sediment mixes immediately before evaluation (on the left), Root length measurement and experiment evaluation (in the middle), Measure the length of roots of six individuals from one container (on the right side).


RESULTS AND DISCUSSION

The results of the growth inhibition test of higher plant roots

To determine the ecotoxicity of the sediment, a limit test was carried out, which was part of the complete test, where the individual concentrations of the sediment – substrate mixture were monitored. The substrate was composed of artificial soil with the following composition: 10% of peat moss, 20% of kaolin clay and 70% of industrial quartz sand.

According to Decree No. 257/2009 Coll. the sediment is ecotoxic if the average plant root length in the mixed sample is significantly lower by at least 30% compared to the control.

It can be seen from Table 1 that the mixed sample of sediment collected from Malonínský brook in part 4 – between Smolenská reservoir and the highway does not reach the required values according to Decree No. 257/2009 Coll. and is ecotoxic. This can be caused by the settling of the sediment from the bottom of Smolenská reservoir at the turn of 2016 and 2017 when it was released. The top layer of the bottom sediment was taken from the reservoir together with the released water into the Malonínský brook.

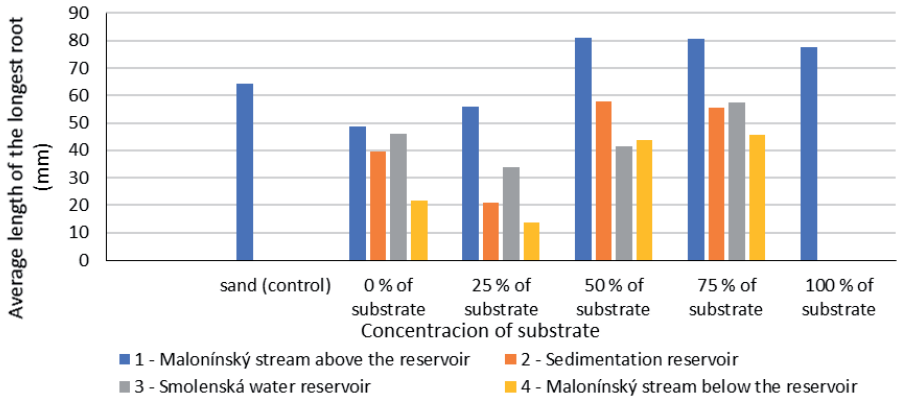
Table 1 Evaluation of the ecotoxicity according to Table 1 in Annex No. 3 of Decree No. 257/2009 Coll.

The average length of the longest root (mm)				
Concentration	1 – Malonínský creek above the reservoir	2 – Sedimentary reservoir	3 – Smolenská reservoir	4 – Malonínský creek below the reservoir
Mixed sample (mm)	81	56	58	46
Control (mm)	78	78	78	78
Percentage (%)	104	72	74	59

Mixed sediment samples from Part 2 and Part 3 are not ecotoxic, but the test results are close to the limit. In the sedimentation reservoir, there was settling of the loops from the surrounding fields until it was completely clogged and Malonínský brook carried sediments further into the Smolenská reservoir.

Sediment from the part 1 – above the Smolenská reservoir is not ecotoxic. The stream has more direct character here, water flows more quickly here and there is no sedimentation of the fine-grained parts, but rather the settling of the sandy and stony fractions.

Figure 5 The average length of the longest root for individual concentrations and parts of the territory



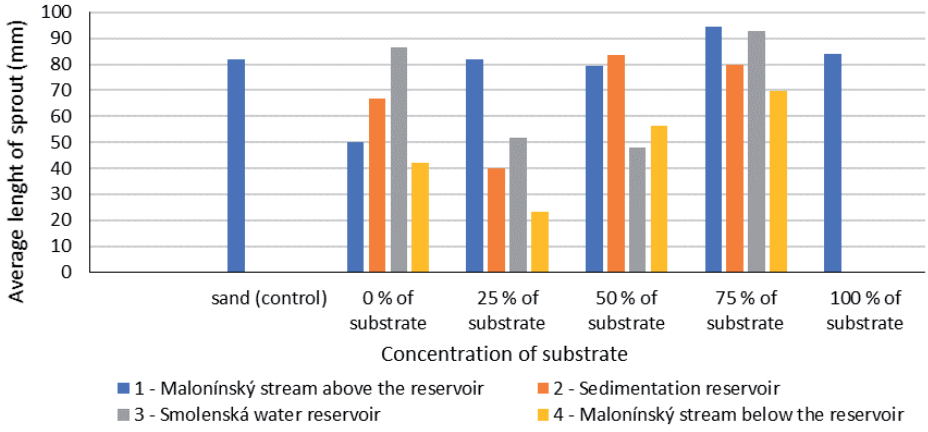
To evaluate the complete ecotoxicity test, a range of substrate concentration values were determined against the sediment sample at 0%, 25%, 50%, 75% and 100% of substrate. For the sake of clarity, ecotoxicity was determined with industrial sand. It can be seen from Figure 5 that the average values of the longest root in mm reached the composite sample from Part 1. At the ratio of substrate to a sample of 1:1 (50% substrate) and 3:1 (75% substrate), the results for part 1 achieves almost the same values as plants at 100% substrate. The sample from Part 4 reaches the lowest root length

values at all concentrations. The values for sedimentation reservoir (Part 2) and Smolenská reservoir (Part 3) are average. It is generally seen from Figure 5 that higher substrate concentrations in relation to the sediment mean higher root increment.

The shoot lengths of each of the individuals and the average values of the lengths in mm for each of the concentrations were calculated were measured as supplemental data. In this case, it is also evident that the sediment from the Malonínský creek below the reservoir (Part 4) inhibits the growth of the shoots more than the sediment from the other parts. Average shoots lengths are shown in Figure 6.

The resulting trend is similar to the length of the longest root (Figure 5). A sample of the Malonínský creek over the reservoir (Part 1) inhibits the growth of the above-ground part less; the sample from the brook under the reservoir (Part 4) inhibits growth more. Samples from sedimentation reservoir (Part 2) and from Smolenská reservoir (Part 3) show interesting results at 0% substrate concentration, where the resulting values exceed the samples from Parts 1 and 2. At a substrate concentration of 25%, the increment values are lowest for all Parts outside 1. It is again apparent from Figure 6 that the higher substrate concentrations in relation to the sediment mean higher elevation of the above-ground portion.

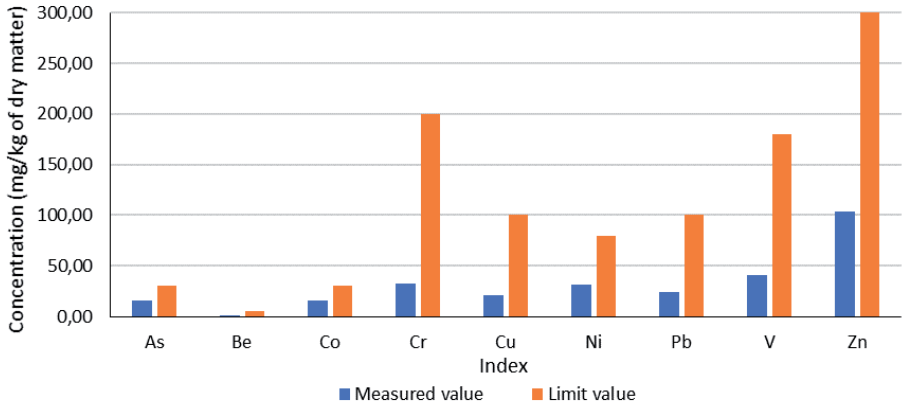
Figure 6 The average length of shoots (mm) for individual concentrations and parts of the area



Chemical analysis of the risk elements

When comparing the measured values of the concentrations of the individual indicators (mg/kg of dry matter) for the mixed sample from the Smolenská water reservoir, which were compared with Annex No. 1 of the Decree No. 257/2009 Coll. it is obvious that the limit values are not exceeded. The values closest to the limit are for arsenic and for cobalt. The arsenic concentration is 15.30 mg/kg of dry matter, representing 51% of the maximum admissible level. Cobalt concentration reaches 16.30 mg/kg of dry matter to reach 54% of the maximum admissible level. The graphically displayed values are shown in Figure 7.

Figure 7 Concentration in dry matter for individual indicators for mixed sample from Smolenská reservoir with comparison with limit values from Annex No. 1 of Decree No. 257/2009 Coll.



CONCLUSION

For the area of interest, part of the area around the Malonínský brook near Jevíčko was selected. The evaluation of the ecotoxicity of the sediment from Malonínský brook, sedimentation reservoir and Smolenská water reservoir was one of the objectives. Comparison of the concentration of the risk elements contained in the sediment from the Smolensk water reservoir with the limit values set out in Decree No. 257/2009 Coll., as amended, was the second objective. The methodology for the collection and evaluation of sediment samples has been developed. The area of interest was divided into 4 Parts. For each part of the territory, one single mixed sample was created. An ecotoxicity test according to CSS EN ISO 11269-1 was carried out in the laboratory. The chemical analysis of the risk elements was processed only for sediment from the Smolenská water reservoir. When comparing the measured values of the concentrations of the individual indicators for the mixed sample from the Smolensk water reservoir, which were compared with Annex No. 1 of the Decree No. 257/2009 Coll. it is obvious that the limit values are not exceeded. From the results of the ecotoxicity test, it can be seen that the mixed sample of sediment collected from the Malonínský brook in Part 4 does not reach the required values according to Decree No. 257/2009 Coll. and is ecotoxic. Mixed sediment samples from Part 2 and Part 3 are not ecotoxic, but the test results are closer to the limit. The sediment from Part 1 – above the Smolenská reservoir is not ecotoxic. The results of the complete test show that the most suitable ratio of substrate to sample mix is 3:1, so 75% substrate volume and 25% sediment. This is exactly what is prescribed by Decree No. 257/2009 Coll. when depositing sediment on farmland. Finally, it should be noted that it is inappropriate to consider the pond sediment as a waste, but to meet the requirements for concentration values of the risk elements, such as fertilizer. The sediment contains nutrients such as nitrogen and mainly phosphorus, which are important for growing crops. Increasing nutrient concentrations in aquatic systems is not the only consequence of sediment accumulation. Reducing water capacity in reservoirs is another consequence, which further reduces the retention capacity of the landscape. Unloading will increase the capacity of the reservoirs and this capacity can be used both in the case of floods and for a higher water supply in case of drought.

ACKNOWLEDGEMENTS

This research was supported by the project IP 35/2017 „Kapková závlaha jako nástroj k řešení problematiky sucha“ which is funded by Internal Grant Agency of Faculty of AgriSciences, Mendel university in Brno.

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CURRENT CONDITION OF IRRIGATION SYSTEMS IN SELECTED TERRITORY

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Abstract: This paper aims to map the state of the irrigation system in the selected area and the evaluation of its components. The area of interest is located near the Malonínský brook near the town Jevíčko. As part of the elaboration of the work methodology historical material has been obtained from the flow manager and the local government, which has been preserved to the present day. In addition, the area of interest was visited (in October 2016, March and August 2017), reconnaissance of the terrain and photo documentation of the current state of the constructions were realized. The collected information was processed and evaluated. In the area of interest there are 6 categories of constructions of surface watering system Malá Haná, which was built in the 1930s. This system is currently largely non-functional outside Smolenská Water Reservoir, which is maintained in a conditional state, and as the only one of the original irrigation system can be used for other functions or possibly incorporated in the future into a new irrigation system based on modern austerity technologies.

Key Words: soil irrigation, water retention, ditch, dam, watercourse

INTRODUCTION

Reasons of irrigation

Agriculture is a significant user of water resources in Europe, accounting for around 30 per cent of total water use. Irrigation can have two main purposes in relation to agricultural production: It can enhance the quantity of output; It can enhance the quality of output.

Allowing sufficient crop yields to provide food for humans and their livestock is the main reason for building the irrigation. According to Czech state standard 75 0140 (2016) irrigation is the artificial supply of irrigation water, sewage, fertilizers and other solutions for the maintenance of water needs and plant nutrition or for other purposes (Dwyer 2000).

The purpose of irrigation is to produce crops that have economic and social value. The continued productivity of irrigated agriculture will be critical as national and world populations grow and demand more food and fiber. Properly managed irrigation can increase crop yields, reduce risks commonly associated with agriculture, increase product quality, reduce pest pressures, and precisely deliver and manage nutrients (USDA 2001).

Division of selected irrigation systems according to CSS 75 0140 (2016):

Furrow irrigation is the way in which the soil is irrigated by soak from any of the irrigation furrows.

Infiltration irrigation is the way of irrigation, where the soil is moistened with any water from channels, ditches, furrows or underground pipes.

Spraying irrigation is a method of irrigation where water is sprayed on the irrigated surface in the form of rain.

Surface watering is a method of irrigation, in which the water flows down the surface of the soil in a thin layer, while enhancing it. There is a distinction between irrigation by belt, sallow, ridge and recesses from channels.

Flood irrigation is a method of irrigation, in which the irrigated plot divided into a lift is flooded with water.

Drip irrigation is a localized irrigation with a water distribution system with small diameters, from which water flows through batch elements such as drippers, streams, capillaries, etc. to the surface of the soil with low intensity, and softens the soil at the root ball of the plants.

MATERIAL AND METHODS

Methodics

This paper aims to map the state of the irrigation system in the selected area and the evaluation of its components

The area of interest is located near the town Jevíčko. The methodology of work and assessment of objects was compiled for the elaboration of the paper. The flow manager and local authorities have provided historical materials that have survived to present. The original design documentation of the irrigation system at the site was not retained. In addition, the area of interest was visited (in October 2016, March and August 2017), reconnaissance of the terrain and photo documentation of the current state of the constructions were realized. The obtained information was processed and evaluated using computer technology at the Department of Applied and Landscape Ecology.

Definition of the interest area

The selected area is situated to the west of the town of Jevíčko, located on the south-eastern edge of the Pardubice Region. Jevíčko is located 15 km south of Moravská Třebová and 16 km north of Boskovice. The area of interest is defined by the area of the area along the Malonínský brook from its confluence with the Jevíčko stream on the III/36612 road bridge, which is run over the Malonínský brook near Smolenská (see Figure 1).

Development of irrigation in the area of interest

Due to the fertility of Malá Haná, agriculture has been developed in this area and, at the same time, land improvement. The first officially recorded planning efforts to implement amelioration measures have been recorded since the mid-18th century. At the beginning of the 20th century, livestock farming was expanded, for which sufficient food had to be ensured, and at the same time the need to increase the protection of the area (especially crops and drying hay in the meadows) from the damaging effect of the floods. It was also necessary to drain the wetlands. This was the reason for the development of projects and the subsequent construction of the surface watering and flush irrigation systems. The irrigation system was built in the 1920s and 1930s (Coufal 1935).

The built system was unique in its time throughout the Czechoslovak Republic. At the same time, the construction of the meadow land was carried out in all the cadastres in Malá Haná, where it was built, and the fields were merged in several other cadastres.

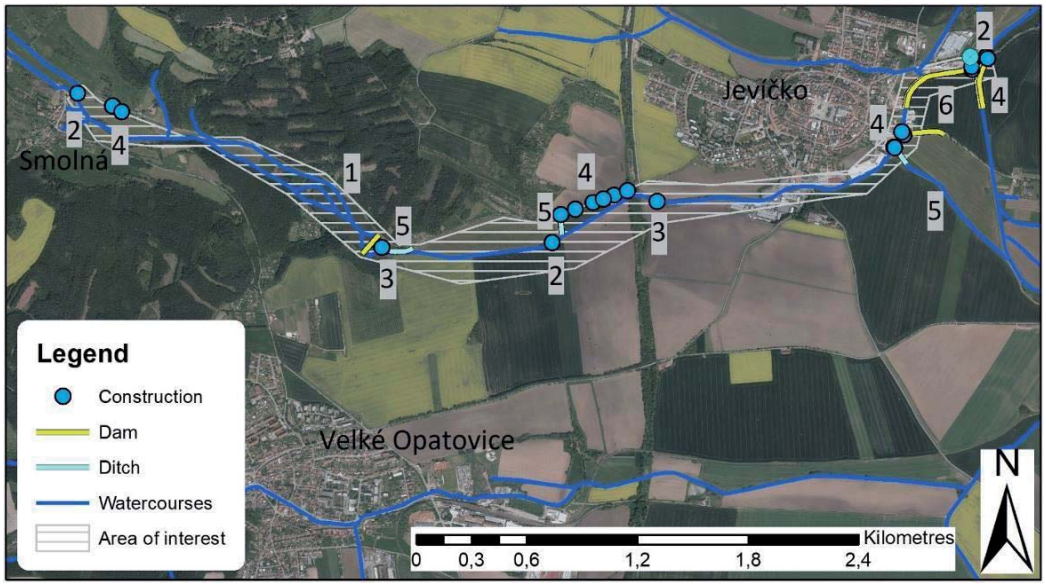
After the Second World War, the water irrigation from the Malá Haná area was abandoned, the built-up channels were mostly covered, but the concrete bays were left both in the flow channels and in the places of the irrigation channels. In the conversion of meadow cultures to arable land, surface watering was abandoned because of the increased use of mechanization and the possible replacement of the system by other modern solutions at that time (Benetin 1979). At present, all the buildings outside the Smolenská reservoir are dilapidated.

RESULTS AND DISCUSSION

Evaluation of irrigation system components

Several components of the surface watering system were identified in the area of interest and divided into six categories. The preserved components are shown in Figure 1.

Figure 1 The interest area with irrigation constructions.



Legend: Orthophotomap: Czech Office for Surveying, Mapping and Cadastre. Modified by author.

1 – Smolenská water reservoir

The Smolenská water reservoir is the main landscape-forming element in the area of interest and served as a source of water for the irrigation system in a period with a lower flow of water in the Malonínský Creek. Smolenská water reservoir in year 1933 is shown in Figure 2 on the left.

Figure 2 Smolenská water reservoir in year 1933 (on the left), Smolenská water reservoir in year 2017 (in the middle), A bridge with a floodgate at Jevíčka stream in 1933 (on the right)

At present, the reservoir is supplemented by a sedimentary pretension, which was established by the State Ameliorative Administration for the detention of the floodplains before entering the Smolenská reservoir. Together with the pretence, a pool was built at the inflow to the reservoir to increase the biodiversity of the area and support amphibians. In 2009, the Agricultural Water Management Administration completed the reconstruction of the security spillway and the discharge facility. At present, the Smolenská reservoir and the preserve are discharged due to the filling of the preload and the subsequent flow of the spills directly into the reservoir. A project to unload the reservoir and to reconstruct the discharge objects is being prepared. The current reservoir manager of adjacent streams is the Povodí Moravy s.p. Smolenská water reservoir in year 2017 is shown in Figure 2 in the middle.

Smolenská reservoir is currently the most conserved part of the original irrigation system. After measures to prevent settling of the sediment in the reservoir and its removal, the capacity of the reservoir will increase and therefore this higher capacity can be used for the supply of any newly built irrigation system or it will be used as a capacity to capture and transform the flood flows on the Malonínský Creek.

2 – The road bridges with the sluices

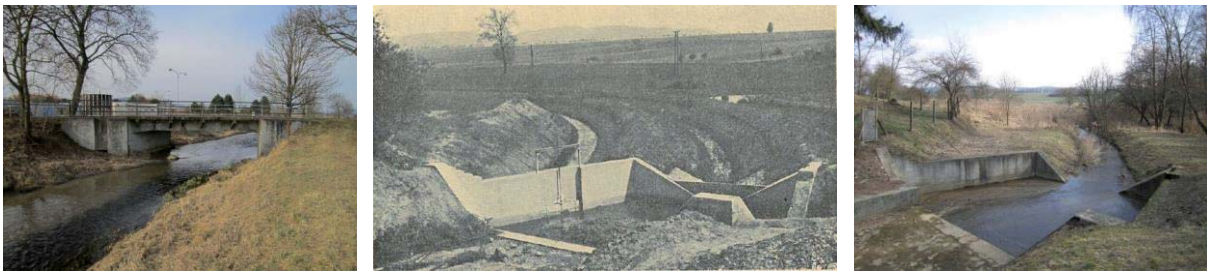
Water gates in road bridges or stand-alone were used to draw water from the watercourse. The bridges are concrete constructions, the control elements of the building were made of steel and a steel frame with a wooden panelling was used for the floodgates. The water was fed to the flood

drifts when the flow was blocked. The floodgate weir on the road bridge of the Jevíčko-Biskupice road had three fields with a height of shutters of 1.1 m. The sluice in the area over the highway had two fields with the lightness of 4.2 m and shutters 1.4 m high. Floodgate on the bridge in Smolenská had two fields with a shutters height of 1.48 m (Pernica 1997). A bridge with a floodgate at Jevíčka stream in 1933 is shown in Figure 2 on the right.

However, after the irrigation system was shut down in the post-war period, the flood shutters remained, as it was part of the road bridges, which were further used. In order to reduce the negative impact of the technical objects in the riverbed and due to the poor condition, during the period of stewardship of the State Melioration Administration, water gates were removed, only the bridges or concrete frame structures remained. For interest, some municipalities have wanted to keep the floodgates on the rivers for possible utilization of the accumulated water as fire water. A bridge with a floodgate at Jevíčka stream in 2017 is shown in Figure 3 on the left.

At present, the sluices in the road bridges are not functional and are not used even for emergency fire water accumulation. Comprehensive reconstruction will be required to ensure operational condition as part of road communications.

Figure 3 A bridge with a floodgate at Jevíčka stream in 2017 (on the left), A floodgate at Malonínský stream in 1933 (in the middle), A floodgate at Malonínský stream in 2017 (on the right)



3 – Independent floodgates

These objects were made up of a simple reinforced concrete structure with embedded steel elements for controlling and the possibility of reserving buoys. The shuttle in the area under the highway had two fields with a lightness of 4.2 m and a window height of 1.1 m. The floodgate at the mill was situated in a location under Smolenská reservoir on the tranquillity reservoir (Pernica 1997). A floodgate at Malonínský stream in 1933 is shown in Figure 3 in the middle.

At present, only a concrete structure that no longer contains steel elements or screens remains at the site under the motorway. The floodgate in the area under the Smolenská reservoir was partly removed during the reconstruction of the safety spill in 2009. A floodgate at Malonínský stream in 2017 is shown in Figure 3 on the right.

At present, these objects are not usable or do not exist. The mill drive was cancelled and the trough was covered.

4 – Setting devices, drains and aqueducts

The setting devices served in their full engagement to retain and drain the water in the pots and to create a drop of water across the meadows. The setting devices were made of concrete with a hole for a wooden stopper. The area around the bucket was tiled with tiles. The length of the overflow edges from the drive was 40–80 m. At present, the trays on the meadows above Smolenská Reservoir and in the area above the highway are preserved. At present, these objects are unjustified because the drives on which they were located no longer exist. Setting device at none exist drive in 2017 is shown in Figure 4 on the left.

Drains were an important part of localities where watercourses were lined with a low hill. So, the water could drain away. Nowadays the drainage is located in the embankments around Jevíčko, they are in a reserved position and do not fulfil their function. They cannot even perform a flood-proof function because the adjacent decks are no longer properly shaped and damaged in several places. They are more or less technically interesting object in the landscape. Example of drain in 2017 is shown in Figure 4 in the middle.

In the area of interest there is the last aqueduct in the area above the highway. This aqueduct served to transfer the drift stream over the waterline at another height level. Currently it does not fulfil its function and its use is not offered. The aqueduct in Jevíčko was removed. An aqueduct across nameless watercourse in 2017 is shown in Figure 4 on the right.

Figure 4 A setting device at none exist drive in 2017 (on the left), Example of a drain in 2017 (in the middle), An aqueduct across nameless watercourse in 2017 (on the right)

5 – Flooding drives

Drives were connected to a flooded property and brought water from streams to irrigated meadows. The drives have a trapezoidal shape with a sloping slope of 1:2 in cross-section to ensure easy maintenance. In the area of Malá Haná, the flood irrigation did not work well and surface watering was the main type of irrigation. The water flowing from the meadows was drained through the wastewaters back into the watercourse. Wastewater also served as drainage at higher blurring of the meadows. Care was taken to ensure that the meadows were not unnecessarily dry. An irrigation millrace with a floodgate in 1933 is shown in Figure 5 on the left.

Drives are mostly covered today. In the area of interest, part of one of them is located in the area below the Smolenská reservoir and in the area above the high. The drive, which enters the area of interest on the southern edge of Jevíčko, is currently part of drainage from the 70s of the 20th century.

Due to the predominant filling of the drives it is not possible to use them. Drills that have not been covered are currently part of a drainage whose functionality needs to be verified.

Figure 5 An irrigation millrace with a floodgate in 1933 (on the left), Damming along the Malonínský brook near Jevíčko in 2017 (on the right)

6 – Damming of flooded soil blocks

Around of the town of Jevíčko there is preserved flowing, which also belonged to the irrigation system. Damming was used to retain the excess water or settling of sediments.

Nowadays, the fence along the Malonínský brook is located close to its confluence with Jevíčka. At present, fencing is counterproductive, because in the case of smaller floods, the water cannot be poured out of the water into the surrounding fields. Damming along the Malonínský brook near Jevíčko in 2017 is shown in Figure 5 on the right.

However, with a higher flood flow, flood overflows may occur and the property is at risk. The drainage and permeable objects of the irrigation system in the dykes are already dilapidated and inoperative. Consequently, the remainder of the irrigation system cannot be considered as an effective flood protection measure.

CONCLUSION

Burton (2010) argues that the irrigation area worldwide has increased threefold over the last 50 years, from 94 million ha in 1950 to over 287 million ha in 2007. According to Čermáková and Mácová (2017) the total irrigated area in the Czech Republic increased from 17 113 hectares in 2015 to 25 003 hectares in 2016. In 2016 Czech agricultural holdings used mostly sprinkler irrigation (702 subjects), then micro-irrigation (382 subjects), surface irrigation (flooding, furrows) (72 subject) and 73 subjects exploited different irrigation methods. According to statistics provided by the Czech Statistical Office, it is evident that the current trend in irrigation of agricultural land in the Czech Republic is increasing in the long run. Today's spraying irrigation will be appropriate in the future to upgrade to more efficient irrigation techniques. Due to the current problems of drought, it will be advisable to carry out a thorough passportization of persistent irrigation systems and assess their usability for future use and incorporation into any of the planned irrigation systems. This will reduce the financial cost of building new systems.

For the area of interest, part of the area around the Malonínský brook near Jevíčko was selected. A survey of the surviving documentation was carried out and a survey of the terrain and the search for the existing irrigation system objects. A sophisticated irrigation system of the 1930s was used in the vicinity of Jevíčko (Coufal 1935). The irrigation was used to irrigate the meadows in the Malonínský stream floodplain and the Jevíčka stream. Fodder was used as a feed for livestock. In the post-war period, the irrigation was dropped and the irrigation channels were partly covered. After the Second World War, the grassland was largely converted to arable land and some soil blocks were drained. The conversion to the arable land in the Malonínský stream floodplain has brought negative consequences in the form of arable land ablation. This is currently visible in the Smolensk reservoir, where the sediment accumulates. Elements of the original surface watering irrigation system are still visible around the Malonínský stream. At present, it would probably not be profitable to restore this type of irrigation, but if it is needed, it will be useful to use its elements for a more modern way of irrigation. This will make it possible to use the Smolenská reservoir, which is currently the best preserved part of the original system. After measures to prevent settling of the sediment in the reservoir and its removal, the capacity of the reservoir will increase and hence this higher capacity will also be used for the supply of any irrigation system.

ACKNOWLEDGEMENTS

This research was supported by the project IP 35/2017 „Kapková závlaha jako nástroj k řešení problematiky sucha“ which is funded by Internal Grant Agency of Faculty of Agrisciences, Mendel university of Brno.

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THE EFFECT OF A WINDBREAK ON THE DEGRADATION OF SOIL AGGREGATES DURING THE WINTER SEASON

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Abstract: We have assessed the potential effect of a windbreak on the degradation of soil aggregates both on the leeward and windward sides. Soil samples were collected at the beginning and end of each winter season at a distance of 3-, 6- and 9-fold windbreak height. After collection, the samples were subjected to aggregate analysis to establish the erodible and non-erodible fractions. The analyses also included evaluation of the effect of meteorological conditions on the soil aggregate degradation. The factors that were evaluated to determine the degradation of soil aggregates were: the action of water and subsequent freezing/thawing of the soil surface. The pressure of the freezing soil moisture followed by washing result in disintegration of soil aggregates into erodible fractions, which in case of erosion-effective winds cause erosion events. Climatic data on the soil surface temperature and soil moisture state were obtained from the nearest professional station of the Czech Hydrometeorological Institute (Kuchařovice). For our study we selected a windbreak in cadastral area Tasovice nad Dyjí. The assessment was performed for three winter seasons, 2014–2015, 2015–2016 and 2016–2017.

Key Words: wind erosion, windbreak microclimate, soil structure, erodible particles

INTRODUCTION

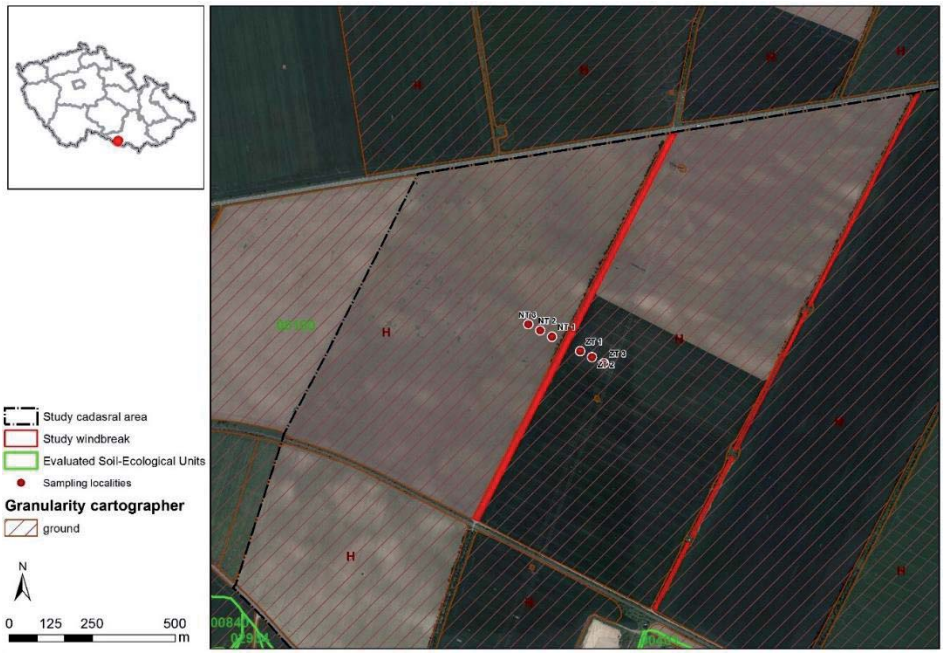
Erosion is a natural process in which the action of water, wind and other factors cause disruption of the soil surface and subsequent transportation of soil particles. Wind erosion thus represents a natural process of erosion consisting in disruption of the soil surface by the mechanical force of wind (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, Chepil 1952). 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 (Bullock et al. 1999). 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 (Logsdail and Webber 1959, DeLuca et al. 1992, Grogan et al. 2004). 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 (Skidmore a Powers 1982, Colazo a Buschiazzo 2010). The key role is played by the size of the soil particles, while differences in the particle shape have only little impact (Pasák 1970). 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 (Chepil 1958, Švehlík 2002, Holý 1994).

MATERIAL AND METHODS

Study area

The study area is situated north-east from the city of Znojmo. This area belongs to a warm, dry zone with mild winters. As a reference station we selected the meteorological station in Kuchařovice near Znojmo. The annual sum of precipitation is around 530 mm, peaking in June to August (monthly sum about 70 mm). The annual average air temperature fluctuates around 9 °C, also peaking in the period June-August around the average of 18 °C. Concerning the wind regimen, the prevailing winds come from north-west or west, with frequent occurrence of drying south-east winds. These climatic conditions show that the area is not adequately supplied by precipitation. The rains mainly display short-term character, and low precipitation is observed particularly during the first months of spring. Soil moisture is additionally reduced by high evaporation. The study area forms part of the ravine of the rivers Dyje-Svratka, characterized by variable undulating plain of altitude 200 m a.s.l. The favourable terrain relief allows intensive agricultural management that has negative impacts on the landscape character, which is monotonous, with large arable land blocks. Non-agricultural land is mainly situated around the meandering river Dyje and its tributaries and close to the urban sites. The area is situated in a territory formed by quarternary rock. The alluvial position of Dyje mostly presents non-calcareous sediments that gave rise to alluvial soils. Some localities show calcareous alluvial sediments, on which chernozems (Phaeozems) have evolved. The study localities in cadastral area of Tasovice nad Dyjí are shown Figure 1.

Figure 1 Survey map of study localities



Brief description of the windbreak in Tasovice nad Dyjí

Windbreak – north-west from the built-in area of village Tasovice, full-grown closed stand of the wooden belt type with a shrub layer, with a field path leading through its middle; the prevailing species are sycamore maple (up to 70%), common oak (25%), aspen (admixture), Norway maple (admixture). The shrub layer is formed by: common elder, canary grass, false acacia, wild brier, hawthorn sp., and filbert. These 5–6 rows of trees represent a high-quality windbreak with favourable species composition and diversity, and a shelter for both small and hoofed game (roe deer).

Pedological analyses of selected soil samples from the investigated localities

The soil samples were collected at the beginning and end of the winter season. The sampling sites were established at both the leeward and windward sides of the windbreak. The distances were determined by the multiples (3x, 6x, and 9x) of the windbreak height. After collection, the soil samples were subjected to aggregate analysis determining the erodible and non-erodible fractions. The soil

samples were collected from a level, smooth soil surface (max. to 2.5 cm). Samples were collected as a mixture at about one metre intervals. To establish the erodible or non-erodible fraction, we used a 0.8 mm sieve. For further assessments we used the percentages of erodible fraction. The results from each period were entered into the database of results. In each processing step we determined the per cent difference between the erodible fraction representation at the beginning and at the end of the winter season. In all the studied seasons, the same agro-technical processing was applied to both the leeward and windward sides of the investigated windbreak in cadastral area of Tasovice nad Dyjí.

Analysis of meteorological conditions

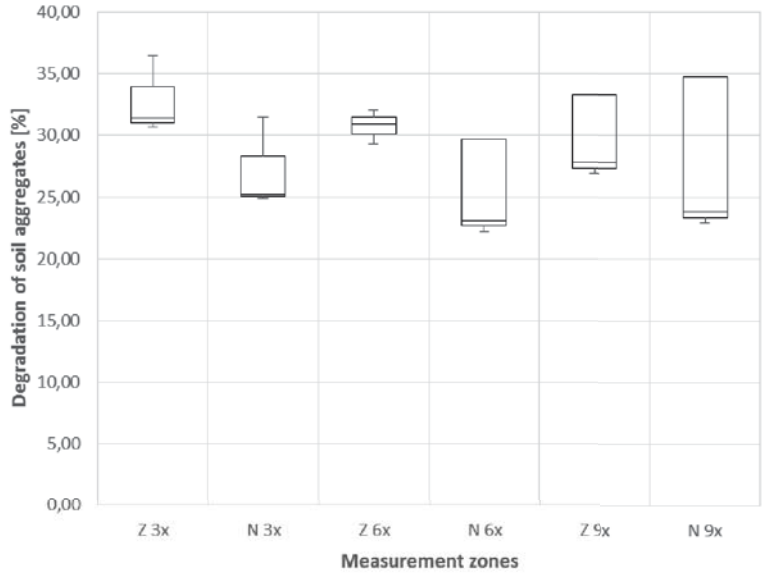
For winter seasons 2014–2015, 2015–2016 and 2016–2017, we performed analysis of the climatic conditions. We obtained hourly data on the temperature at the soil surface and on the soil moisture in these seasons from the Czech Hydrometeorological Institute (CHMI). The climatic data were obtained from the closest professional CHMI station in Kuchařovice.

RESULTS AND DISCUSSION

Pedological analyses of selected soil samples from the investigated localities

For the final analysis we particularly selected the windbreak in cadastral area Tasovice. The reason was the comparable agro-technical soil processing from both the leeward and windward sides of the windbreak. The analysis of the obtained values was performed for three seasons, 2014/2015, 2015/2016 and 2016/2017. The outputs of the analysis are represented in a box graph (Figure 2) showing an increase in erodible fraction in the indicated distances from the windbreak (Z- leeward, N-windward). Regardless of the windbreak side and comparison of medians, the highest increase in erodible particles was observed on the leeward side at the distance 3x (31.4%). Comparable median values were also observed on the leeward side at the distance 6x (30.9%). In contrast, the lowest increase in erodible fraction was recorded on the windward side at the distance 6x (23.10%) and 9x (23.8%). The median values at these distances have shown a minimum difference. When comparing the sites closest to the windbreak (Z 3x and N 3x), the difference in the median values reached 6.2%. The difference was also observed in both maximum and minimum values.

Figure 2 Analysis of degradation of soil aggregates at the studied windbreak Tasovice



A further step in the evaluation consisted in correlation analysis between the distance from the windbreak and the per cent increase in the erodible fraction. The correlation analysis was processed for both the windward and leeward sides of the windbreak. Analysis for the windward side showed correlation 0.19 and for the leeward side -0.19. The correlation analysis was also applied to the distance from the windbreak regardless of the windbreak side. The correlation value was found to be 0.06. These correlation values do not suggest an unequivocal effect of the distance from the windbreak on the soil particle degradation.

Analysis of meteorological conditions in relation to the soil aggregate degradation

For analysis of the climatic conditions we used data from CHMI (professional station Kuchařovice). For the analysed winter seasons, we obtained hourly recordings of the temperature above the soil surface (TPM). Based on these data we determined the number of periods of freezing/thawing in particular months and seasons. The beginning of the period was determined by the initial value -5 °C (number of events of -5 °C). Each period was defined by duration in hours. The analysed periods were terminated when the temperature exceeded 0 °C. The temperature of -5 °C was set as the average value of freezing of the soil solution (Šantrůčková 2014). The Table 1 below contains information on the number of soil states facilitating degradation of the aggregates. These soil states are: wet soil surface (soaked – stagnant water in smaller or larger puddles), snow or melting snow (with or without ice) covering less than half of the soil, and snow or melting snow (with or without ice) covering more than half of the soil but not all of it. The highest number of the evaluated soil states (14) was observed in the season 2015–2016. This season also recorded the longest duration of TPM events (361 hr). The value for erodible aggregates was obtained from the results of aggregate analysis based on samples collected at the distance equal to 9-fold windbreak height for both the leeward and windward sides. The distance 9x was selected because the effect of the windbreak on the degradation of soil aggregates was eliminated.

Table 1 Analysis of the number of periods from the data measured on the surface (TPM), including soil state – CHMI station (Kuchařovice)

Season	Erodible aggregates [%]	Number of TPM periods	Duration of TPM periods [hr]	Number of soil states
2014–2015	25.8	19	283	11
2015–2016	42.3	18	361	14
2016–2017	24.9	18	270	10

For analysis of the effect of selected meteorological conditions we calculated the correlation matrix in Table 2. The correlation analysis was performed for the number of TPM periods, duration of TPM periods and number of soil states. The results of the analysis show that the most significant correlation was found for the duration of TPM periods, 0.9963, and for the number of soil states, 0.9807. The correlation for the number of TPM periods reached a negative value, -0.4597. The correlation results unequivocally show an association between the TPM duration and number of soil states on the one hand and the increase in erodible aggregates on the other hand. It should be mentioned that this analysis was only performed for three winter seasons. For complete demonstration, the analysis should include more winter seasons and more study localities.

Table 2 Correlation matrix of the effect of monitored meteorological conditions on the soil aggregate degradation

Evaluated factors	Erodible aggregates – correlation factor
Number of TPM periods	-0.4597
Duration of TPM periods [hr]	0.9963
Number of soil states	0.9807

CONCLUSION

The process of wind erosion is also influenced by winter seasons, when meteorological conditions lead to degradation of soil aggregates. This degradation is particularly influenced by the action of water with subsequent repeated freezing/thawing. The activity of these factors leads to disintegration of the soil aggregates into erodible fractions, which in the case of erosion-effective wind cause erosion events. We analysed the dependence of soil aggregate degradation in the winter season on the indicated distances (3x, 6x, and 9x) from the windbreak for both the leeward and windward sides. The correlation analysis showed correlation 0.19 for the windward side and -0.19 for the leeward side. This analysis was also applied to the distance from the windbreak regardless of the windbreak side. In this case, the correlation value was found to be 0.06. These correlation values do not suggest an unequivocal effect of the distance from the windbreak on the soil aggregate degradation. The detailed analysis

of the windbreak effect on the degradation of soil aggregates must also include data on the meteorological factors related to the localities of the soil sampling. The correlation analysis of the effect of selected meteorological conditions shows that the most significant correlation was observed for the duration of the TPM period, 0.9963, and the number of soil states, 0.9807. For the number of TPM periods, the correlation reached a negative value, -0.4597. The correlation analysis results show unequivocally that there is a link between the TPM duration and number of soil states on the one hand and the increase in erodible aggregates on the other hand. The analysis was only performed for three winter seasons. To validate the hypothesis, data for more winter seasons and more study localities will have to be statistically assessed.

ACKNOWLEDGEMENTS
The results of this work are part of projects NAZV Nos. QJ1330121 and QK1710197.

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CLIMATE CHANGE AND ADAPTATION STRATEGIES IN BANGLADESH

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Abstract: Bangladesh as a country lying in the most attackable place to climate hazards – delta of three large rivers with a high population density and high poverty, is one of the most vulnerable areas to impacts of climate change in the future. There are three main natural climate hazards which are very likely to be worse in the future: the floods, the tropical cyclone activity connected with the storm surges and the droughts. The Government of Bangladesh has published the strategic document *Bangladesh Climate Change Strategy and Action Plan 2009* with six main issues to ensure decreasing the adverse impacts of climate changes. It focuses on ensuring basic needs of its inhabitants, developing infrastructure and urbanization with emphasis on low carbon emissions, improving disaster management, research and strengthening institutions. In this research there is critically analysed the main strategic document for adaptation plan as well as other studies and discussed strengths as well as weaknesses and limitation of the strategic plan. Some of the weaknesses may be unsuitability for entire area, disregarding local conditions and local people needs and their abilities to adapt and also unappropriated money distribution along right prioritizing of the adaptation plans.

Key Words: strategic policy, floods, tropical cyclones, storm surges, droughts

INTRODUCTION

Bangladesh as a relatively small country is nowadays considered as one of the most affected country by the climate changes (Huq 2001, MoEF 2009, Huq 2011, Kreft et al. 2016). With its high density of inhabitants, who are living in relatively poor conditions, situated in a vulnerable zone to natural disasters, it becomes one of the most vulnerable areas to climate conditions in the world (MoEF 2009, Huq 2011). Moreover, according to IPCC (2013) it is very likely that bad climate conditions in this area will worse in the future.

Bangladesh is situated on one of the largest deltas in the world – in the mouth of three large rivers: the Brahmaputra, the Ganges and the Meghna. The fertile alluvium soils attracted people in ancient history as there were good conditions for agriculture. Bangladesh made independent from Pakistan in 1971 (MoEF 2009), so it is a relatively young country, but with a long settlement history. In its old empire history, it has been connected with India and surroundings for a long time known as a Bengal. Actually, it was the first empire built in this area (Abu´l-Fazl 1902). One can notice a similarity of the names Bengal and Bangladesh. According to Abu´l-Fazl (1902) the name Bengal was originated from the name Bung and "the suffix ´al´ at the Bengali language means an embankment or raised ground, which is placed around a garden or cultivation, so that floods may not enter it". This refers to the fact, that already ancient people living here were accommodated to the local ´disastrous´ climate conditions unsuitable for agriculture and living.

Since Bangladesh is independent of Pakistan after Bangladesh Liberation War in 1971, it has achieved couple of economic and human developmental goals. Its GDP has more than tripled, food production has increased, the population growth rate has declined, the percentage of people living in poverty has also declined and its Human Development Index has improved (MoEF 2009). In spite of these successes, the poverty in the country is still enormous. With a population of more than 150 million people in 2012 in a relatively small area of 58 000 square miles, it has one of the biggest population density in the world – more than 1 200 persons per square kilometer in 2012 (Younus 2014). With a high-growth rate the population in the country is expected to rise above 180 million

by 2025 (Younus 2014). According to Ministry of Environment and Forests, Government of the People´s Republic of Bangladesh (2009) more than 50 million of Bangladeshis still live in poverty. According to another author, Huq (2011), nearly 50% of them live below the poverty line. This poverty and high population density makes the country one of the most vulnerable areas to nature hazards.

MATERIAL AND METHODS

In this research, the planning document of strategic action plans to adaptation to climate change in Bangladesh was analyzed by critical thinking as an act to determine the validity of a given claim, views and opinions. It is a process of conceptualizing, analyzing, synthesizing, and evaluating information to reach an answer or conclusion. The strategic document *Bangladesh Climate Change Strategy and Action Plan 2009* (BCCSAP) was published in 2009 by Ministry of Environment and Forests (MoEF), Government of the People´s Republic of Bangladesh and it was downloaded from official page of International Union for Conservation of Nature in English language. As well as the planning document BCCSAP, there were critically analyzed also other studies and researches of natural climate hazards, climate change, adaptation options and situation in Bangladesh published by scientists all over the world.

Next, by inductive thinking drawing on different facts, concepts and opinions in scientific papers, there were determined main natural climate hazards in Bangladesh, evidence and impacts of climate change in the country and who is the most affected by climate change in Bangladesh. Then, the planning document of adaptation actions is also analyzed and discussed in its six issues, its strengths as well as weaknesses are critically determined and there are also given some suggestions for improve or mention other relevant issues and facts.

RESULTS AND DISCUSSION

Natural climate hazards in Bangladesh

According to the statement of Ministry of Environment and Forests (2009) there are several climate hazards in this country like floods, tropical cyclones and storm surges and droughts.

- **Floods**

Floods are often considered as the biggest hazard in Bangladesh (Younus 2014). According to certain authors they are the main obstacle to development of the country (Younus 2014). About 70% of the area is cultivated (Younus 2014, MoEF 2009). Bangladesh is mostly low and flat, two-thirds of the land lies less than five metres above sea level (MoEF 2009). There are changing dry and wet seasons (monsoon rainfalls). These consequences lead to seasonal floods once a year. In an average year, approximately 25% of the country is under the floods (MoEF 2009). People who live in those areas have adapted through the years by building their houses on raised mounds and cultivating special varieties of crops, mainly rice during various seasons. However, once in four or five years there is a serious flood which covers more than 60% of the area and causes several damages to housing, agriculture and infrastructure. Also river bank erosion can occur and it causes losses on agricultural land (MoEF 2009).

- **Tropical cyclones and storm surges**

Another big climate hazard in the area is a tropical cyclone activity and storm surges. The strong winds which occur approximately once in three years can result in storm surges up to seven metres high (MoEF 2009). There are losses in human lives, their housings and livelihoods. Mostly affected are coastal communities. In last three decades the government has invested an effort in building of cyclone shelters, and improving early warning systems, which seems to be effective way as it has been seen in the huge tropical cyclone Sidr in 2007 when the number of fatalities was ´only´ 3 500 compared to the similar cyclone in 1991 of 140 000 lives lost (MoEF 2009).

- **Droughts**

In the northwestern region of the country there is dominant another climate hazard – droughts. This part of the country has, in general, lower rainfall that the rest one. Droughts occur also seasonally and can cause high losses of the crop yields, thus a lot of poor people can suffer from losing their livelihood (MoEF 2009).

Climate change – evidence and impacts

In last decades there is a changing in the global climate system caused by anthropogenic releasing of greenhouse gases to the atmosphere, according to IPCC (2013). In the future, it is very likely that warming of the surface atmosphere temperature and ocean temperature will continue, contrasts between wet and dry seasons will increase, the glaciers will continue melting down and sea level will rise (IPCC 2013). Bangladesh as the country suffering from climate hazard very frequently, with a very high population density of poor inhabitants will be very likely affected in a great extent by the anthropogenic-caused climate changes.

- **Floods**

One of the expected changes in the future are heavier and more erratic rainfalls connecting with the major climate hazard in the country, floods (MoEF 2009). According to Nishat and Mukherjee (2013), there is already an increasing trend in an amount of rainfall during 1950–2010 in a lot of parties of the country and it is likely that it will be further increasing. The higher rainfalls during monsoonal seasons will cause higher river flows and river bank erosions resulting in damages to agricultural land, housing, infrastructure and livelihood (MoEF 2009). Next, according to Younus (2014), farmers adapted to the natural conditions make their decisions about crop production based on traditional predictive factors relation to natural phenomena of flooding duration, extent and timing. With more erratic rainfalls the farmers cannot be able to grow their crop. Also, melting of the Himalayan glaciers leading to higher river flows during the warmer months of the year will be another non-natural phenomena very likely occurring during the next decades and discredit the farmer´s traditional predictions.

- **Tropical cyclones and storm surges**

There is also expected rising sea level and increasing in frequency and severity of tropical cyclones (MoEF 2009) caused by warming of the land and the ocean in coastal areas. It is estimated that there is a threat of displacement of 6 to 8 million people by 2050 due to sea level rise and increase in both cyclone and storm surges (MoEF 2009). The other effect of rising sea level is saline water intrusion up coastal areas, which gets into groundwater and leads to loss of drinking water (MoEF 2009). There is a study about correlation between high salinity levels in drinking water and impacts on health of people, where especially pregnant women are threatened (Khan et al. 2011). However, the rising sea level is probably the least threaten impact of climate change in Bangladesh with its long time scale and certain uncertainty, because it will be probably seen maybe after more than 20 years (MoEF 2009).

- **Droughts**

The last enormous climate hazard in Bangladesh, the drought in northwestern part of the country will also be very likely occurring more frequently or in a higher intensity (MoEF 2009). According to measurements of Nishat and Mukherjee (2013), there is an increased trend in both minimum and maximum temperatures in selected observed stations in the country and it is very likely that increasing will continue in the future. The higher temperatures or longer durations of the droughts in certain parts of the country will increase the disability of the local communities to grow the crop and provide their livelihood. Next, increasing the droughts and also a humidity of the weather in some parts of the country can also cause increasing of occurrence of some diseases, for example, water-borne and air-borne diseases as bacteria and parasites breed faster in warmer and wetter conditions (MoEF 2009).

- **Who is the most affected?**

The poorest people often live in slums and informal settlements, which do not give required protection against extreme climate events and their settlements are often situated in low-lying

and the most threatened parts of the coastal areas and river sides. Thus, the poorest people are most threatened by adverse impacts of climate change. It is very likely that millions of people will be forced to migrate due to floods, river bank erosions and losses of coastal areas.

Adaptation strategy in Bangladesh – strengths, weaknesses and suggestions

Despite all these huge risk consequences of climate change on lives of tens of millions people in Bangladesh, the Government of Bangladesh is aware of importance of adaptation strategy policies. According to Huq (2011), "Bangladeshi scientists were among the first in the developing world to study the potential impacts of climate change". In 2009 the MoEF published the *Bangladesh Climate Change Strategy and Action Plan 2009* (BCCSAP) where they are focusing on six main issues to be improved.

- **BCCSAP issue 1: Food security, social protection, and health**

The first issue concerns food security, social protection, and health of the people in the country (MoEF 2009). It concludes the developing of climate-resilient crops, such as varieties of rice that are resistant to floods, salinity and drought, access to drinking water, basic health and education services and insurance policies (Huq 2011). The farming process and crop choice is highly discussed in scientific researches according to changing climate in Bangladesh. There are couple of researches about growing crops in the country with climate changing conditions, for example Ruane at al. (2012) and Moniruzzaman (2014) in their research they are comparing growing traditional varieties of rice Boro, Aman and Aus according to changed climate conditions with a results of Boro needing more irrigation and Aman being the most resilient to climate change. However, it is important not to focus only on common (modern) methods of growing crops based on high yields, but also to inspire from some local farmer´s age-old agricultural practices in wetland areas of the country like floating gardening or shrim farming. In their study, Al Pavel et al. (2014) suggest floating bed cultivation as very suitable in areas under flood water for 7 or 8 months, which gives another opportunity how to mine from longer-lasting floods in the future. To conclude, it is really necessary to put the issue of ensuring basic human rights like livelihood, protection and health on the first position of the strategy plan in any country. Although, in specific actions how to reach such a goal, it is also necessary to look at the local communities, learn from them, build on their knowledge of their local environments and ensure that proposed changes meet their needs (MoEF 2009).

- **BCCSAP issue 2: Comprehensive disaster management**

The second issue of the country´s adaptation strategy plan is to improve disaster management with focusing on building cyclone shelters along the coastal belt and improving forecasting of disaster climate events (MoEF 2009). According to government of Bangladesh, country has already built over 2 000 cyclone shelters and improved forecasting of disaster events (mainly cyclones) and early warnings, and it has succeeded in saving lives of people in cyclone activities in last 10–15 years (MoEF 2009). Although, the weaknesses of this issue are uncertainties in forecasting and a very short time for preparing for the event before, from the point in forecasting, it is likely to happen. Another fragile point is that people are usually unwilling to go to the shelters and leave their croplands and houses behind (MoEF 2009).

- **BCCSAP issue 3: Infrastructure development**

The third issue is to develop and repair existing infrastructure to provide people protection. The infrastructure concludes coastal and river embankments and urban drainage systems (MoEF 2009). However, there is widely discussed the coastal and river embankments adverse impacts on environment and their long-term effectiveness in Bangladesh. While in some areas the social and environmental effects of embankments are positive, embankments along some rivers have caused couple of problems, for example bank erosions and lack of cropland, reducing capture fisheries, changes in aquatic ecosystems and water quality in some areas (Nishat and Mukherjee 2013).

- **BCCSAP issue 4: Research and knowledge management**

In its fourth issue the country is focusing on improving research mainly in the area of climate change impacts and adaptations and improving knowledge management. It focuses on modelling

climate change scenarios by applying global, regional and national climate change models (MoEF 2009). Improving modelling of climate change scenarios can improve preparedness of the government on taking such actions to protect its inhabitants as well as its environment. However, the scenarios, mainly those based on global models, can have big uncertainties and large-scale analysis can have its limitation when used in local condition. The integration of local people knowledge and their experiences with scientific knowledge can improve disaster risk management very much (IPCC 2012).

- **BCCSAP issue 5: Mitigation and low-carbon development**

Next, country with its very low contribution to the generation of greenhouse gases compared to developed countries wants to ensure that its urban development and energy supply development will have low greenhouse gas emissions in the future. Also they are taking part in a reforestation programme and expanding of mangrove forests along the shoreline (MoEF 2009). However, it can be a huge obstacle for such poor country like Bangladesh in its further development to build that relatively more expensive infrastructure with low carbon emissions. For example, in their study, Bala et al. (2014) propose transition to using renewable energy in the future energy supply development of the country, however it would depend on large technology imports in its first phase and thus, a lot of money needs to be invested in it.

- **BCCSAP issue 6: Capacity building and institutional strengthening**

The last issue is focusing on strengthening the position of government, ministries and agencies in Bangladesh to take a leadership position on matters related to climate change both in national and international level (Huq 2011). However, worldwide, there are also various opinions about who has to or has not to take a leadership role in adaptation to climate changes. For instance, according to ex-president of the Czech Republic, Klaus (2010), it is important not to push any climate changes above fundamental issues of liberty and democracy. It is also important to be humble of spontaneous evolution of human society and be aware of importance of perception of every person to nature and local environment.

- **Weaknesses of the adaptation plan and proposed suggestions**

Finally, in its BCCSAP, the government does not analyse the issue of possible future migration of people affected by river floods and rising sea level. In BCCSAP it states that several million people could be displaced by 2050 (MoEF 2009). However, due to high population density, it is not so easy to rehouse people inside the country. The connection between climate change and migration of people mainly living in low-lying coastal zones with high population densities has rose among scientists and policy-makers in last years (Stojanov 2016). A migration adaptation strategy would be recommended for Bangladesh. For example, adaptation policy of the other country, Maldives, which is very likely in more extreme position than Bangladesh with its threat of losing the entire area situated on low-lying islands, is to buy a land in another country and in such way to prevent being environmental refugees living in tents for decades in the future (Ramesh 2008).

CONCLUSION

To conclude, Bangladesh as a country lying in the most attackable place to climate hazards, is one of the most vulnerable area to impacts of climate change in the future. The three natural climate hazards, the floods, the tropical cyclone activity connected with the storm surges and the droughts, are very likely to be worse in the future. The Government of Bangladesh is aware of those increasing risk hazards in its area and has planned to do couple of adaptation actions decreasing the adverse impacts of climate changes. In its strategy action plan BCCSAP it focuses on ensuring basic needs of its inhabitants, developing infrastructure and urbanization with emphasis on low carbon emissions, improving disaster management, research and strengthening institutions. Along these strategy planning actions it is also important to be aware of their limitation, e.g. unsuitability for entire area, disregarding local conditions and local people needs and their abilities to adapt and also unappropriated money distribution along right prioritizing of the adaptation plans. It would be also recommended to have a migration adaptation strategy for Bangladesh due to high probability of losing of inhabitants´ homes and country´s already very high population density.

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A COMPARATIVE ANALYSIS OF THE DYNAMICS OF CHANGES IN WASTE ACCUMULATION INDICATORS IN SELECTED SUBURBAN COMMUNES – CASE STUDY

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Abstract: Knowledge of the technological characteristics of municipal solid waste (mixed and segregated) such as accumulation indicators provides the necessary information indispensable, for example, in the choice of appropriate waste treatment method and is essential in the planning process of the waste management system. The aim of the study was to carry out a comparative analysis of changes in mass accumulation index of mixed and segregated municipal solid waste in selected rural (suburban) communes in Poland in order to identify trends of changes in the accumulation of waste. In addition, the objective of the study was to determine the relationship between the calculated indicators and the socio-economic factors that can impacted on them. Data on waste for the period 2013–2015 was obtained from the MIKI Company in Krakow (Poland). In all analysed communes, the indicators of mixed municipal solid waste showed an upward trend; however, the efficiency of segregation in rural areas decreased which negatively reflects the ecological awareness of the inhabitants.

Key Words: waste accumulation, municipal solid waste, dynamics of change

INTRODUCTION

Municipal solid waste is household waste and waste from other generators which do not contain hazardous wastes which, by their nature or composition, are similar to household waste (JoL 2013, No 21 as amended). Other generators of municipal solid waste include *infrastructure facilities* such as government agencies, offices, educational institutions as well as small trading and service companies. In 2013, an average resident of the European Union produced 481 kg of municipal solid waste. Poland, with the rate of 273 kg/person/year, is one of those countries in Europe that produces significantly less waste than the EU average (Szul and Nęcka 2016). In Poland, the amount of municipal solid waste generated is assessed on the basis of the data published by the Central Statistical Office (CSO), which come from the reporting, the register as well as the population balance and housing stock in a given calendar year (Zbroński 2014). Waste treatment and disposal plants are an additional source of information on the mass of generated waste (den Boer et al. 2009).

Studies on the properties of municipal solid waste, including accumulation indicators, are regularly performed mainly for urban waste in large cities (Felice 2014, Gomez et al. 2008, Sieja 2006, Tran et al. 2014). The analyses and tests of municipal solid waste are also carried out in rural communes (Adamcova et al., 2016, Jamróz and Generowicz 2012, Malinowski and Kopytko 2014, Przydatek 2016, Przydatek et al 2017, Świerk and Twardy 2008); however, on a significantly lower scale and much less frequently due to the high costs of such studies (resulting primarily from the necessity of parallel analysis of the morphological composition of waste). Some trends in changes in the waste accumulation indicators can be identified for cities where research was repeated. Sieja (2006) concluded that the indicators of mass accumulation in large cities (over 500 000 inhabitants) increased systematically. In Poland about 77% of collected municipal solid waste was generated in urban areas, whereas in rural areas it was about 23% (NWMP 2016). The lower share of waste from the rural areas may result, among other things, from the management

of some waste groups within residents' property (Malinowski 2013). Typical suburban areas that is rural communes directly adjacent to the main city of the agglomeration were often overlooked in these studies. The analysis of the accumulated waste in these communes, mainly inhabited by people who commute to work in the main city of the agglomeration, is interesting from a cognitive perspective and demonstrates the inhabitants' behaviour of these communes. The research determining the impact of socio-economic factors on the amount of municipal solid waste produced are often based on the calculated indicators (Lebersorger and Beigl 2011, Purcell and Magette 2009, Szul et al. 2017) to better understand the waste generation mechanisms and trends that occur in this regard.

The aim of the study was to analyse the mass accumulation indicators of mixed municipal solid waste and selectively collected waste in 3 rural communes (Figure 1) located in the agglomeration of Krakow (Liszki, Skawina and Biskupice) so as to determine the dynamics of changes in the accumulation of waste on a monthly basis. In addition, the research attempted to show the influence of selected socio-economic factors (which characterize these communes) on the mass of generated waste.

Figure 1 Location of the analysed suburban communes

MATERIAL AND METHODS

Data on waste was obtained from the MIKI company in Krakow which deals with the transport and management of waste from the area of the Krakow agglomeration. The analysis of raw data provided by the company allowed to determine the mass of municipal solid waste (mixed and segregated) collected from individual communes (on a monthly and yearly basis) which was then utilised to calculate the mass accumulation indicators of mixed waste $a_{ij(z)}$ and selectively collected waste $a_{ij(s)}$. The indicators determine the mass of solid waste collected in a given unit expressed in units of mass per capita. The mass accumulation indicator was calculated on the basis of the dependence (1):

$$a_{ij(z,s)} = \frac{\sum_{i=1}^{d} m_{ij(z,s)}}{M_i \cdot d_{i(z,s)}} \cdot 365 \ [kg/M/rok] \qquad (1)$$

Legend: m – the mass of collected municipal solid waste mixed (z) or selectively collected waste (s) from a given commune (i) during a given month/year (j) [kg]; M – number of residents [-]; d – number of days of collection (for month d = 30.8); 365 – number of days per year.

The efficiency of segregation e_{ij}, which is expressed as the proportion of waste selectively collected in relation to all collected municipal solid waste. The efficiency of segregation was calculated on a monthly and yearly basis (2):

$$e_{ij(s)} = \frac{m_{ij(s)}}{m_{ij(z)} + m_{ij(s)}} \cdot 100 \ \ [\%] \qquad (2)$$

The average chain index of dynamics of changes $\bar{i}$ in waste accumulation (Zeliaś 1988) was used to define trends and determine the dynamics of changes in waste accumulation (3):

$$\bar{i} = \sqrt[n-1]{\frac{y_1}{y_0} \cdot \frac{y_2}{y_1} \cdot \frac{y_3}{y_2} \cdot \ldots \cdot \frac{y_n}{y_{n-1}}} = \sqrt[n-1]{\frac{y_n}{y_0}} \ [-] \qquad (3)$$

Legend: y_0 – *value of the studied phenomenon in the period taken as a basis for comparison;* $y_1, y_2, y_3, \ldots, y_n$ – *value of the waste accumulation indicator in subsequent years.*

Based on the above calculated indicator, the average annual change rate $\overline{T}$ was determined (4):

$$\overline{T} = (\bar{i} - 1) \cdot 100 \ [\%] \qquad (4)$$

The average rate is interpreted as the average increase (+) or (–) in waste mass expressed as a percentage. The calculation of the indicator best illustrates a multi-month trend.

The two-dimensional statistical analysis, the r-Pearson correlation coefficient in the STATISTICA 11 program was utilized to determine the relationship between the calculated indicators of waste accumulation and the selected factors characterizing the investigated communes (among others: population density, age and employment structure, water and gas consumption).

RESULTS AND DISCUSSION

Indices of waste accumulation

Table 1 shows the results for individual communes from the study period. Among the examined communes, the highest average mass accumulation indicator of both mixed and segregated waste was noted in the municipality of Skawina. The lowest average mass accumulation indicator for mixed and segregated waste was observed in the Biskupice commune. The values obtained for the mass accumulation indicators of mixed municipal solid waste did not exceed the country's average of 273 kg/M/year in 2013. Taking into account the ranges of mass accumulation indicators for different types of settlement units, only the Liszki and Biskupice communes fell into the range typical of rural areas. The accumulation of waste in the municipality of Skawina was representative of small and medium-sized towns.

Table 1 Basic characteristics of mass accumulation indicators of mixed and segregated waste collected in communes questioned

Parametr	Unit	Mixed municipal solid waste			Segregated municipal solid waste		
		Liszki	Skawina	Biskupice	Liszki	Skawina	Biskupice
Minimum	kg/M/year	59.5	196.3	63.0	37.6	62.3	25.4
Maximum		168.0	345.2	103.3	65.2	101.4	49.8
Average		98.2	260.6	86.3	52.9	80.1	39.8
Standard deviation		27.2	47.8	12.3	7.7	10.1	6.2
Coefficient of variation	-	0.28	0.18	0.14	0.15	0.13	0.16

In the Liszki commune, the largest accumulation of mixed waste occurred in August 2015 (168.0 kg/M/year), while the lowest in November 2013 (59.5 kg/M/year). The highest accumulation indicator of segregated waste was observed in August 2014 and the lowest in July 2013. The average mass indicator of mixed waste in the Liszki commune was significantly lower than the value obtained in the research by Malinowski and Kopytko (2014). Their calculated indicator for the same commune for the turn of 2012 and 2013 was virtually twice as high and amounted to 166.1 kg/M/year.

In the municipality of Skawina the average indicator of mass accumulation of mixed waste for the period considered came to 260.6 kg/M/year. In the case of selectively collected waste, this indicator was more than three times lower and amounted to 80.1 kg/M/year. The highest indicator value for mixed waste was recorded in September 2015 (345.2 kg/M/year), while the lowest in December 2013 (196.3 kg/M/year). In the case of selectively collected waste, the highest indicator occurred in August 2014 (as in the Liszki commune) and the lowest in January 2015. The accumulation of mixed waste shows an upward trend. Comparing the results of the study to Malinowski's (2013) research, it was noted that the calculated average accumulation indicators for both mixed and segregated waste were higher than in 2011 and 2012. The difference between

the accumulation of mixed waste amounted to about 78 kg/M/year and between the accumulation of selectively collected waste came to approximately 15 kg/M/year. In the Biskupice commune, the highest accumulation indicator for both mixed and selectively collected waste was recorded in August 2015 and the lowest in July 2013 (as in the Liszki commune). The values of average biomass accumulation indicators collected for the Biskupice commune were lower than those obtained in Malinowski's (2013) research. In the case of mixed municipal solid waste, the difference came to nearly 11 kg/M/year. For segregated waste, mass accumulation in years 2010-2012 was lower, on average, by 17 kg/M/year.

The efficiency of segregation

The average share of segregated waste in all waste collected for the surveyed communes came to 27.5%. In the analysed period the value of the segregation efficiency for the whole country increased from 13.5 to 19.8%. The highest share of segregated waste was in the Liszki commune and amounted to 35.7%, while the smallest share of waste, 24%, was observed in the municipality of Skawina (Table 2).

It is disturbing that the proportion of selectively collected waste is decreasing in all municipal solid waste accumulated in every investigated commune (Figure 2). The share of selectively collected waste in the Liszki commune in 2015 was as much as 10% lower than in 2013. The main cause of this alarming trend is the increasing mass of mixed municipal solid waste.

Figure 2 Segregation efficiency in an annual term

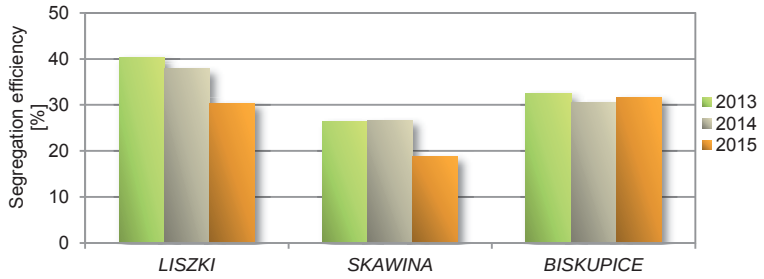

Table 2 Segregation efficiency in the investigated communes and average annual change rate

Parametr	Unit	Liszki	Skawina	Biskupice
Minimum	%	24.6	16.6	28.7
Maximum		47.5	30.6	40.0
Average		35.7	24.0	31.6
Standard deviation		5.1	4.0	3.1
Coefficient of variation	-	0.15	0.18	0.09
Average annual change	%	-6.9	-8.3	-0.6

The dynamics of changes in waste accumulation

A significant increase in the mass of mixed municipal waste per capita was observed in all communes. The largest increase occurred in the Liszki commune where the average rate of growth dynamics of mixed waste was highest and amounted to 15.6% and 13.3%, respectively (in 2015 in the Liszki commune 125.7 kg/M of waste was collected, almost twice as high as in 2013). In the remaining communes the rate of change dynamics (for mixed waste) came to 8.2% in the case of the Skawina municipality and 7.8% for the Biskupice commune.

The increase in the mass of segregated waste was observed only in the communes of Biskupice (6.7%) and Liszki (3.0%). In the Biskupice commune 47 kg/M was produced in 2015. This is 11 kg/M more than in 2013. In the municipality of Skawina the mass of waste selectively collected decreased in comparison to previous years. The average rate of the dynamics of changes for segregated waste in this municipality amounted to -3.1%.

Significant linear relationships between the analysed indicators were observed for the following socio-economic factors (matrix of Pearson's linear correlation coefficients between selected

socio-economic factors describing the investigated communes and calculated accumulation indicators as well as segregation efficiency):

a) Population density – the higher the analysed area is, the higher the accumulation indicators of mixed and segregated waste are;
b) The share of the pre-working population – in areas where the percentage of the pre-working population is high, there is less likelihood of obtaining high rates of mixed and segregated waste accumulation. On the other hand, the greater the share, the higher the efficiency of segregation;
c) Percentage of the working-age population – a higher proportion of the population of this group positively affects segregation efficiency;
d) As the share of the post-working population increases, the accumulation of mixed and segregated waste rises, but the efficiency of segregation decreases;
e) Working class strongly influences the indicators of the mass accumulation of selectively collected waste. The higher the percentage of the group, the higher the accumulation of segregated waste;
f) The higher the proportion of dwellings with central heating and bathrooms, the higher the indicator of mass accumulation of mixed and segregated waste;
g) The percentage of inhabitants utilising wastewater treatment plants positively correlates with the indicators tested. It shows that the more people use the sewage treatment services, the higher the accumulation indicators are;
h) Factors such as water consumption per capita and the proportion of women in society influence significantly the indicators of waste accumulation.

Table 3 Linear correlation matrix

Indices	Mass accumulation indicator of mixed waste	Mass accumulation indicator of segregated waste	Segregation efficiency
Population density	0.71	0.77	-0.42
Percentage of population of pre-working age	-0.91	-0.74	0.72
Percentage of population of working age	-0.59	-0.21	0.82
Percentage of population of potst working age	0.87	0.61	-0.81
Percentage of employees	0.47	0.84	-0.01
Percentage share of unemployed persons	0.32	0.00	-0.48
Share of dwellings equipped with central heating	0.79	0.98	-0.35
Share of dwellings equipped with bathroom	0.75	0.85	-0.51
Share of the population using wastewater treatment	0.64	0.85	-0.47
Gas consumption per capita	-0.02	0.27	0.25
Water consumption per capita	0.70	0.86	-0.35
Density of water supply network	-0.31	0.11	0.54
Density of the sewage network	0.06	0.17	-0.09
Share of women in population	0.85	0.65	-0.83

Legend: key correlation coefficients with values $|R| > 0.6$ were marked in blue.

CONCLUSION

Over the review period, a noticeable increase in the indicators of mixed municipal solid waste is noted. The communes in question are characterized by a similar share of segregated waste in all generated municipal solid waste. There was an increase in the mass of segregated waste collected in all communes except for Skawina. Regrettably, during the period considered, the efficiency of segregation declined. There are significant relationships between the mass of waste generated and the socio-economic factors. These factors include, for example, population density and the share of dwellings with central heating or bathrooms.

ACKNOWLEDGEMENTS

This Research was financed by the Ministry of Science and Higher Education of the Republic of Poland – statutory activity no. DS 3600/WIPIE.

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COMMERCIAL SUBURBANIZATION OF NITRA CITY (CASE STUDY ČERMÁŇ DISTRICT)

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Abstract: Social-economic transformation of Nitra city is significantly influenced by the process of commercial suburbanization. Main localization factors affecting progress of this process within the observed area are the very good traffic accessibility with good connection to the highway R1, large industrial buildings in this area as well as large available land for commercial purposes. The analysis of the development of commercial suburbanization in Čermáň district was based on comparison of the amount of commercial objects in 1998 and 2017. Commercial suburbanization marked an increase in 76 objects (starting with 11 in 1998 to 87 in 2017). The basis for this analysis was field research that provided data for real number of objects. Additionally, comparison of aerial photographs identified the growth of areas used for commercial purposes in the analyzed period as well as circumstances affecting localization of individual objects. Based on the research, it is foreseeable that the process of commercial suburbanization will continue to record steep increase in the number of objects. The presumption is associated with continuous thickening of the built-up area, with the revitalization of abandoned buildings as well as with significant change in the function of fertile agricultural land.

Key Words: Nitra city, city district Čermáň, commercial suburbanization, commercial objects

INTRODUCTION

Many scientific papers are focused on research of cities as they are complex objects of geographic studies. Large scale of economic, social and cultural processes happen in cities. Cities can be seen as centers of social changes, cultural transformation and economic innovation (Slavík and Grac 2009). Currently, a remarkable increase in business activities that bring new commercial purposes such as retail and wholesale areas, logistic zones, production areas, warehouses and administrative buildings to originally agricultural cities can be observed in Slovakia. These processes similarly to other cities in Slovakia also in Nitra lead to transformation of social-economic structure of Nitra city. They appear mainly in peripheral urban areas with well-built transport infrastructure, close links to the R1 highway, plenty of relatively cheap uncultivated plots, existing industrial buildings, and family homes with original residential functions. Due to the significant development of the analyzed process, there is a significant change in the function of the fertile agricultural land, the thickening of the built-up area, the revitalization of abandoned buildings used in the past especially for industrial production, but also the change of the original residential function to the detriment of the commercial one. Newly localized objects have positive as well as negative impacts. The positive consequences are e.g. new employment opportunities and better access to diverse services. On the contrary, the negative consequences are the significant increase in the traffic congestion, the increase in emissions from motor vehicles, the advertising smog and the aesthetic changes due to the construction of new commercial buildings.

MATERIAL AND METHODS

There have been significant changes in the suburban areas since the mid of 20th century. While in the 1950s suburban areas were perceived as "no-fit, homogeneous night-time communities" (Gober 1989), a number of industrial and service entities have been deployed in recent decades (Hahn 2014). Today, this process is known as commercial suburbanization. According to Champion

(2001) commercial suburbanization is linked to industry, retail, administration as well as high tech business decentralization. Ouředníček (2002) includes to the commercial suburbanization also the spatial extension of business functions linked to the central traffic lines. According to Sedláková (2005), transport accessibility is the basic localization premise of commercial objects. Ouředníček et al. (2008) argue that commercial suburbanization is part of a suburbanization process that includes the transfer of commercial activities (trade, production, storage, entertainment and administration) from the core of the city to their rural areas. The process of commercial suburbanization also refers to the non-residential suburbanization, including the transfer of non-commercial functions, schools, offices and museums. The number of authors dealing with and analyzing commercial suburbanization process in Slovakia is gradually growing, e.g. Sedláková (2005), Danielová (2008), Masný and Dubcová (2010), Šveda and Križan (2012), Repaská and Masárová (2014), Midler and Dubcová (2016).

The aim of this paper is to analyze development and forms of the process of commercial suburbanization in the territory of the city district Čermáň in the period of 1998 and 2017. The number of commercial objects in 1998 was based on the data from the publication Nitra-detailed atlas. It was compiled by a field survey of the Department of Geography and Regional Development in Nitra. The status of commercial objects in 2017 was analyzed during actual field research. The comparison of aerial photos between 1991 and 2017 brought data showing the increase in areas used for commercial purposes. Due to data privacy and personal data protection, information about individual businesses and their operations from www.finstat.sk web portal are delivered in intervals.

The standard geographic methods such as analysis, synthesis, comparative method, mathematical-statistical and cartographic method were applied to process the data.

RESULTS AND DISCUSSION

All rural districts of the Nitra city including city district Čermáň show significant impact of commercial suburbanization process. Commercial objects are located in the observed district along the main traffic line Cabajská ulica. Cabajská ulica connects the Čermáň city district to the R1 highway. Čermáň city district is also directly connected to the town Šaľa (Nitra region). Development of commercial suburbanization in this area is remarkably affected by good traffic availability and a direct connection of the R1 highway to the capital city Bratislava as well as the county town Banská Bystrica (Banská Bystrica region). Besides the well-developed traffic infrastructure, the city district disposes of available land used for agricultural purposes, existing large-scale industrial centers as well as large buit-up areas of family houses.

Beginnings of the commercial suburbanization process in Nitra district Čermáň are observed already in the beginning of the post war period when Czechoslovak Automobile Company (ČSAD) and state-owned factory Western Slovak butchery were based in Čermáň city district. Commercial objects remain this way until the transformation period that significantly affected all industrial areas in Slovakia. Looking at ČSAD, transformation process lead to a split into passenger transport and freight in 1993. Hence, Freight Nitra is established (NAD Nitra). Former Western Slovak butchery undergone similar but more complex transformation process. Company ended its activities in 1993 and Schärdinger-Milex plc. was established instead. It was renamed to AGROMILK plc. later. Due to small domestic milk demand, the operations closed. AGRO TAMI has dominated the industrial zone since 2008. Further important commercial objects in the city district are TAURIS SIESTA ltd. (headquarters are in Rimavská Sobota), Bramac – roofing systems ltd. (headquarters are in Ivanka pri Nitre), LEMUS and Aika. Only Bramac and LEMUS exist today. However, Aika transferred its operations to the city district Old Town.

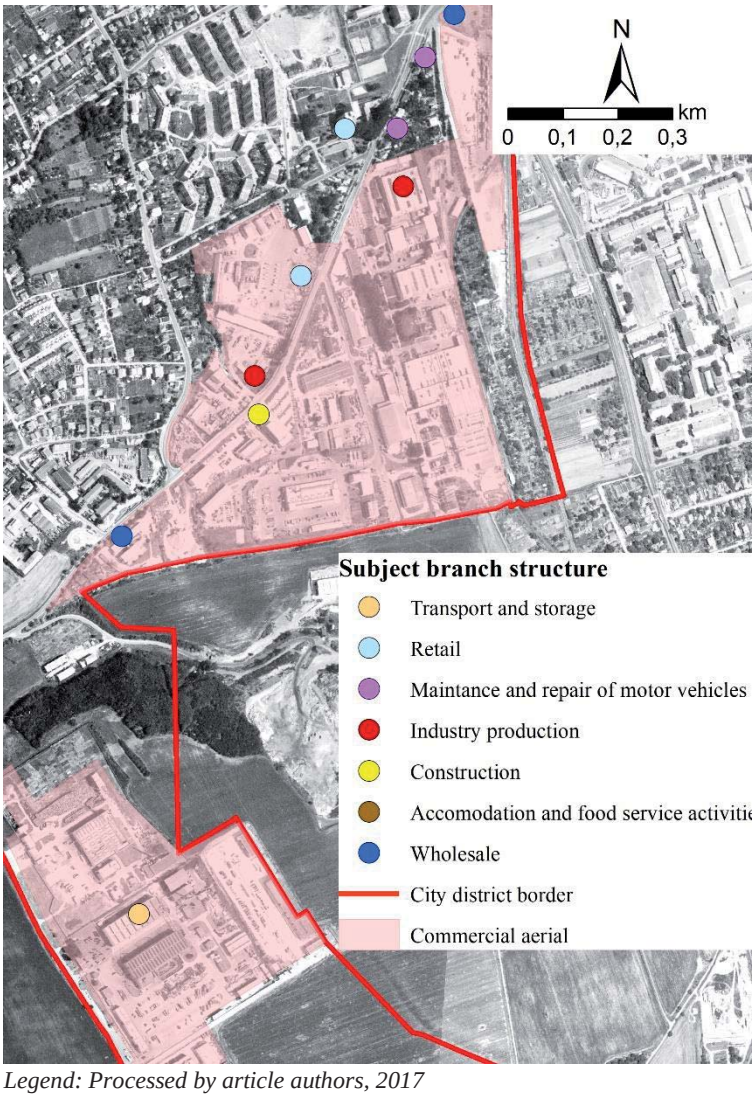
Commercial objects in Čermáň city district grew by 76 entities between 1998 and 2017 (11 in 1998, 87 in 2017). They are mainly located along the traffic line (road II/562) and they use large industrial area established by Western Slovak butchery. 41 objects were located on its premises in 2017. The most significant entity is AGRO TAMI ltd. focused on milk processing. AGRO TAMI ltd. employed the most employees 2017 (more than 200 employees) in the area. Dominant entity is PHARMAGAL-BIO ltd. that covers with its premises the largest area. Though the large plot coverage it has only around 20 employees. The highest number of operations are micro-organizations with less

than 10 employees. On the one hand, they are located in already existing premises. On the other hand, they rearrange already existing premises for rent.

Industrial zone left by the former Czechoslovak State Automobile Company is facing significant suburbanization process. However, the dominant entity is Freight Nitra in this industrial zone. Although, it does not use as large areas as the former Czechoslovak State Automobile Company. As a consequence, it rents the majority of the area to other objects. 6 commercial objects were located in this zone (incl. Freight Nitra) in 2017. Existing buildings are used for industrial production (production of metal constructions and its parts – SANZELL, ltd., lighting production – SEC ltd., production of mining machines and building construction – Menzi MUCK Slovakia, ltd.) as well as construction entity (STAVEX Nitra ltd.) and wholesale store (HEDONIA ltd.).

Relatively large area was used by BRAMAC with headquarters in Ivanka pri Nitre in the past. Although the area is relatively large, only 3 objects are located there (HRIADEĽ ltd., Ing. Miroslav Sádovský STAVEBNÁ FIRMA SÁDOVSKÝ and AUTO-DUMI ltd.). The largest part of the zone belongs to HRIADEĽ ltd. specialized in wholesale of agricultural machines and accessories with more than 50 employees.

Figure 1 Objects of commercial suburbanization in city district Čermáň in 1998



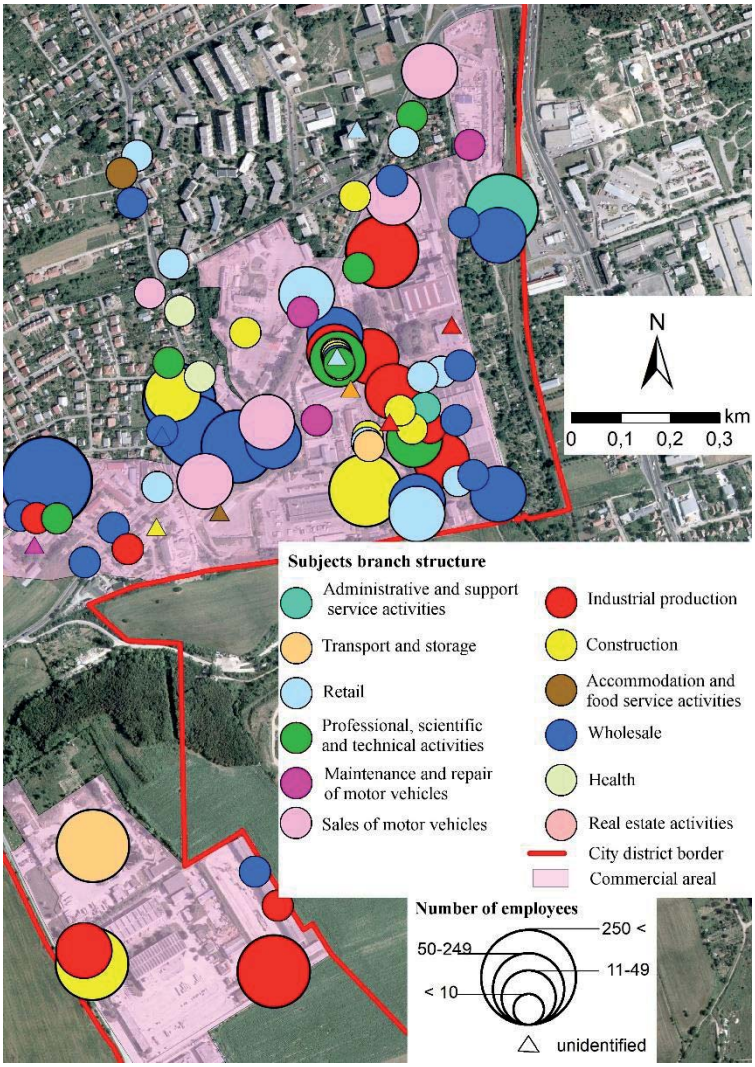
Legend: Processed by article authors, 2017

Čermáň's suburbanization process does not develop at the cost of agricultural land. The spread of commercial zones increased by 5.7 ha (57.9 ha in 1998, 63.6 ha in 2017). Growth of the commercial zone was marked mainly in the Southwestern zone of the Čermáň that was dedicated to residential buildings. Due to good accessibility of the R1 highway some private entities moved their operations to this area. Consequently, it lead to the transformation from residential to industrial function. Similar

transformation processes were observed along the Dolnočermánska Street where new entities were established in the family houses. However, here residential function remains.

Development of commercial suburbanization brings with remarkable changes in functional use of the area. Whereas the areas were used by mono-industrial commercial zones in the past (Figure 1), poly-industrial zones prevail today (Figure 2). Single-industrial zone is only the area used by the company Roads Nitra focusing on bitumen production. This zone is isolated from the city built-up-area and from other commercial zones.

Figure 2 Objects of commercial suburbanization in city district Čermáň in 2017



Legend: Processed by article authors, 2017

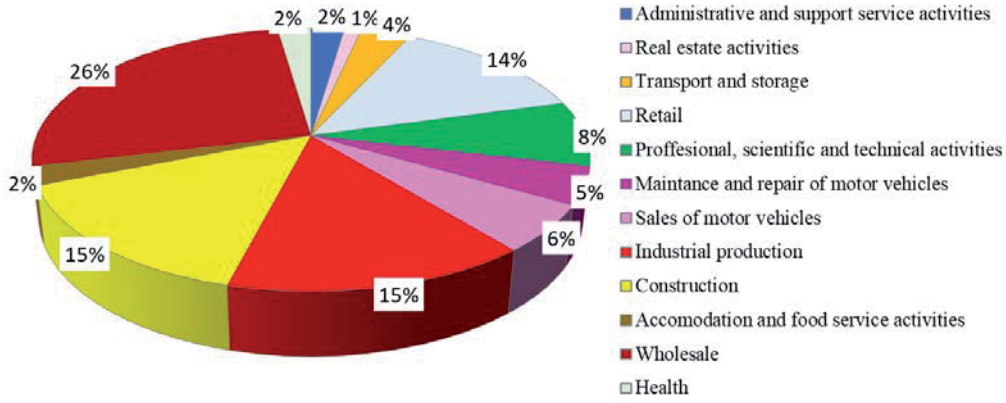
There is a strong differentiation process between 1998 and 2017 based on the industrial specialization of the commercial objects between 1998 and 2017. Individual branches were represented in relatively balanced portion in 1998 (Figure 1). However, in 2017 (Figure 2) wholesale dominates (23 objects). The most important wholesale commercial subject was company MED – ART ltd. (more than 250 employees). It is specialized in pharmaceutical wholesale business. Other significant wholesale companies in the district are MPL building materials, Vaillant – MORA and Hriadeľ with 50–99 employees. Further entities have less than 10 employees.

Additionally, intensively represented is also the building industry (13 objects), the industrial production (13 objects) and the retail business (12 objects) (Figure 3). Companies focused on road and highway construction dominate the construction industry. Moreover, construction of residential and commercial buildings play an important role in the construction industry. STAVEX Nitra plc. is one of the most powerful companies in this area with 200–249 employees. Branch industrial

production is dominated by SEC ltd. with 200–249 employees. All retail shops have less than 10 employees.

On the contrary to other rural city districts, Čermáň city district is dominated by service and maintenance of motor vehicles (4 objects). Car dealers are rather rare (5 objects: AUTO-DUMI, PP CARS, ŠKODA MOTO JAS, Car spare parts TF).

Figure 3 Objects of commercial suburbanization in city district Čermáň in 2017



CONCLUSION

Commercial suburbanization is present in all major cities of Slovakia. However, their intensity and form of expression significantly differ. Capital city and the county town Košice (Košice region) follow a similar suburbanization process as other Western European cities. Many entities in these areas transfer their premises outside of the city centers in form of wholesale, sports and relax centers. Smaller but administratively significant cities observe the process of commercial suburbanization in the peripheral parts along major traffic lines, such as Nitra city. Commercial objects are localized mainly along main traffic roads in large industrial zone. Centers are built on "green field" or they occupy family houses with former residential function. Suburbanization boom in Nitra was significantly influenced by the construction of the R1 highway that connects Nitra city with the capital city Bratislava as well as with the county town Banská Bystrica (Banská Bystrica region). Moreover, the large number of built and unused industrial buildings as well as a large number of inexpensive family houses played an important role.

ACKNOWLEDGEMENTS

The research was financially supported by the University Grant Agency of Constantine the Philosopher University in Nitra VIII/15/2017– Lokalizácia komerčnej suburbanizácie v meste Nitra.

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ELEMENTARY GORELIEF FORMS AS A TOOL FOR DELINEATION OF SOIL AREAS INFLUENCED BY WATER EROSION

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Abstract: In the model area of intensively used agricultural landscape was realized the detailed pedologic survey. The aim of the survey was to explore the area by 37 ha, in the cadastral area of Rišňovce and Rumanová municipalities, affected by water erosion. The complication of the study area was multiplied by the heterogeneity of substrate subsoil and transition zone of the Chernozem area to the area of Haplic Luvisols. The model area was explored by 111 probes spaced at nearly regular network. By using digital elevation model were created 25 sites of elementary georelief forms. Their detailed survey was carried out in relation to the identification of soil units (soil type, soil class, variety, form). In the paper, we point out to the fact that the composition of the soil cover is different from the Complex Survey of Agricultural Soils (Němeček 1966) it´s reflecting not only the development of erosion-accumulation processes, but also mapping methods in the past and today. These differences were demonstrated on the example of small model area.

Key Words: water erosion, erosion-accumulation processes, elementary georelief forms, soil units, erosion factors

INTRODUCTION

Soil is one of the most important landscape component. Not only natural, but also anthropogenic processes affect it and if they are dominant, can lead to the soil degradation to the level of total loss of the basic soil quality - fertility. The anthropogenic impact to the soil is manifesting by intensive agricultural landscape using. Inappropriate agricultural land use causes a number of degradation processes, including soil erosion.

Soil erosion, as reported (Fulajtár and Janský 2001), is manifested by continuous movement of soil mass and can be very dangerous. In some cases, soil erosion can be considered as a natural threat that leads to total soil degradation, to the occurrence of degraded soil as understood it (Midriak 2004, 2007). The intensity of this process is due to direct (mostly water and wind) but also to indirect factors and conditions (geological basis, surface and vegetation). However, only human activity is one of the factor that initiates erosion processes (including tillage erosion). So it is clear, that the soil needs to be protected by frequent monitoring. However, this is an economic and time-consuming process. Therefore we have decided to apply our research at the topical level in agricultural land with an area of 36.77 hectares, which lies in a strongly eroded area of Slovakia (Fulajtár and Janský 2001). It lies on the border between Rišňovce and Rumanová and has short border with Veľké Zálužie. It belongs to the Nitra region and the Nitra district. In a non-homogeneous landscape environment, there is a need for more accurate mapping by using more modern means such as the digital surface model (DMR) by the Geographic Information System (GIS).

The aim is to identify the erosive areas of the selected area and to determine the dominant influencing factor to the intensity erosion activity. To point out the differentiation of soil genesis in relation to the localization of soil units and surface in the erosion-accumulation environment. It includes a proposal for a more detailed method of mapping, defining and analyzing soil areas

in a non-homogeneous landscape, which is under the constant influence of water surface erosion through the concept of creating elementary surface forms. We would like to propose a way of creating areas that should be monitored more frequently on the basis of this concept.

MATERIAL AND METHODS

To achieve the aim, we chosed two methodologies: *Terrain mapping of soils* - in accordance with proposed standard soil survey procedures in the paper (Čurlík and Šurina 1998) we created contour line network of 111 soil probes uniformly spaced about 70 m (Balkovič, Rampašeková, Hutár, Sobocká and Skalský 2013). On average, there were 3 probes per hectare, which confirms detailed mapping at a scale of 1 : 10 000. The probes were made by Edelman soil drill to a depth of 120–130 cm respectively after reaching the soil-forming substrate. Each point in the network was geographically identified by the ArcGIS™, i.e. by latitude and longitude. The terrain was subsequently searched by using the eTrex Venture Cx GPS. The real geographic coordinates of the point (probe) were recorded in the soil notepad. The location of soil probes was digitized in ArcGIS™ and vectorized georeferenced data layer was created (Figure 1).

The concept of surface elementary forms was selected on the basis of factors analysis (substrate, vegetation) and conditions (surface) after field research. It uses surface in the form of boundary-forming factor (Minár 1995, 1998a, 1998b, Minár and Evans 2008). The morphometric properties of the georelief (Figure 2) were created by digital surface model (DMR). The basis for creation were topographical maps of 1:10 000 (map sheets 35-34-24 and 45-12-04), which were orthorectified into the S-JTSK coordinate system (Křovák). In the R2V (© Albe Software Corp) software vector layout of the contour raster layer was created and by ArcGISTM (© Esri) was generated an altitude input field. On the basis of DMR were generated morphometric parameters of georelief, such as slope, orientation (exposure), georelief normal (slope line) curvature and horizontal (contour line) curvature of the georelief in ArcGIS™ by using the Spatial Analyst and saved as raster data layer. Based on the expert visual analysis of DMR were generated the areas of elementary surface. In the context of this analysis were taken into account sudden changes in surface as a slope, horizontal and normal curvature, as well as exposure. The obtained boundaries of the surface elementary forms were finally adjusted on the basis of field verification and correction. In the mapped area were created 25 areas of surface elementary forms (Figure 3).

The real erosion-accumulation processes taking place in the model area are presented in the paper by a map of erosion-accumulation areas (Figure 4). Areas were created on the basis of 111 field research probes, identified in the sense (Societas Pedologica Slovaca 2014, Čurlík and Šurina 1998) in soil eroded (E), accumulated (H), overlaid (Y) and bounded by elementary forms of georelief. The mentioned areas were identified based on the depth of the surface humus horizon (A - horizon) of the individual soil types. The average depth of A horizon of the Chernozems (CM) was 50 cm, the Arenosols (RM) 33 cm and Cambisols (HM) 28 cm. The boundaries were set as follows:

CM: CM/e (eroded: < 40 cm), CM/h (accumulated: > 50 cm), CM/y (covered:120 cm),

RM: RM/e (eroded: < 20 cm), RM / h (accumulated: > 30 cm), RM/y (covered:120 cm),

HM: HM/e (eroded: < 20 cm), HM/h (accumulated: > 30 cm).

RESULTS AND DISCUSSION

Based on field research, we have found that long-term use of agricultural land leads to a change in soil-forming processes, i.e. soil development. What impact did the substrate, surface and vegetation have we found by the analysis of these factors.

The mapped area was considered from the point of view of the substrate by analysing the results of the field research as a homogeneous environment because 90% (33.08 ha) are dusty-clay loess, 5% (1.98 ha) polygenetic and loess sediments, 2% (0.62 ha) coluvial sediments and 3% (1.09 ha) of neogene (limnetic to brackish) sediments. So we thought, that this factor would have a negligible impact on water erosion.

Figure 1 Location of soil probes in the area of interest

Figure 2 Morphometric properties of surface of interest area

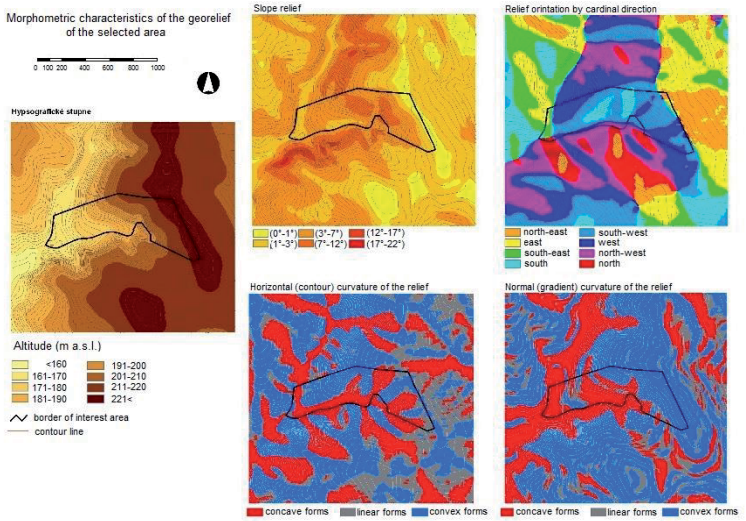


Figure 3 Areas of surface elementary forms of interest area

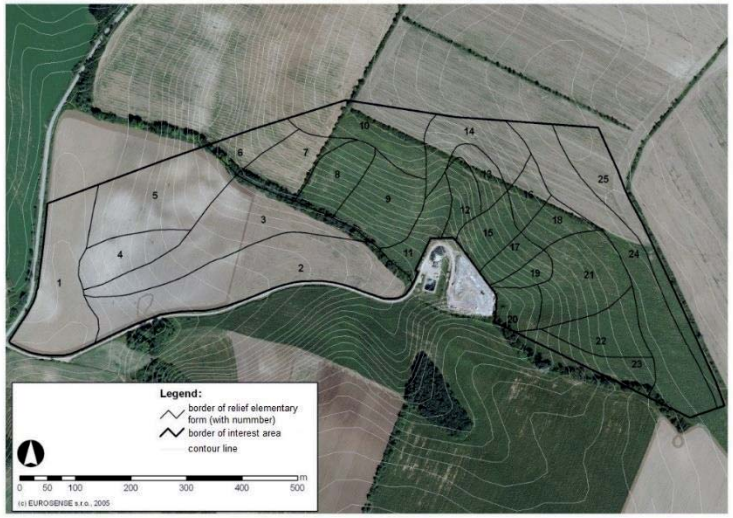
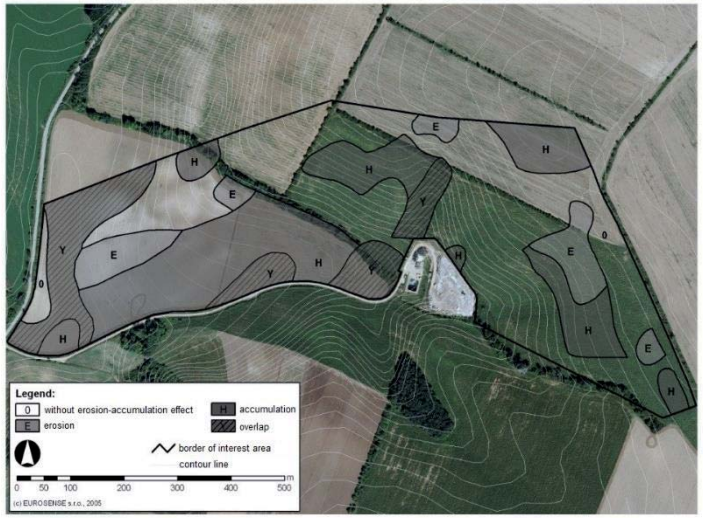


Figure 4 Erosion - accumulation areas of interest area



For these reasons, we considered the surface based on morphometric properties as the dominant condition influencing the intensity of water erosion. We excluded the impact of exposure as the slope was mostly south-west. We assumed that the highest intensity of erosive activity would have a slope, which in two cases exceeded 7°. Based on the work (Zachar 1970) we thought, that the greatest erosion would occur on deluvial materials, i.e.on the longest slopes and with the listed slope and accumulation on the alluvial materials under the slope of the mapped area with the concave-concave forms of the surface.

Based on the maps of the Soil Comprehensive Survey of 1966, we expected the eastern part will form the HM and the western ČM. By comparing with the results of the mapping of bonitated soil-based units (BSBU), we found that the boundary between soil types is shifted to the northeast. Chernozems on the territory of Slovakia have a humus horizon - molluscic (Am) extending to a depth of 60–80 cm, cambisols and arenosols ochric (Ao) reaching to a depth of 30 cm. From our own field research, we found, that soil on slopes with a slope of less than 3° would be accumulated and on slopes with a slope greater than 3° eroded. As we observed the dominant influence on erosive activity, we determined the boundary of erosion-accumulation activity from the average depth of humus horizons of individual soil types. Based on the adjusted depths, we have located areas with significant erosion-accumulation processes.

Through using surface elementary forms method, we have specified areas with the greatest potential for water erosion. It was the northeastern part of areas 3 and 5, then 7, 8, 9, further 12, 13, 15, 16, 17, 18. Based on the field research of the soils and areas created by this method, we generated real **erosive areas** ČMa with depth of humus horizon less than 40 cm. Their area is 3.12 ha, which is 7% of the territory. They were created on loess, on slopes with a slope of 3–7° and from morphometric point of view on convex-convex (VV) surface forms. By comparing the results of the soil field survey and the specified number of areas, there was only very little match of areas 3 and 5 and 18. In other specified complexes we even found significant accumulation processes. This phenomenon is explained not only by the geometric shape of the surface but also by the sudden passage of the loam material into more clayey and skeletal material with up to 10% content in the tillage and under tillage. It has been shown that the sudden change in granularity and skelet mitigates and in some cases, eliminates erosive processes. Thus, we agree with the theory (Nestroya 2001 and Antala 1998), they claiming that the soil with a higher slope and a higher content of clay shows a lower degree of erodibility than the soil with a lower slope and a lower clay content (area 4 and the peak of area 23).

We also found a rate of 30% (10.95h) **accumulated areas (H)**. They were covered mainly by accumulation forms of ČMa with a depth of humus horizon more than 50 cm, RMa and HMa with a depth of humus horizon more than 30 cm. These areas were formed mainly on loess, on slopes with a slope of 3 - 7°. From a morphometric point of view, in the western part of the area of interest, on a larger slope with a predominantly homogeneous substrate, it binds to the concave-concave (KK) to concave-convex (KV) surface forms. In the eastern to the south-eastern part, on a slope of smaller length, with non-homogeneous substrate material, they bind to convex-linear (VL) and in some cases to convex-convex (VV) surface forms. However, this result contradicts the generally accepted theory of soil accumulation. As stated by the authors (Stankoviansky 1997, 2001, Sobocká and Jambor 1998) in VV and VL forms there is erosion processes and not accumulation. This is explained by a sudden slope change and a change in the grain size of substrate material.

The intensified accumulation process created **overlapped areas (Y)** ČMa and RMa with a depth of humus horizon of about 120 cm, with an area of 5.07 ha, which is 13% of interest area. These areas have been formed on loess and coluvial floodplains of long bottom forms of slopes bounding the western and southern parts of the area of interest, with a slope of 1–3°, but also in depression in the central part, with slope inclines of 3–7°. According to the Soil Morphogenetic Classification System (Societas Pedologica Slovaca 2014) some of them had to be mapped as arenosols, although the stratification of the soil profiles showed, that it is a coluvial material overlapping the buried humus horizon, and so it is rather the arenosols as understand them authors (Sobocká 2002, Zádorová and Penížek 2008). Since these were areas created in depressed forms, these overlaps are bound from a morphometric point of view, exclusively concave-concave (KK) surface forms. This result

confirms the theory of authors (Fulajtár and Janský 2001, Gallay 2002), it show that with slope length increases the washing out and transport capacity and in the lower part increases accumulation or overlap.

From a *vegetation* perspective during the mapping, we noticed that the eastern cragied slope was sown with a low erosive crop – *Zea mays* and western part by less grain cereals (wheat – *Triticum aestivum*).

We found, as well as in the work (Petlušová et al. 2016), that all the observed factors intensively affect the erosion-accumulation processes in the selected area.

CONCLUSION

The problem of soil erosion has been known since the 19th century and continues to the present day. It´s reflected in change of soil properties such as, for example organic matter composition in the form of humus, grain and soil skeleton, pH ratios and others. Usually, if it is not only the initial stage of the erosion process, soil degradation occurs. There is only 0.25 ha available of arable land per capita in Slovakia (Sobocká 2007) it is necessary to protect it. Therefore, in this paper we call for more frequent mapping in areas affected by erosion. We realize that this is a time and economically very difficult. At the present time there are different prediction models of soil erosion, which however only draw attention to the susceptibility of the soil erosion and are mostly applied to larger areas. Also have been created various countermeasures, which should be observed in areas with increased susceptibility to erosion. But as they are observed and as they actually act on the soil cover, we can verify only by field survey of soils. Therefore, we propose that the mapping of soils in erosion-accumulation areas should be carried out through surface elementary forms (in terms of slope, exposure, horizontal and normal curvature) because, as our field research confirms (as well as many authors' researches), the surface is one of the dominant indirect factors of soil erosion. At the same time, we have shown that the erosive processes in the heterogeneous territory in terms of substrate, surface and soil do not only change the soil cover at the level of the soil form, but also act to the level of soil types, which greatly disrupts the landscape development. We have deliberately chosen heterogeneous territory of substrate, surface and soil to achieve the aim.

From the point of view of erosion-accumulation processes induced by water activity, which clearly influenced the present appearance and the soil cover of studied area, we consider this territory as a territory with a manifestation of surface water erosion which, through direct intervention of the human being into the soil passes into a directly altered (*anthropogenic*), *accelerated*, harmful (*malignant*) erosion, respectively into tillage erosion. In the mapped area, this process is manifested by the existence of erosive chernozems and formation of arenosols. The accumulation processes of the relief are bound to deep chernozems and arenosols, on erosive surface processes are bound by shallow arenosols in the largest slope area of 7–12°. The substrate has decreased to eliminate erosion in the mapped area in terms of grain and skeletal change.

Thus, we can say that in the assessment of the factors, it was found, that the principles of soil protection were not observed at all. In the category of slope of 7 ° and above there is a large-scale arable land, which is intensively used for the cultivation of dispersed crops. Following the valid guidelines, it is necessary to cultivate on these areas crops with predominantly for example lantern clover-grass mixtures and so on.

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SCREENING ANALYSIS OF TOXIC METALS ON SPECIFIC ALLOTMENT GARDEN AREAS IN THE CITY OF BRNO AND ITS SURROUNDINGS

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Abstract: Currently, introducing harmful substances into the environment forms a frequently discussed issue. This includes toxic metals that occur in the soil. In the Czech Republic, cultivating your own crops in allotment gardens is a widespread activity. This paper aims at assessing amounts of toxic metals occurring in the soil while determining the basic soil attributes in specific allotment garden areas; the soil include pH, ORP (oxidation-reduction potential) and conductivity as factors for the mobility of metals since such mobility is closely linked to the potential risk of transfer of toxic metals in food chains. The study also includes analysing the measured values against the applicable legal norm in the Czech Republic and identifying the level of soil contamination at specific gardening sites. The XRF analysis was used to determine metal levels in soil samples; the BASS DELTA device was used for this method. Out of the eight specified metals (As, Cd, Cr, Cu, Ni, Pb, V, and Zn), four metals (Cd, Cu, V, and Zn) were found to exceed the legal norms in terms of content. Cd was found to be the most hazardous element within the set with its level reaching 12.69 mg/kg whereas 0.5 mg/kg is provided by the legal norm. It should be noted, however, that considering the detected soil attributes, mobility of metals is expected to be low in the given settings.

Key Words: soil contamination, legislation, XRF, pH, ORP

INTRODUCTION

Soil forms one of the key natural resources. It is considered a comprehensive component of the environment that performs a number of functions. Of these, generating biomass, transforming substances, filtering, and forming habitats for organisms are of utmost importance (Jandák et al. 2010).

Cultivating agricultural products by individuals for their own use has been a long-term tradition in the Czech Republic. Setting up the first allotment gardens dates back to the 1920s. This involved areas featuring a great portion of greenery and rich biodiversity. While formerly such sites were located in suburban areas of cities creating intermediary zones between urban areas and natural settings, they were gradually integrated into the former as the process of urbanisation evolved. The gardening areas started to be neighbours with structures such as roads, industrial sites and municipal incineration plants. All of these structures and the related anthropogenic activities affect the quality and soil structure in allotment gardens. Due to the anthropogenic factors, there is an extreme amount of harmful substances being released into the components of the environment. European Environment Agency (2009) reports that heavy metals and mineral oils form the largest proportion of pollution in the Member States of European Economic Area (EEA).

While some of the metals are immobile in their nature, thus are subject to accumulation in soils, others are mobile. Metal mobility is affected by soil qualities such as organic matter, oxides, pH, ORP, soil structure, etc. (Mehes-Smith 2013, Violante and Cozzolino 2013). In some cases, metals can pass through the rootstock of plant into fruits and, eventually, enter food chains. Individuals active in allotment gardens, however, lack information on amounts of toxic metals in the soil and the associated hazards. This makes it necessary to identify the composition of soils at such sites and – where possible – predict possible risks of toxic metals.

In the Czech Republic, the protection of soils is addressed under multiple legal regulations. The limits of contamination of agricultural soil by toxic metals are provided under Regulation No. 153/2016 Coll. providing the details of protecting quality of agricultural land. The legislation determines *preventive* and *indicative* figures for the levels of each of the elements.

MATERIAL AND METHODS

Screening toxic metal levels involved five allotment garden areas located within the territory of Brno. Choosing the sites specifically aimed at achieving the maximum diversity of possible resources of soil contamination by toxic metals.

The sites of choice are listed below:

1 - Brno Řečkovice & Mokrá Hora, Podhájí Street

The site is located close to E461 and R43 roads, the Svitavy direction. A chemical plant (Erba Lachema) is found to the north of this area.

2 - Brno Lesná, the street of Seifertova

The site is not located close to a possible source of soil contamination by toxic metals such as industrial plants or busy roads.

3 - Brno Černovice, the street of Charbulova

The site is located close to the road #374 that is used as a Brno ring road. In the northwest, there is a textile production plant (Nová Musilana).

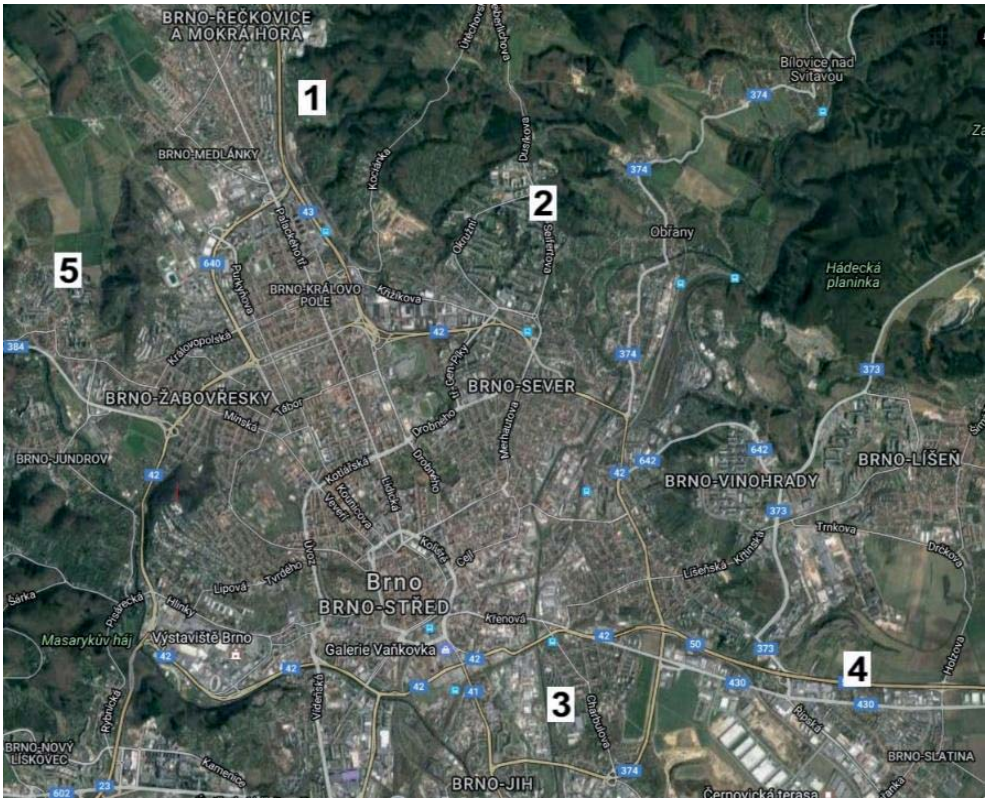
4 - Brno Slatina, the Ostravská and Podstránská streets

There is R50 Road leading along the southern side of the area. To the north of the site there are industrial premises of the Zetor company; to the south-west there is an incineration plant (SAKO Brno).

5 - Brno Komín, the street of Řezáčova

Adjacent to the site is arable land used for growing agricultural crops.

Figure 1 Map showing the allotment garden areas



The samples were taken in autumn 2016. A total of 5 soil samples were taken from each of the sites; the distance of the points of sampling was 80 to 100 m approximately. The samples were taken after removing the turf layer at the depth of 5 to 20 cm using a gardening shovel. Subsequently, the samples were put into labelled polyethylene bags and transported to laboratories of the Institute of Agricultural, Food and Environmental Technology of Mendel University in Brno for processing, which included the removal of gross impurities, drying, homogenisation and sieving to achieve grain size of 2 mm. The pre-treated soil samples underwent the XRF analysis using the BAS DELTA device to determine the quantity of elements per sample of soil. Data were extracted from the data set relating to the toxic metals that are simultaneously identified as hazardous elements by legislation (Regulation No. 153/2016 Coll. providing the details of protecting quality of agricultural land). The studied elements were as follows: As, Cd, Cr, Cu, Ni, Pb, V, and Zn.

Exceeded limits of metal content as provided by the legislation may not necessarily mean a risk associated with a transfer in food chains since metal intake by the plant has a linear character depending on the availability of the metal rather than on the quantity of the same in the soil. The mobility of elements and transportation into the plant body is influenced by chemical and mineralogical properties of soils; examples of such properties include pH and ORP (Violante and Cozzolino 2010). Determining soil pH is essential to identify the mobility of the metal. In neutral and alkaline soils, elements are bound more tightly, while in acid soils they are dissolved in water when it is raining, then washed out and made accessible for the rootstock of plants. The literature indicates that mobility of toxic metals in soils decreases in the following order: Cd > Ni > Zn > Cu > Pb (Tlustoš and Szaková 2007, Makovníková and Barančíková 2006). For these reasons, measurements took place in the laboratories to identify characteristics of the soils with pH, ORP and electrical conductivity selected to be the observed attributes for their influence on mobility of metals in the environment.

RESULTS AND DISCUSSION

Within all of the sites, As, Cr, Ni and Pb levels comply with the legal criteria established by the Regulation No. 153/2016 Coll. The maximum preventive values of the remaining hazardous elements are as follows: Cd = 0.5 mg/kg, Cu = 60 mg/kg, V = 130 mg/kg, and Zn = 120 mg/kg. The preventive value of Cd was exceeded at all of the sites except Site 5 (Brno - Komín). The highest value was measured at Site 4 (Brno - Slatina): Cd = 12.69 mg/kg. Cd can be denoted as the most hazardous element throughout the set. With its indicator value being 2 mg/kg, the element exceeds not only preventive values, but also indicator values. Exceeding indicator values may threaten health of both humans and animals.

The value of copper (Cu) was exceeded at Site 2 (Brno - Lesná), where the amount of Cu was detected to be 92 mg/kg. The remainder of the sites were in compliance with the norm for Cu.

The level of V was exceeded at all of the sites; Site 1 (Brno - Řečkovice & Mokrá Hora) was found to be the area of the greatest excess with V = 166.53 mg/kg.

The preventive levels of Zn were exceeded at each of the sites except Site 5 (Brno - Komín). Site 1 (Brno - Řečkovice & Mokrá Hora) was found to be an area of the highest excess with Zn = 314.18 mg/kg.

Table 1 Average values of toxic metal contents in soils, part 1

Site	Element							
	As		Cd		Cr		Cu	
	mg/kg	Standard deviation	mg/kg	Standard deviation	mg/kg	Standard deviation	mg/kg	Standard deviation
1	16.80	0.92	**2.88**	5.76	30.28	9.36	37.41	6.14
2	14.73	2.13	**1.92**	3.84	41.26	19.33	**92.90**	55.94
3	14.68	0.89	**10.35**	5.63	49.81	29.69	46.63	9.07
4	15.95	1.71	**12.69**	5.08	45.40	6.88	42.16	6.74
5	14.19	0.34	0.00	0.00	50.49	8.66	33.79	3.06

Legend: The figures in bold exceed the legal norms for the level of the element in the soil.

Table 2 Average values of toxic metal contents in soils, part 2

Site	Element							
	Ni		Pb		V		Zn	
	mg/kg	Standard deviation	mg/kg	Standard deviation	mg/kg	Standard deviation	mg/kg	Standard deviation
1	15.05	6.88	41.64	7.58	**166.53**	28.83	**314.18**	55.18
2	31.29	14.29	48.47	22.73	**152.27**	17.03	**183.07**	53.37
3	24.84	6.06	45.82	7.14	**144.23**	12.43	**209.35**	89.31
4	32.15	2.52	43.78	17.26	**138.82**	20.88	**279.40**	75.71
5	33.58	4.18	28.34	0.56	**155.07**	15.43	111.61	10.94

Legend: The figures in bold exceed the legal norms for the level of the element in the soil.

The measurement of characteristics of the soils shows that they are neutral to slightly alkaline soils. pH of soil samples ranges from 7.03 (Řečkovice & Mokrá Hora) to 7.78 (Komín) (see Table 3). The measured values suggest that throughout the set the mobility of toxic metals will be similar in relation to pH, i.e. low.

The ORP levels are different. Ranging from 285.15 mV_H (Slatina) to 292.03 mV_H (Komín), they reach positive values at all times, meaning an aerobic environment. Potential hazards of increased metal mobility can be expected at Site 4 (Slatina) and Site 5 (Komín). These locations were measured to have the lowest ORP values that are closely linked to higher mobility of metals.

Table 3. The summary of characteristics of soil extracts at selected sites

Site	**pH**	**ORP** [mV_H]	**κ** [μS/cm]
1	7.03 ± 0.16	419.78 ± 9.28	100.18 ± 23.02
2	7.71 ± 0.16	389.04 ± 5.87	177.90 ± 72.92
3	7.29 ± 0.20	339.32 ± 44.31	195.73 ± 73.11
4	7.64 ± 0.11	285.18 ± 5.07	168.16 ± 47.14
5	7.78 ± 0.03	292.03 ± 1.83	147.62 ± 9.02

CONCLUSION

The paper addresses the analysis of soils in specific allotment garden areas. It focuses on the composition of soils in terms of elements, mainly toxic metals. Studying the amount of toxic metals involved the following elements: As, Cd, Cr, Cu, Ni, Pb, V, and Zn. The measured values were analysed against the applicable Czech legislation (Regulation No. 153/2016 Coll.). The results warn about increased levels of Cd, V, and Zn at all of the sites. The level of Cu was exceeded only at Site 2 (Lesná). The highest excess of Cd (12.69 mg/kg) was found at Site 5 (Slatina) while Cd has to be denoted as the most hazardous element in that it exceeds not only preventive values, but also indicator values. Site 1 (Řečkovice & Mokrá Hora) was found to have the highest levels of Zn and V with Zn = 314.18 mg/kg and V = 166.53 mg/kg.

There are a considerable number of sources of soil contamination neighbouring the sites of interest. Therefore, it is difficult to identify the prevailing source of contamination of soil by toxic metals. For Site 1 and Site 4, the proximity to a frequented motorway is probably a common source of soil contamination, since combustion of fossil fuels is predominant as the source of air pollution caused by Cd and V. The increased content of Zn may be due to overuse of pesticides by the gardeners alone. However, no conclusive evidence can be presented for the assumption.

ACKNOWLEDGEMENTS

The research was financially supported by the Funkční úkol MZe "Posouzení vhodnosti terénního analyzátoru prvkového složení pro rentgenovou fluorescenční analýzu vybraných materiálů rostlinného původu."

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GOOGLE STREET VIEW AS A TOOL FOR FAUNISTIC RESEARCH: CASE OF *BRIGITTEA CIVICA* AND ITS OCCURRENCE IN MORAVIA AND SILESIA (CZECH REPUBLIC)

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Abstract: This study focuses on mapping the occurrence of the *Brigittea civica* spider (Lucas 1850) in Moravia and Silesia using Google Street View. With this spider we can meet exclusively on the walls of human dwellings within the Czech Republic, where it creates characteristic circular webs, allowing it to be monitored using a computer with internet connection. The method of observation using Google Street View has not yet been applied to any species of spider. In this case, therefore, this is the first published study, where the presence of a particular spider species was monitored through this application. In total, the observations were done in 128 of faunistic squares. The presence of *B. civica* was recorded in 47 faunistic squares in total, with 45 new cases in the given square, and in two cases an earlier finding was confirmed. Based on the findings, we can say that *B. civica* is much wider in our country than we thought.

Key Words: Brigittea civica, species expansion, Google Street View, Araneae, synantropic species

INTRODUCTION

It is relatively difficult to assign most of spiders to certain species in the wild but also in the urban environment from a distance. However, *B. civica* is one of the few exceptions. This spider is exclusively synanthropic in central Europe (Billaudelle 1957). *B. civica* is easily recognizable according to a typical circular webs on the walls of buildings (Samu et al. 2002). A very common phenomenon is ten or even more spiders per $1m^2$. The whole cobweb colonies are also not the exception (Krumpálová 2001). The effect on the size of the cobweb has, in particular, the surface of the wall. On smooth walls, the cobweb size is usually larger and can range up to 100 cm^2 in some cases (Billaudelle 1957). The size of the cobwebs is usually about 5 cm (Kostanjšek and Celestina 2008). The spider itself then reaches a size of 2.5–3 mm in males, females are slightly larger with 3–3.5 mm (Kostanjšek and Celestina 2008). Besides the size, there is another important determining feature of a gray ass with a black drawing (Billaudelle 1957).

So far, all published findings from human dwelling environments have been made only by direct on-site observations, for example (Van Keer et al. 2010, Marusik et al. 2011, Dandria et al. 2005, Havlová 2008). There has not yet been published a single article dealing with mapping this spider using Google Street View, although in this case, thanks to the unmistakable shape and density of cobwebs, it is directly available. Google Street View is one of the geographic information services that can be used to explore the places that we are currently interested in. The advantage of this system is that the images in this application are equipped with precise geographic coordinates and they are digitally interconnected with each other and with a map base. Then you just need choose the place you want to see on the map that is linked to Google Street View. If it is scanned, a tour is displayed. This is the same as when we take a closer look on the map (Tomíšek 2012). However, this application is not entirely unknown in biological mapping. For example, Olea and Mateo-Tomás (2013) using Google Street View mapped the habitats of two cliff-nesting vultures (the griffon vulture and the globally endangered Egyptian vulture)

in northwestern Spain. With help of this application, Deus et al. (2015) studied the spread of invasive plants in Portugal where similar mapping is carried out by car.

MATERIAL AND METHODS

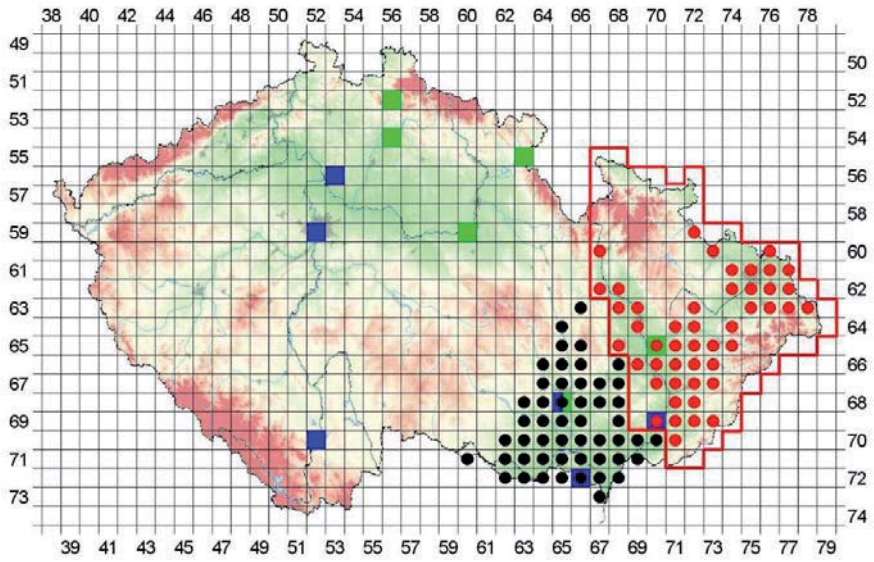
The area of southeastern, central, eastern and northern Moravia as well as Silesia were chosen as a model territory for mapping the occurrence of *B. civica* in our territory using Google Street View.

Research was done on the computer monitor using Google Street View, where you can view panoramic views taken from a height of approximately 2.5 meters every 10 meters of the track (Google 2017). The observation itself took place for 20 days, from 10 July 2017 to 30 July 2017. In order to comply with the uniform methodology, the cobwebs were always observed in the same and unchanging way. Firstly, a faunistic square had been selected, and then all the permanently inhabited villages and towns located in the square were reviewed in this application. The observation was always begun at the center of the settlement, and all the available scattered streets were traversed all the way to the end of these settlements. Observations were made on both sides of the street, left and right at the same time. *B. civica* is easily recognizable according to a typical circular webs on the walls of buildings We did not record the real presence of spider individuals. We assume that the presence of its web means the presence of the species. The detected data was recorded in a pre-prepared form. Drawings of occurrences into particular faunistic squares on maps were done according to Pruner and Mika (1996). For the creation of the map, we used the free applications of BioLib (2017).

RESULTS

A total of 128 faunistic squares were examined using Google Street View. Before this study, *B. civica* was found only in two faunistic squares in this area of interest (Czech Arachnological Society 2017). By observation, the presence of the cobwebs was newly found in another 45 faunistic squares of the area of interest, and in two other faunistic squares the incidence from previous years was confirmed. In total, this spider was newly found in 45 housing units. The survey data were plotted on map.

Figure 1: *Map of occurrence of B. civica with faunistic squares (BioLib 2017)*



Legend: The red line indicates the area of interest on which the observation was done. Red dots are the current Google Street View mapping. Squares with no finds remain without fill. The black dots then represent the occurrence of a spider in the South Moravian Region where mapping was performed by observation directly on the sites (Novotný et al. 2017). Squares with no occurrence recorded remain without padding. Blue square indicates the incidence recorded in the years 1951–2000. Green square indicates the findings from the years 2001–2015 (based on data of the Czech Arachnological Society 2017, Havlová 2008, Macek 2006).

Table 1 Faunistic squares with places of finding

Number faunistic square	City
7071	Hluk
6973	Slavičín
6972	Luhačovice, Bojkovice
6971	Uherský Brod
6970	Uherské Hradiště, Staré Město, Kunovice
6872	Luhačovice
6871	Napajedla
6773	Vizovice
6772	Zlín, Fryšták
6771	Zlín, Otrokovice, Tlumačov
6774	Kopřivnice, Nový Jičín
6472	Hranice
6469	Olomouc
6267	Mohelnice
6369	Olomouc
6268	Litovel, Uničov
6368	Litovel
6770	Kroměříž, Tlumačov
6673	Vsetín
6672	Bystřice pod Hostýnem
6671	Holešov
6670	Hulín, Chropyně, Kroměříž
6669	Kojetín
6574	Rožnov pod Radhoštěm
6573	Valašské Meziříčí
6572	Bystřice pod Hostýnem
6571	Bystřice pod Hostýnem
6570	Přerov
6568	Prostějov
6471	Lipník nad Bečvou
6378	Třinec
6377	Třinec
6376	Frýdek-Místek
6375	Frýdek-Místek
6374	Kopřivnice, Příbor
6372	Odry
6174	Ostrava
6076	Bohumín
6277	Český Těšín
6276	Havířov
6275	Ostrava
6274	Studénka
6177	Karviná
6176	Bohumín
6175	Ostrava, Hlučín

DISCUSSION

The observation in the interest areas of Moravia and Silesia showed that the spider spread not only in this territory, but throughout the Czech Republic, is considerably underestimated. The Czech Arachnological Society (2017) officially reports only 7 faunistic squares in the Czech Republic, of which only two are in the area of interest. To this number Macek (2006) adds one finding from East Bohemia and Havlová (2008) another three faunistic squares from northern Bohemia. However, as it has already been observed from earlier observations directly on sites in the South Moravian region, this spider is very abundant in this region. Newly, 48 faunistic squares have been identified in this area (Novotný et al. 2017).

As it can be seen from Figure 1, altitude plays an important role in spreading. Buchar and Růžička (2002) states that *B. civica* is most commonly found at altitudes of 200–400 m above sea level. This assertion can be accepted. The observation revealed that the optimal altitude for its existence is indeed within this range of values, but it is also abundant in altitudes below 200 m above sea level. On the contrary, at altitudes above 400 m above sea level, we can not practically meet it. This is due to the fact that it is extremely delicate for frost and it does not like also rain and dampness (Billaudelle 1957). On the contrary, it complies with the conditions of the environment where it is warm and dry (Krumpálová 2001). That is why we can only meet with this spider in the lowlands. Up to 400 m above sea level, it penetrates only sporadically and only into larger cities, which can be explained by the effect of the city thermal island. According to Bednář (1985), this is reflected by the fact that we can observe a higher temperature inside the city than in the surrounding undeveloped landscape. This frost-sensitive spider seems to use this and thus it can survive the winter and the unfavorable conditions. An important role in spreading appears to be the presence of major road journeys that lead through cities and villages. Here is a big prerequisite for spreading through automotive and freight transport on the chassis of the means of transport. A similar effect is probably the presence of the railroad near human settlements, which would explain the abundant occurrence of cobwebs in the railway stations, where there is probably the spread of this type by train sets.

CONCLUSION

Mapping using Google Street View, which has never been used to spider sightings, has brought new insights into the spread of the species in the territory of Moravia and Silesia. From the area of interest, only two faunistic squares were recorded before this observation, but it was newly found out of in another 45 faunistic squares. However, it is likely that this extension is far from definitive and the spider will continue to spread to places where its occurrence has not yet been recorded.

ACKNOWLEDGEMENT

The research was financially supported by the grant IGA FA MENDELU Brno No. IP_8/2017. Special thanks to Ing. Andrea Lešková for help with translation of the article.

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THE PHENOMENON OF SUBURBANIZATION AND SATISFACTION OF THE POPULATION IN SELECTED VILLAGES

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Abstract: The paper analyses the influence of counterurbanization on the contemporary face and nature of the countryside. The research was focused on identifying problems associated with the countryside. It presents several different examples of countrerurbanization in some rural areas. Average statistical indicators such as the number and age of population, highest level of education, and others were compiled and evaluated. Possibilities of transport accessibility to the nearest towns were also included and analysed. Comparative methods were used in selected areas not only for statistical data but also for map data. The comparative methods were complemented with the results of interviews with local residents. The results show differences not only in the statistical data for the individual villages but also allow an insight into different cultural life and life quality in rural areas, and its perception by residents. Based on the obtained data, we could identify different factors influencing the process of counterurbanization/suburbanization in the individual areas.

Key Words: land appropriation, countryside, counterurbanization, land use, quality of life

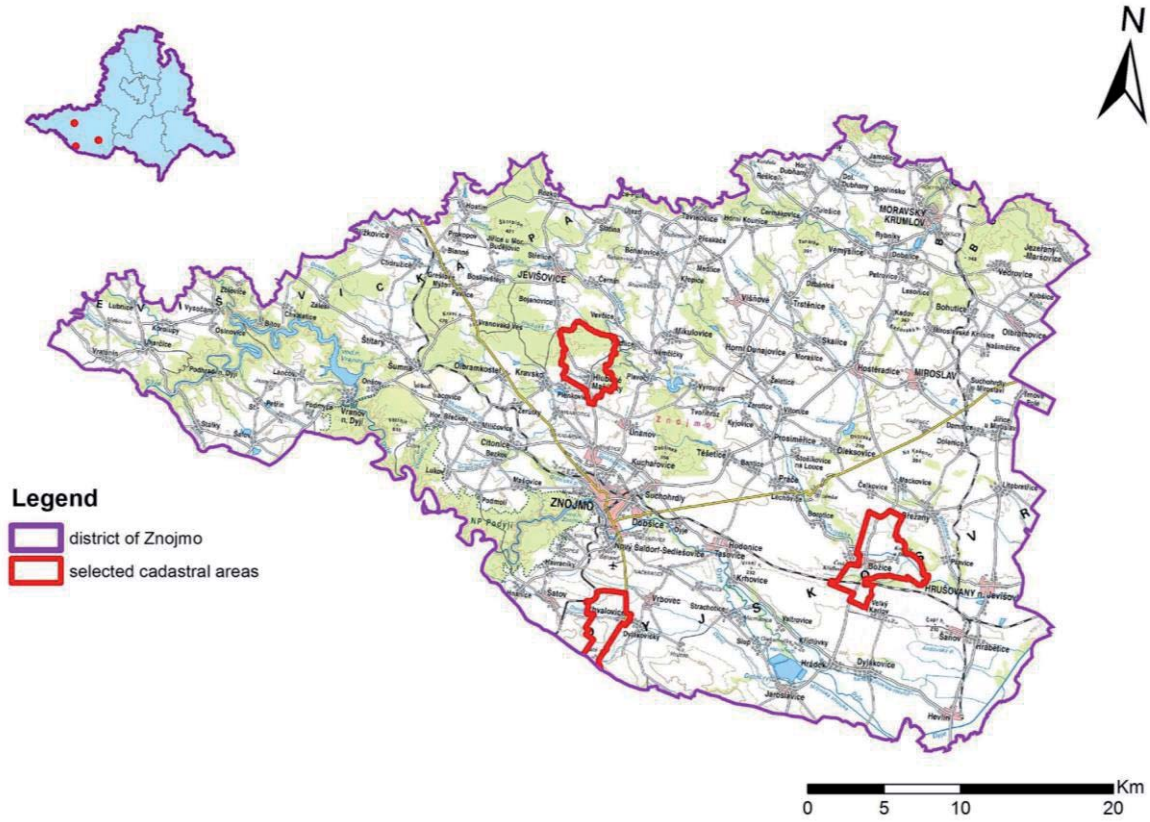
INTRODUCTION

Counterurbanization and suburbanization are demographic and social processes whereby people move from urban areas to rural areas (Berry 1980). Another definition of suburbanization as a process of moving residential, commercial and other functions from the core town to its suburbs, and moving inhabitants and their activities from the core to suburban and rural areas still does not make it possible to distinguish precisely between counterurbanization and suburbanization. At all the above-mentioned definitions, suburbanization will be perceived as a part of counterurbanization with the population structure being a key to the understanding of post-socialist urbanization (Krisjane and Berzins 2012). For the purposes of our research, we anticipated that suburbanization could be understood as a component of counterurbanization. Rural areas constitute 73–82% of the Czech Republic's land area (depending on specific criteria) (Novotná et al. 2013). Residential decentralization is a major trend in the Czech Republic after 1990. Under the development of suburbanization, according to Guest and Brown (2015), there are two main causes, namely transport technologies that increasingly make it possible to travel over long distances for a short time span, although congestion in urban centers is partly limiting. Second, improving electronic communications. In post-capitalist countries, according to Stanilov and Sýkora (2014), fast suburbanization is facilitated mainly by the privatization of state assets, deregulation of economic activities and decentralization. At the same time, we must also take into account the new quality of suburbanization in Central and Eastern Europe, especially after 2000. It is not just about migrating people but also jobs and suburban work. This means changing the relationship between the core and the suburban area. Nevertheless, the motivation for suburbanization and its interpretation can not be generalized. The features of this process differ from case to case and depend on the context (Brade and Kovacs 2014).

Researched areas were three villages situated in the Znojmo district (Figure 1). First written references to these three villages date back to the 13th century. Their different development resulted primarily from historical events in the period 1938–1989. The village of Hluboké Mašůvky

is situated 8 km north of Znojmo town. The village of Chvalovice, which is the smallest of the three villages chosen for our research, is situated 8 km south of Znojmo. The village of Božice is situated 20 km east of Znojmo town and in our research, it represents the largest and most populated village

Figure 1 Znojmo district depicting the selected cadastral areas. ArcGIS Programme, Author (ÚAKE, FA MENDELU 2017)



MATERIAL AND METHODS

For this study, we chose two municipalities neighboring their cadastre with the city of Znojmo (33 823 inhabitants) and one municipality over 1 000 inhabitants for comparison of developments not located in the neighborhood of the town of Znojmo. Chvalovice and Hluboká Mašůvky were chosen due to the different population development after 1945.

Comparative method

Using the comparative method, we compared the aspects of individual villages. Similarly, we used this method to compare changes in maps, orthophotomaps and statistical documents for individual areas.

Hypothesis 1: In our opinion, suburbanization and its recognition is based primarily on the age and on the educational structure of inhabitants along with the distance of commuting to job/school.

Hypothesis 2: We also presume that the number of inhabitants and houses accomplished in the period between 2001–2016 increased due to the fact that the village cadastre neighbours with a town.

Interviews with villagers

Semi-standardized interviews with citizens in the respective villages combine the comparative method with data on the perception of cultural life and life quality in the village.

Processing of map outputs

ArcGIS 10.3.1. software was used to process the map outputs.

RESULTS AND DISCUSSION

Development of inhabitants and houses

Chvalovice, Božice and Hluboké Mašůvky villages are most likely of old Slavonic origin and the first written records date back to the 13th century. All the selected villages with the exception of Hluboké Mašůvky were adjoined to the Reich in 1938 and situated in the so-called Deutschsüdmähren. Hluboké Mašůvky was a typically Czech village which is demonstrated by for instance local names from the facsimile of maps of the stable cadastre dated 1824. The trend in the number of inhabitants and houses from 1869 provides an insight into the development of the villages. The village of Božice recorded the highest number of inhabitants and houses in 1930 (2 593 inhabitants and 596 houses). Following the events connected with the resettlement of local population in 1945 and subsequent repopulation of the area, the number of inhabitants dropped to 1 366 and the number of houses to 374 in 1950. In 1961, the population slightly increased to 1 606 persons. From that time, it was continually decreasing up to 1991, when the number of inhabitants started to grow again.

In the period that was most interesting for us (2001–2016), Božice attained a growth in inhabitants by 14.4% up to 1 544 and in houses by 14.3% up to 455. The village of Hluboké Mašůvky reached the highest number of population and houses in 2016 (814 inhabitants and 339 houses). The village that did not experience the mass displacement of population shows a continuous population development since 1869, with slight drops in 1950, apparently related to the evacuation of a few inhabitants and to leaving for resettled areas. Another small population decrease was recorded in 1980–1991 in connection with the departure of inhabitants to the nearby town.

In the period from 2001–2016, the number of inhabitants and houses increased by 15.8% and 23.3%, respectively. The highest population growth in the period from 2001–2016 was recorded in Chvalovice, by 55% for inhabitants (646 in 2016) and by 43.7% for houses (171 houses in 2016). Yet the village failed to equal the maximum number of inhabitants (808) reached in 1890 or the maximum number of houses (177) reached in 1930.

The village of Chvalovice is an example of village that was not only stigmatized by the displacement of inhabitants but also by the subsequent "iron curtain" and this is why its population was continuously diminishing until 1980. A significant population growth was recorded only after 2001; it was however connected with a considerable financial subsidy granted to the villagers.

Farmland appropriation

These municipalities were gradually losing their farmland area. This shows best in the village of Hluboké Mašůvky (Table 1), where the scale of total cadastre area changed very little over time and thus the decline of agricultural land can be best observed. We have used the definition that agricultural land is land used for agriculture or pasture. Agricultural land consists of arable land, hop fields, vineyards, gardens, orchards, meadows and pastures. The remaining part of the surface is referred to as non-agricultural soil. It is further subdivided into forest soil, water areas, built-up areas and other areas (eg roads, playgrounds, pavements, gorges, parks) (Pozemky a farmy 2014). While the farmland constituted 53.4% of the cadastre area in 1945, it was merely 39.5% in 2016. At the same time, the built-up land segment increased from 0.4% in 1945 to 1.1% in 2016.

This development is evident also from the development of soils in the Czech Republic, where from 31. 12. 2000 was 54.3% of agricultural land and 45.7% of non-agricultural land. The situation in the Znojmo district on the same date was slightly different due to the prevailing agricultural character. Of the total acreage as of 31. 12. 2000, 69.3% of the land was agricultural and 30.7% of the land was non-agricultural, while 21.5% of the total area of the area was forest land and 1.2% of the water area. The situation as of 31. 12. 2016 confirms the decline of agricultural land. The situation in the Czech Republic is 53.4% of agricultural land and 46.6% of non-agricultural land. In the Znojmo district there are 67.5% of agricultural soils and 32.5% of non-agricultural soils. Of the total area of the district there is also an increase compared to 2000 in forest soils to 22.2% and water areas to 1.9%.

Table 1 Appropriation of farmland in Hluboké Mašůvky. ÚAZK 2016, Bičík 2008, ČSÚ 2017

Year	1945	1948	1990	2000	2016
Non-agricultural land (in ha)	559.90	698.20	752.00	769.60	776.39
Agricultural land (in ha)	608.70	580.7	528.10	508.20	507.35

Social and economic aspects

Our selected aspects include: percentage of inhabitants over 65 years of age (Table 2), percentage of economically active people (since 2011), percentage of inhabitants commuting to school or job (since 2011) and percentage of inhabitants over the age of 15 who have attained complete secondary or higher education (since 2011). We also surveyed current services in the concerned villages such as the general practitioner, shop, post office, primary and nursery school.

Table 2 Percentage of population over the age of 65 in the village. ČSÚ 2017

Villages 2016	Population 0–13 years	Population 14–54 years	Population over 65 years	Population total	Percentage of population over the age of 65 in the village
Hluboké Mašůvky	139	524	151	814	18.60%
Chvalovice	127	437	82	646	12.70%
Božice	267	1015	262	1 544	17.00%

Age structure of the population closely relates to the ever-increasing mean age. In 2016, the mean age in the villages of Chvalovice, Hluboké Mašůvky and Božice was 37.9, 42.0 and 39.9 years respectively. Mean age and age structure combine with the increasing population and number of houses. In 2011, the percentage of economically active population in the village of Chvalovice was 50% and according to actual data on the age structure, age and economic status of respondents, we can assume that the percentage is between 55–60% in 2016. In the village of Božice, it was 44% in 2011 and the assumption for 2016 is 50–52%. In the village of Hluboké Mašůvky, it was 45% in 2011 and the assumption for 2016 is 50–55%. The percentage of commuters in 2011 was 26% in Chvalovice, 18% in Božice and 25% in Hluboké Mašůvky. The commuting of citizens to the nearest town of Znojmo/Hrušovany nad Jevišovkou is corroborated also by maps according to Ouředníček et al. 2008, who inform that the percentage of commuters over 45 minutes does not exceed 15% in the villages. The percentage of commuters in 2016 is definitely higher (over 30%); however, apart from the interviews, which reported 80%, the authors do not have other demonstrable results. The percentage of persons over 15 years with the completed secondary and higher education in 2011 in Chvalovice, Hluboké Mašůvky and Božice was respectively 28%, 24% and 20%.

Life quality and its perception by residents

In the interviews were included 65 people from all three municipalities (24 Chvalovice, 22 Božice and 19 Hluboké Mašůvky). The most prominent group was women aged 25–35 with full secondary and tertiary education, who live in the community for a lifetime or for over 17 years. The interviews with individual residents indicate that their most frequent point rating (scale 1–6:1 very poor, 6 excellent) was at the level of 5, i.e. good. An opposite result we received when the mutual interconnection was assessed of old residents and new comers where the most frequent rating was 2–3 points with the interconnection being evaluated as poor and inadequate. Perception of the development of intravillan and extravillan of villages in the last 17 years gave clear results only in the case of intravillan, when the citizens assessed the adequacy of new changes and development. As to extravillans, results of the interviews were not so clear; the residents were not able to evaluate the development of their village extravillan and focused most often only on the appropriation of land

connected with the new housing. This was assessed especially in the village of Hluboké Mašůvky as dislocated only to one site where a separate part of the village emerged without any continuous interconnection with the original built-up area. A quite opposite perception was recorded in Chvalovice, where the new housing is broken into several parts of which all are connected with the core built-up area. Notwithstanding the appropriate/inappropriate division of the new housing estates, a majority of respondents agreed that the location of buildings on the site was inappropriate as well as the size of building sites themselves, infrastructure was inadequate or missing, greenery lacking and road network inadequate, namely in terms of road width. Although the problem of the width of roads is felt only in the new developments, it should be pointed out that it is very similar in both new and old housing areas. We consider this negative perception as due to the increasing number of private cars. The fact also directly connects with the commuting to work/school where 65% of respondents stated to use only the car and only 20% regularly use public transport. The residents also expressed opinions about their possible engagement in organizing cultural events, about the frequency of such events and traditions in the village. Inhabitants of all villages miss "common village entertainments", especially in the summer time. They also gave explanation what cultural events are important for them and what are not. Interesting was that for example in the village of Chvalovice, residents consider the St. Margaret Pilgrimage (organized already for the seventh time in 2017) unimportant because unoriginal. At the same time, however, they take the event of open cellars (younger than the St. Margaret Pilgrimage) as a valuable mass event linking up with the tradition of wine growing and making in the village.

CONCLUSION

It is very difficult to distinguish between suburbanization and counterurbanization in real life. Suburbanization can be viewed as a change in the population distribution and in the spatial structure of suburban areas as well as a transformation in the life style of "suburbanizing" inhabitants (Ouředníček and Temelová 2008). The problems of not only suburbanization and counterurbanization, but also the development of the concept and contemporary concept of counterurbanization are described in detail by Šimon (2011). Based on the comparative method, it is possible to say that the limit for suburbanized area is considered population increase by more than 25% and increase in the number of accomplished houses by 20% in 2016 as compared with 2001. Furthermore, the percentage of people over the age of 65 does not exceed 20%, the percentage of economically active people in the local population is over 50%, at least 1/3 of inhabitants over the age of 15 have completed secondary or higher education and over 1/3 of inhabitants commute to work or to school.

If the results of the comparative method also yield the results of interviews with citizens, we can say the following: The village of Chvalovice could be considered suburbanized because it met all criteria selected by us. The village cadastre neighbours with the town cadastre, and in the period from 2001–2016 the number of inhabitants increased by more than 25% and the number of houses increased by more than 20%. Specifically in Chvalovice, the increase in population and number of completed new houses amounted to 55% and 43.7%, respectively. At the same time, the number of inhabitants over 65 years of age does not exceed 20% (Chvalovice 12.7%) and the economically active residents represent over 50% of the population (Chvalovice 50%). Although the last two parameters and the fact that at least 1/3 of inhabitants over the age of 15 let have had completed the secondary or higher education (Chvalovice 28%) and more than 1/3 residents commute to work or to school (Chvalovice 26%) were not exceeded, it is assumed -with respect to some older data (2011) – they were exceeded in 2016. In spite of all this, we consider the village of Chvalovice as a counterurbanized area, primarily due to the decentralized new housing in the village, involvement of newcomers into the local cultural life, assurance of population's basic needs by the village shop, post office, general practitioner, kindergarten and other services for which the residents do not have to travel to the town. Although the village of Hluboké Mašůvky does not meet all criteria, we can consider it as a suburbanized area with regard to unavailability of all data for 2016.

In Hluboké Mašůvky, a clear problem emerged with the new housing on ill-thought layout of sites and with the missing connection with the original built-up area when the new development constitutes in fact a separate "satellite". Furthermore, the new development is perceived negatively by the old residents and the number of cultural events there, which would facilitate establishment

of mutual relations between the old residents and newcomers, forming also an important factor of perceiving life quality in the village, is decreasing. The village of Božice does not meet more criteria, which will presumably be not met in 2017 either and this why we consider the area as counterurbanized based on the results of interviews.

Both of our hypotheses have been confirmed and the results will be used for further suburbanization research in municipalities located in the neighborhood or near the town of Znojmo. Based on map outputs we can visually observe individual municipalities, changes in them and their location within not only the Znojmo district but also the South Moravian Region.

ACKNOWLEDGEMENTS

The research presented in this publication was supported from the Internal Grant Agency of the Faculty of AgriSciences, Mendel University in Brno. IGA grant no. IP_14/2017 "The phenomenon of suburbanization and it manifestations in the countryside".

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DIFFERENT IMPACTS OF DROUGHT IN PROTECTED AREAS OF THE MOHELNO SERPENTINE STEPPE AND THE MORAVIAN KARST

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Abstract: Moisture and temperature conditions are among the crucial abiotic factors of the environment, which are fundamental for the occurrence of a particular species. The aim of this work is to assess two different protected areas, where drought has a completely different impact. With the use of water balance characteristics, a map of drought threat for the Czech Republic was created. Based on the knowledge of the moisture conditions, two contrasting localities were selected, where the impact of drought is completely different. It was a locality, for which drought represents an environmental problem – a protected landscape area the Moravian Karst, and a locality, which is not threatened by drought – a national nature preserve of the Mohelno Serpentine Steppe. Further assessment of the localities is focused on the relationship of drought (a change of moisture and temperature conditions) and the existence and function of the ecosystems.

Key Words: the Mohelno Serpentine Steppe, the Moravian Karst, drought, water balance

INTRODUCTION

Individual species and populations can only reproduce, grow and survive within the range of specific conditions. The set of requirements of a particular organism of the environmental conditions is called ecological niche. The range of the tolerance of a particular species of a specific factor is called ecological valence. Based on this, species can be either stenovalent (species with a very narrow valence), or euryvalent (species with a wide valence). For the survival of an unbalanced or damaged ecosystem (i.e. by a change of a particular abiotic factor), its ecological stability is crucial, which is determined by its resistance and resilience. Resistance is an ability of the ecosystem to withstand a disruption without visible changes, resilience enables the unbalanced ecosystem to remedy andre-establish its functions. A change of abiotic conditions of a biotope of a particular biocenosis can also cause further disruption with biotic changes. In connection with this, populations of species can expand to areas, where they did not originally occur, resulting in suppressing of the original species typical for the area (Laštůvka and Krejčová 2000).

There is a number of various ecosystems within our country, differing in moisture as well as temperature requirements. Drought is a threat for those valuable localities, where the existence of animals, plants and other important biodiversity elements, is closely dependent on water environment. On the other hand, there are localities, where drought and high temperatures are crucial for the occurrence of specific plant and animal species. Contrasting conditions, such as these, can be found in the Mohelno Serpentine Steppe and the Moravian Karst. While in the Mohelno Serpentine Steppe, the climate is predominantly warm and dry throughout all the year, the Moravian Karst is a locality rich in caves with a very specific climate (cool and humid), which is, however, dependent on the outer climate, affecting the occurrence and process of the karstic phenomena.

MATERIAL AND METHODS

Moisture conditions at the selected localities were measured with characteristics of water balance, which shows the difference between precipitation and potential evapo-transpiration. The data was provided by the Czech Hydrometeorological Institute. Based on this data, a map was created,

which categorizes individual localities according to the level of water balance. Two localities stroke by drought were selected, where the effect of the drought is completely different.

A map of drought layer

The characteristic of water balance of grass herbage in a vegetation period, which is characteristic for potential climatic drought, was selected for the evaluation of drought. It is a difference between precipitation and potential evapo-transpiration. The potential evapo-transpiration is a total amount of water, which can evaporate from the surface with grass herbage while it is ideally saturated. The map of drought threat was created on the basis of the entry data from the years 1961–2010. The entry values for the calculation of the potential evapo-transpiration derives from Technical Data Series (TDS), which is a database of daily values of climatic elements created since 1961 for 787 grid points in the Czech Republic in a network of grid points 10 km far from one another. TDS are based on the station network of The Czech Hydrometeorological institute and were calculated in the grid points of the outcomes of the climatic model ALADIN-Climate/CZ with 10 km resolution. A data quality check of the entry data was performed before the calculation of the TDS using ProClimDB software (Štěpánek 2012). The values of the potential evapo-transpiration were calculated using the agro-meteorological model AVISO, which works on the basis of a modified algorithm Penman-Monteith. This model has been used at a branch of the Czech Hydrometeorological Institute since1992 and was modified and adjusted to the conditions in the Czech Republic (Kohut 2007).

Based on this data, a map of the drought threat was created for the Czech Republic. On this map layer, a map layer of specially protected areas of the Czech Republic was added, which was provided by the Nature Conservation Agency of the Czech Republic. On the map of drought, the small-scale and large-scale specially protected areas were thus projected. Then two of the drought-affected localities were selected, where drought has a very different impact. They are Mohelno Serpentine Steppe and the protected area of the Moravian Karst. A detailed description of the localities is focused on the relationship of drought (a change of moisture and temperature conditions) to the existence and functionality of local ecosystems.

The protected area of the Moravian Karst

The protected area of the Moravian Karst is situated in the South Moravian Region and covers the area of a 3–6 km wide and 25 km long belt of devonian limestone from the city part Brno-Lisen northwards to the town of Sloup and the village of Holstejn. Currently, the area of the Moravian Karst is 92 km^2. It is the largest and the most remarkable karst area in the Czech Republic. More than 1100 caverns have been discovered till these days; there are 68 species of protected Embryophyta, and it is a home of more than 100 protected animals, including 22 out of 24 known bat species found around the Czech Republic. In the Moravian Karst also endemic species (i.e. *Cortusa matthioli subsp. Moravica*), and more than 5 species of cavern invertebrates can be found (Mackovčin 2007).

National nature preserve of the Mohelno Serpentine Steppe

The Mohelno Serpentine Steppe is located in the Vysocina region (Trebic District) about 150 m southwards from Mohelno. The area of the preserve covers 50.34 ha, it was established in 1933 and nowadays it is one of the most valuable and remarkable preserves within the area of the Czech Republic. The preserve was established due to the wide-ranging fauna and flora, which benefits from the serpentine subsoil. The subject of preservation at this locality is a large group of xerothermic associations of the serpentine steppe, thermophilic lawns, and serpentine pinewoods. The variety of species is caused by a number of factors, among others mainly abiotic factors, geographical location, as well as human activity. Hence, more than 620 plant species were found at this locality, which is about 1/5 of all the flora in the area of the Czech Republic. As to fauna, the locality is unique for the occurrence of invertebrates (Čech 2002).

RESULTS AND DISCUSSION

Figure 1 The Mohelno Serpentine Steppe (no. 1), and the Moravian Karst (no. 2) on the base map of the water balance

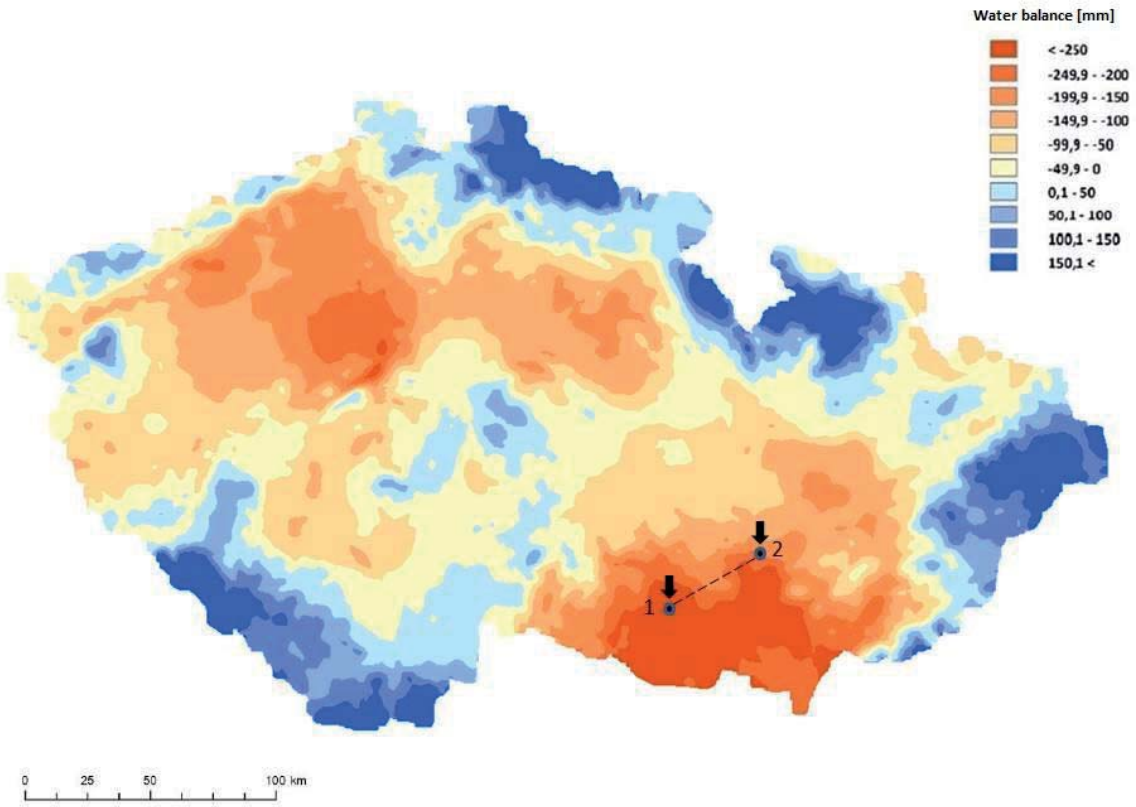

Figure 1 shows the map of the drought threat in the Czech Republic. Numbers 1 and 2 mark the location of the Mohelno Serpentine Steppe (1) and the Moravian Karst (2). Both localities are in the currently most drought-stricken areas of the Czech Republic. The whole area of the Mohelno Serpentine Steppe belongs to the lowest level of the water balance (< -250 mm). Within the area of the Moravian Karst, 3 moisture levels can be found, from -149,9 mm to -249,9 mm. Even though these localities are only 60 km apart, drought has a completely different impact on local ecosystems. It is further observed in the following part of the work.

The effects of drought in the protected landscape area of the Moravian Karst

Caves in general have a very specific micro-climate, which is formed by a number of factors. The thermal regime is mainly affected by heat coming from topsoil, subsoil, airflow and waterflow from the outside environment. Compared to the outside environment, the average temperature of the air in a cave has only a small seasonal and daily amplitude and their value is close to the average annual temperature of the outside environment. Moisture conditions of the caves are, similarly to the air temperature, characterized by smaller daily as well as annual amplitudes, the relative and absolute air humidity and low vaporization. Also daily and annual amplitudes of speed and direction of wind flow (Hebelka and Rožnovský 2011).

Because the temperature inside the cave correlates with the average annual temperature of the outside environment, it is possible to claim that in case of the rise of the outside temperature due to a climatic change, the temperature inside the cave will rise as well. The temperature inside the cave is also affected by flowing water (underground rivers). The impact of the water flowing in the cave is fairly significant, and affects the climate in the cave more than the airflow. The location and orientation of the cave influences the inner temperature as well. If the slopes of the cave face south and the thickness of the topsoil is low, it can cause an increase of the temperature inside the cave.

On the contrary, north facing slopes can cause a decrease of the temperature in the cave (Pflitsch and Piasecki 2003).

Recorded changes caused by drought periods

Due to the drought periods, a number of unusual phenomena has occurred in the Moravian Karst. The cave Pikova dama (Queen of Spades), which is a part of a cave complex called Amaterske jeskyne (Amateur Caves) in the Moravian Karst, is the largest cave in the Czech Republic. It is famous for its giant, several metres long icicles. They begin to form in March and last until October. Their formation is dependent on a strong, cold draught. But because of mild winters and warm springs of the last few years, there has been no ice in this cave since 2006 (Česká tisková kancelář 2015). Another evidence of climate change is the low levels of rivers. The low level of Sloupsky potok (Sloup Brook) revealed Wanklovy komory (Wankel's Chambers, also known as Wankel's Siphons or Wankel's Lake) in 2015, which are not accessible dry-shod. The low level enabled the exploration of places, which can only be observed several times a century (Osouch 2015).

Possible future changes caused by the dry periods

Provided that the caves in the Moravian Karst are of karstic origin (formed by limestone, gypsum, dolomite and others), typical karstic phenomena occur inside them – one of which is speleothem formation (the national nature preserve of the Moravian Karst is the largest and the most well-developed karst area in the Czech Republic). The speleothem formation is dependent on the outside conditions. With the contribution of water and carbon dioxide, the calcium carbonate on the surface rock turns to calcium bicarbonate, which is soluble in water. This compound then flows with water through cracks and pores to the cave. Due to the lower carbon dioxide concentration inside the cave, calcium bicarbonate turns back to calcium carbonate and thus speleothems are formed (Přibyl et al. 1992). Therefore, with the lack of precipitation or its unsuitable distribution in time, the speleothem formation is inhibited.

Another possible phenomenon is a shortening or shifting of the winter dormancy of bats. Currently, the bats only move to the caverns in order to hibernate, while their young are born outside the caves. In case of a temperature rise, caves might be inhabited by summer colonies of bats and their young would then be born inside the caves (Křenková 2015). Apart from the bats, other changes in the cave fauna may be expected.

The effects of drought in the Mohelno Serpentine Steppe

Because of chemical and physical characteristics of the serpentine, segmentation of the georelief and variability of micro-climatic conditions, local flora and fauna are remarkably rich. Dry micro-climate is caused by a combination of several factors, which are the subsoil, geographical orientation of the steppe, and the climatic area. The subsoil of the Mohelno Serpentine Steppe consists mainly of serpentine, which is formed by the metamorphosis of ultrabasic rocks. Serpentine contains a lot of magnesium oxides, which have a significant impact on local plants. Serpentine soil also contains a high amount of heavy metals (nickel, chrome, cobalt). The soil is poor in nutrients, and toxic, due to the heavy metals. Physical characteristics of serpentine and serpentine soil also contribute to the effect. Serpentine is a dark rock with low conductivity and bad weathering. Due to this fact, ridges, rocks and steep slopes can be found at such localities, which contributes to the soil erosion and nutrient loss. Such shallow and skeleton soil leaks water quickly. There are several reasons why serpentine soils are stressful for plants. It is the low amount of nutrients, toxicity and drought, caused by overheating of the dark, serpentine rock (in summer the temperature of the surface can reach 50 °C), and the water leak (Fornůsková and Polákova 2014).

From the geographical point of view, the slopes of the steppe are located in 120 m deep slopes of the canyon of the river Jihlava. Whole steppe is south-oriented, but there are significant differences in exposition, thus steep slopes face south as well as north, which has a considerable impact on the composition of the vegetation. The slopes facing south-east or south-west have a lot of solarification and the sun rays are almost perpendicular to the surface all day and all year. With the contribution of the dark colour of serpentine, the subsoil at these localities can be 18–24 °C warmer than the air. There is also a specific micro-climate in winter months (Fornůsková and Poláková 2014). Quitte (1971) claims that the Mohelno Serpentine Steppe belongs

to temperate climatic area MT11, which is characterized by warm, dry, and long summer, short, mild, and dry winter, and temperate spring and autumn. The precipitation totals in the vegetation period are 350–400 mm. The average annual temperature in the steppe is up to 10 °C higher than in the town of Mohelno (Novák and Fruhwirtová 2012).

A change of moisture conditions at the Mohelno Serpentine Steppe

The area of the preserve was earlier used as a local pasture for cattle, sheep and goats. Since 1933, when the preserve was established, pasturage was prohibited and the area was left unused. Since then, a secondary succession has occurred. Woody species have started to grow there (mainly pine, black locust and thermophilic bushes), along with grasses and various plant species. This flora started to shade and change the characteristics of the station (moisture changes, soil chemism, humus formation etc.), as a result, rare steppe species were suppressed. Therefore, in the 80s a systematic reduction of the secondary succession plants was introduced and in 1997 pasturage was reintroduced as well. Both these activities are crucial for the occurrence and preservation of the steppe (Čech 2002). It is possible that the expansion of the unwanted species as also supported by the construction (1970–1978) of a near pump-storaged hydroelectricity (PSH) – Dalesice reservoir – for the Dukovany nuclear power plant. In that case, micro-climat ic conditions would have been influenced by the cumulation of a huge amount of water close to this locality (Fornůsková and Poláková, 2014). Quitt (1996) deals with the change of the micro-climate caused by the construction of the Dalesice PSH. It was found that the temperature maximums in Jihlava floodplain were lowered, daily amplitude of the air temperature also decreased, and micro-circulation occurs between the levels of both reservoirs, which pushes the cooler and more humid air up to 10 m far from the banks.

Both localities are in the most drought-stricken area. Středová et al. (2016) claims that it is possible to expect another increase in the annual air temperature as well as the air temperature in the vegetation period. This theory is also supported by the fact that when the two past half-centuries (1901–1950 and 1961–2010), the average air temperature in the vegetation period increased from 14–16 °C in the first half-century to 16–18 °C in the second half-century. According to Svejkovská (2016) dry periods are to be expected more frequently and with higher intensity all over the area of the Czech Republic by 2100, causing also the expansion of the dry areas. Svejkovská (2017) also claims that by 2100 the values of potential evapo-transpiration in the vegetation period will increase in the Mohelno Serpentine Steppe from 635 mm to 683 mm.

Středova et al. (2016) states that by 2100 a decrease in precipitation will occur along with a change of its distribution in time. More intensive precipitation and longer dry periods are to be expected. Svejkovská and Prochazková (2016) say that regardless of the kind of drought, biological variability, ecosystem resistance and ecosystem services will be affected.

CONCLUSION

Using the data of the basic water balance, a map of drought threat was created for the Czech Republic. Based on this map, two localities were selected from the drought-stricken areas for more detailed description, this factor, however, has a completely different impact on each of them. The localities were the Mohelno Serpentine Steppe and the Moravian Karst. In the Mohelno Serpentine Steppe, drought is a vital for the occurrence of rare plant and animal species. The water balance value in the vegetation period reaches < -250 mm. The historical context shows that wrong care of this area led to an expansion of unwanted species, changing thus the micro-climate to unfavourable for the rare species, which were then suppressed. A contrastive locality to the Mohelno Serpentine Steppe is the Moravian Karst, which is the largest and the most significant karst area in the Czech Republic. It was proven that the micro-climate inside the caves was dependent on the outer climatic conditions. Due to the dry periods, unusual phenomena were recorded in these caves. It was observed that the two ecosystems have different climatic requirements and thus drought does not have to only have a negative impact. The other authors predict an increase in drought intensity as well as an expansion of dry areas. Further impact on the ecosystems is to be expected.

ACKNOWLEDGEMENTS

This article was written at Mendel University in Brno as a part of the projects IGA FA MENDELU no. IP_25/2017 and IP_1/2017 and with the support of the Specific University Research Grant, provided by the Ministry of Education, Youth and Sports of the Czech Republic in the year of 2017.

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AN ACCESSIBILITY STUDY OF SELECTED FORESTED AREA REGARDING PERSONS WITH REDUCED MOBILITY

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Abstract: The Training Forest Enterprise Masaryk Forest Křtiny is an organizational part of Mendel University in Brno, and it serves training as well as other purposes. This area is also heavily used by hikers, bikers, horseback riders and other target groups. This article focuses in particular on wheelchair users. From the perspective of the general use of the area and possible conflicts between users of the forest trails, we have analysed the related fundamental legal standards such as the Forest Act, the Civil Code, the Roads and Motorways Act, and others. A questionnaire was prepared in order to obtain information about the wheelchair users' preferences. Among other things, our questionnaire survey helped identify the most significant obstacles that people in a wheelchair face in the woodlands. The obtained results have been used for preparing particular proposals concerning a selected location – the forest road from Rudice towards the Dyk Tree Nurseries and their surroundings.

Key Words: wheelchair users, forest area, accesibility, legal conditions, Training Forest Enterprise Masaryk Forest Křtiny

INTRODUCTION

The Training Forest Enterprise Masaryk Forest Křtiny is an organizational part of Mendel University in Brno, and it serves training as well as other purposes. This area is also heavily used by hikers, bikers, horseback riders and other target groups. A recent development are locations for single-track mountain biking. The aim of the project implemented at the Department of Landscape Management (Faculty of Forestry and Wood Technology, Mendel University in Brno) and financed with the support of Internal Grant Agency (Mendel University in Brno) is to identify locations suitable for people with reduced mobility. The aim of this paper is to present the results of a questionnaire survey carried out among selected target group of wheelchair users, to discuss possible conflicts between different user groups and legal restrictions concerning the use of woodlands for recreation, and to propose particular measures for the selected location reflecting the results of previous investigations.

MATERIAL AND METHODS

The current trend is to discover new routes which will allow people with physical disabilities to choose a home and way of life to their liking and live as independently as possible (without relying on the help of others, needing constantly to ask for aid). In this context, we must realise that the basic prerequisite for active participation of a person in social life is the accessibility of areas and buildings, and the ability to use them and move freely in them. It is the fulfilment of the right to freedom

of movement in the broadest sense of the word (http://www.czp-msk.cz). Making landscape accessible by designing tourist routes allowing mainly wheelchair users an independent, safe, easy and smooth motion and their passing with other pedestrians or even bicycles in a natural environment has been discussed by Loučková and Fialová (2010), Jakubis and Jakubisová (2012), Junek and Fialová (2012), Kotásková and Hrůza (2013), Fialová et al. (2015), Rollová (2010), Janeczko et al. (2016). Leisure time spending by people with disabilities has been dealt with by e.g., Pagán (2012), Figueiredo et al. (2012), Kastenholz et al. (2015), Eichhorn et al. (2013), or Blichfeldt and Nicolaisen (2011). The results of their research were used for the proposal of measures. From the perspective of the general use of the area and possible conflicts between users of the forest trails, we have analysed the related fundamental legal standards such as the Forest Act, the Civil Code, the Roads and Motorways Act, and others. Following the principle of universal design (Rollová 2010), wheelchair users were selected as the target group for the purposes of this project. The suggested proposals for people in a wheelchair are also applicable to other reduced mobility groups (elderly people, mothers with prams, etc.) A questionnaire was prepared in order to obtain information about the wheelchair users' preferences. The questionnaire survey was promoted through several channels, also with the help of the Czech Wheelchair Users League and the Metropolitan magazine. The questionnaire was created electronically in Google Documents. Among other things, our questionnaire survey helped identify the most significant obstacles that people in a wheelchair face in the woodlands. The obtained results have been used for preparing particular proposals concerning a selected location – the forest road from Rudice ("K Hlinkovým dolům") towards the Dyk Tree Nurseries and their surroundings.

RESULTS AND DISCUSSION

Questionnaire survey

The answers were collected electronically and were statistically evaluated. A total of 67 questionnaires were filled in. The respondents clearly prefer the summer season, whereas winter is almost invariably regarded as unsuitable. The spring and autumn seasons were identified as partially usable, with a slight preference of spring. Nearly all respondents consider asphalt roads to be the most appropriate. As regards paths and roads with more natural treatment, compacted surface roads as well as wooden pathways were rated positively. Paths and roads with pebble, cobble and grass surface were identified as unsuitable. According to the degree of interest, the requirements can be divided into several groups. The first group (considered by the respondents as the most important) includes features that allow accessing the trail and then spending extended time on it: parking and toilets. Information boards were, too, frequently asked for. Powered-wheelchair users require charging stations. The second group of features, seen as less urgent, mainly includes those making the use of the trail more comfortable: shelters, tables, restaurants or other refreshment facilities, and fire rings. The most important are factors affecting the actual usability of the trail for people in a wheelchair: transverse and longitudinal slope, width, and general visibility. Trail marking, slope or direction changes, and trail length were regarded as less important (Table 1). All obstacles listed in the questionnaire were assessed as very serious. The most serious reservations appear to concern steps and stairs, water crossings, and deep holes (Table 2).

Table 1 A statistical evaluation of factors influencing the choice of trail

Factor	Diameter	Median	Factor	Diameter	Median
marking	2.463	2.5	visibility	2.889	3
equipment	2.611	3	width	3.148	3
changes	2.704	3	transverse slope	3.352	4
length	2.870	3	longitudinal slope	3.389	4

Legend: 1 – unimportant, 2 – hardly important, 3 – rather important, 4 – very important

Table 2 A statistical evaluation of obstacles along the trail

Obstacle	Diameter	Median	Obstacle	Diameter	Median
snow	3.130	3	roots	3.593	4
tree branches	3.148	3	frequent holes	3.685	4
rutted surface	3.481	4	deep holes	3.852	4
narrow trail	3.519	4	water crossing	3.852	4
stones	3.556	4	steps/stairs	3.926	4

Legend: 1 – unimportant, 2 – hardly important, 3 – rather important, 4 – very important

Minimizing conflicts between different user groups

The right to visit woods and forests is referred to as "the public use of woodland". Apart from other legislation, it is enshrined in Section 19 of Act No. 289/1995, on forests (as amended). The Act also says that this right can only be exercised at one's own risk. Thus, the forest represents a place where different groups of users and visitors meet to pursue different aims and needs. Their activities are, of course, governed by the same law; specifically by its Section 20. Even though the entrance to the forest is granted at one's own risk, the general prevention obligation continues to apply according to Section 2900 of Act No. 89/2010 (the Civil Code), as amended. This legislation stipulates that each individual should act so as not to inflict injury to life, health or property.

Above all, the general prevention obligation guarantees the safety of the visitors, and is based on preventing or averting damages should they appear imminent. However, it is always necessary to individually examine what you can expect from these persons, what can be predicted, and to what extent (if at all) it is possible to prevent damages from occurring. Thus, a safe use of woodlands is rooted in a good maintenance of growth and of the path network on the part of the owner. In order to meet the prevention obligations it is necessary to inspect the condition of the paths and the health of the growth and vegetation, especially in the vicinity of the paths and in the most frequently visited locations. Further, it is necessary to comply with the working and technological procedures or occupational health and safety requirements.

Speaking of forest owners, it is also important to mention that the new Civil Code has introduced the institute of notification obligations. This entails warning about potential damage. If an obligation has been (or is going to be) broken, this information must be passed on to the people who might suffer damage as a result. Therefore, if a person authorized by the owner suitably warns the visitors about potential dangers (damaged road surface, obstacles blocking the road, risk of falling trees, etc.), the injured party will lose the right of compensation because the damage could have been prevented based on the warning. Further risks are associated with using the woodland path and road network. The general use of this network is governed by Act No. 13/1997, on roads and motorways (as amended), which classifies both paved and non-paved forest roads as access roads. According to Section 2 Subs. 1 a) of Regulation No. 433/2001, a forest road is an access road designated for the transport of timber, persons or material solely in the interest of the owner, and for the passage of special-purpose vehicles. So forest roads and paths are primarily intended for forest maintenance and management, yet their use by other people and for other purposes can be expected.

On the part of the owner it is necessary to ensure public safety during work in the forest, by displaying due warnings and by putting the roads into a suitable condition when the work is over. The warnings should be displayed not only at all access points to the area where work is in progress, but also on the website of the Training Forest Enterprise. On the part of the visitor it is particularly important to take extra care in areas where work is under way or has recently taken place, and to see the website before taking the trip. Along the proposed trails in the Dyk Tree Nurseries area disabled users will mingle with other visitor groups – hikers, joggers, cyclists, etc. Significant risks arise especially where disabled people and cyclists use the same routes. In the case of the proposed trails, the highest risk is present in sections where the paths are part of the cycle track. In these places it is necessary to provide a road sign for the cyclists to inform about the possible presence of persons

with reduced mobility, wheelchair users in particular. These shared-use sections also need to be indicated in the forest management company's road network maps, both printed and electronic. This will allow both user groups to familiarise themselves with the trail before visiting it. Also, marking the beginnings of cycle tracks will help improve the orientation of disabled as well as other visitors.

A loop trail proposal for reduced-mobility users

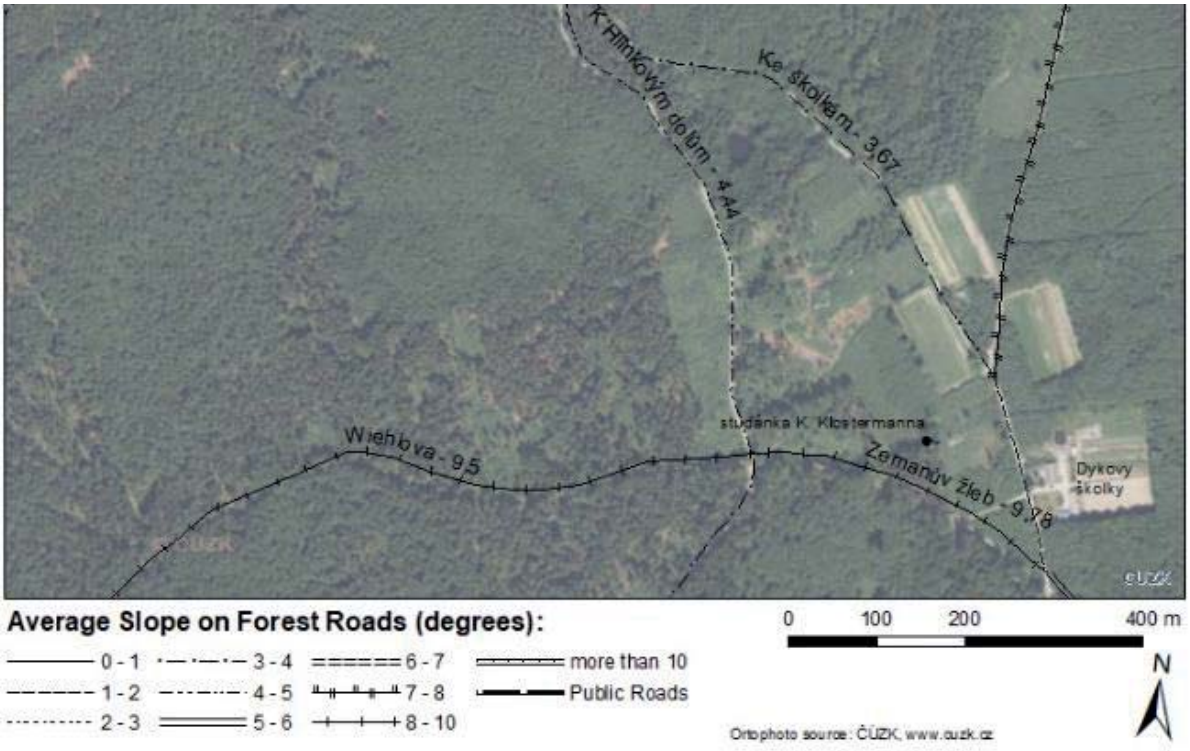
Based on the above-mentioned findings, a trail route has been proposed that leads from the village of Rudice towards the Dyk Tree Nurseries. This route can partly be organised as a loop trail. The same route can be taken to travel there and back. It will not be necessary to travel the entire route. Several alternatives of making the route longer or shorter have been suggested. The longitudinal sloping of the forest service roads can be seen in Figure 1.

The proposal takes into account the requirements for parking and trail access points. The Rudice village is accessible by a local bus. A request would have to be filed to the transport company to deploy wheelchair accessible buses. Cars can be parked in the car park near the forest cemetery, in the close vicinity of the trail beginning at the boom gate on the "K Hlinkovým dolům" forest road. Additional parking space is available in Rudice.A low-traffic road leads from the village car park to the cemetery, with a minimum slope and flanked by trees, so the route can be extended by this section. The trail route can include the service roads "K Hlinkovým dolům", "Pokojná" and "Ke školkám". At the beginning of the "K Hlinkovým dolům" road a bypass needs to be provided to allow access when the boom gate is closed. The longitudinal slope of the roads along the proposed route does not exceed the required 8.33% (i.e. 1:12). The transverse slopes do not exceed 2% (1:50), which again is in compliance with legal requirements for wheelchair accessibility. Wheelchair users regard asphalt as the most suitable road surface for its flatness, lower risk of skidding, and riding comfort. The best quality asphalt-based covers in use today are asphalt concrete and tarmac. Along most sections of the trail, the surface of the selected forest roads is from compacted stone aggregate, which – if conveniently graded – is a surface that suits wheelchair users as well. The service roads are wide enough for wheelchairs. 900 mm are enough to allow one wheelchair, but where passing a pedestrian or making a U-turn is necessary, a width of 1500 mm needs to be accounted for. In order for two wheelchair users to pass each other, a width of 1800 mm is required.

A natural water spring is located near the Dyk Tree Nurseries. Along a stretch of about 200 metres the access road is currently not suitable for wheelchair access, but as part of the above mentioned project (financed by the Internal Grant Agency) a solution is being looked for to improve the accessibility of this water spring and to build a place for resting. Additional place for relaxation has been proposed in the vicinity of the Dyk Tree Nurseries. The staff of the TFE have been consulted about the possibility to provide wheelchair accessible toilets behind the tree nursery. As part of the trail design, it is possible to make use of certain existing service paths with a plausible slope to establish a loop trail serving educational purposes. In the future several stop points with educational boards will be provided along the trail. Concerning the proximity of the Dyk Tree Nurseries, the boards will thematically focus on silviculture. However, all boards must be positioned so as to be easily readable even for a person sitting in a wheelchair. The boards must be put up right next to the trail, or it must be possible for wheelchair users to access them and make a U-turn to return back to the trail. A circular area of 1500 mm in diameter is required to turn the wheelchair, which represents the minimum required manoeuvring space to allow multi-direction rotation of the wheelchair within an angle greater than 180°.

Although the TFE MF Křtiny is not situated in a flatland area, there are locations where persons with reduced mobility (such as people in a wheelchair) can seek relaxation. Forest service roads in the area have a suitable longitudinal slope and a suitable surface; there is a dedicated car park, parking space, or a public transport stop near the trail access points; and at the same time, there are tourist attractions (a reservoir, a natural water spring, tree nurseries, a memorial, lookout points, geocaches, etc.). Therefore, it is advisable to promote these places in a way described above. The Dyk Tree Nurseries area meets more or less all of the above-mentioned requirements, and so we shall continue dealing with the idea of proposing particular additional facilities and landscaping to make the area accessible for persons with reduced mobility to the greatest possible extent.

Figure 1 Averages Slopes on Forest Roads Near Rudice – a section of the map



CONCLUSION

The objectives of the research presented in this article included an analysis of the existing legislation governing the use of woodlands, an analysis of the needs of people using a wheelchair for trips to wooded areas, and a discussion of possibilities to design a trail (ideally, a loop trail) in the area of the Dyk Tree Nurseries at the Training Forest Enterprise in Křtiny. All of these objectives have been met, and moreover, suitable locations were selected for placing additional facilities that could also be utilized by wheelchair users.

ACKNOWLEDGEMENTS

The article was created with support of the Internal Grant Agency of the Faculty of Forestry and Wood Technology, Mendel University in Brno, project no. LDF PSV 2016016 Making forest accessible in the changing social requirements and conditions.

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EFFECT OF IRRIGATION ON PLANT DEVELOPMENT AND FLOWERING PERIOD OF CHOSEN PLANT MIXTURE

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Abstract: Decreasing biodiversity is actual and serious problem of recent years. Protection and re-planting of non-crop vegetation is one of several ways how to support ecosystem services, which are e.g. nutrient and water cycling, soil formation, pest and disease control, pollination. Non-crop vegetation can be either permanent (woods, forest, meadows) or temporary (flowering plant strip as intercrop or in between the rows that can alternate after few years). The study was focused on monitoring of overall integration and flowering period of commercial plant mixture for greening vineyards, orchards and fields and effect of the irrigation. Chosen mixture of plants contains flowers that were continuously blooming from 23. calendar week till the end of experiment (46. calendar week). Irrigation had effect on appearance of several plants species (e.g. *T. repens, P. lanceoalata*) and overall integration of vegetation. Most of the taxons had higher appearance and longer flowering period on non-irrigated variation (e.g. *T. incarnatum, P. tanacetifolia, F. esculentum*), two species had same length of flowering period on both variations and only one taxon (*Onobrychis vicifolia*) have one week longer flowering period on irrigated variation.

Key Words: biodiversity, flowering plant strips, ecosystem service, habitat management

INTRODUCTION

Agrobiodiversity, Non-crop vegetation and Habitat Management

Generally, biodiversity is variability and diversity in between the living organisms and ecological complexes where these organisms exist (Rod et al. 2005). Sustainability of plant production is adherent to active protection and support of biodiversity. In last decades biodiversity is suppressed by monocultural production, liquidation of non-crop vegetation and wild species, melioration, application of chemical pesticides, synthetic fertilizers and growing varieties susceptible to pests and diseases (Rod et al. 2005).

Agroecosystems represent specific kind of environment for which is defined so-called agrobiodiversity, that consist (from UNEP 2000) of biological variability of agricultural ecosystems, genetic plant and animal resources used in agriculture, genetic resources of microorganisms and fungi. Agrobiodiversity has two components: proper grown or breed species chosen by man for agriculture production; and species allied in agroecosystems (weeds, natural species, species in margin parts). Actual biodiversity of agroecosystem depends on diversity of vegetation in proper agroecosystem and surrounding biotops, grown species, level of isolation of agricultural areas from natural ecosystems (Šarapatka 2010).

Function of non-production vegetation inheres in providing so-called ecosystem service. In natural ecosystems, vegetation in woods and meadows protects soil from erosion, helps spare underground water resources, enhances infiltration of water to the soil and reduce run-off by what helps control floods (Harlan 1995). The aim of non-crop vegetation inheres mostly in regulation service (e.g. regulation of air and water pollution, soil erosion and mainly in control of pest and diseases (Haines-Young 2016).

Comparison of ecological and conventional agricultural companies in Switzerland (Schader et al. 2008) and England (Gibson and Pearce 2007) has shown that ratio of the natural areas

is in ecological production higher than in conventional. From analysis of all Swiss agricultural companies ensue that ecological production set apart 22% compare to non-ecological production 13% of area for natural areas (Schader et al. 2008). Diversity in species is 3 times higher in ecological production systems than in conventional production areas (Holzschuh et al. 2007).

Habitat management deals with designed planting of non-crop vegetation and protection of biodiversity in countryside (Fiedler et al. 2008). Such a vegetation is important for reducing negative effect of agriculture such as water and nutrient run-off, erosion and also it is important biotope for beneficial organisms (Šarapatka 2010). According to size of ground, sort of grown plants, local climate conditions is possible to create different forms of non-crop vegetation e.g. flowering plant strips within the crop area or margin parts, hedge fences, large and small patches of perennial plants and trees or various large corridors. By the type of non-crop vegetation there is perennial vegetation (shrubs, trees, perennial plants) and temporary vegetation, that can be after year or few years used as green manure or mulch (Bentrup 2008). The aim of the experiment was to assess the effect of irrigation on development of flowering plant vegetation.

GreenMix multi mixture is mixed from perennial and annual plants. Annual plants require sowing every year so they become less likely a weed in a field. Perennial plants are more likely to spread beyond their designated areas or harbour invasive weeds (Wratten et al. 2003). However, some plant species with potential to enhance biological control may also suppress weeds. The prostrate growth habitat of the *Lobularia maritima* is known to suppress weeds as well being an abundant source of pollen and nectar (Landis et al. 2000).

Beside correct timing of flowering period, compatibility with cropping system and crop is also important. Rotational systems are suitable for annual floral resources within area and perennial species around it, while permanent crop can incorporate either annual or perennial floral resources (Landis et al. 2000). Floral resource used needs to be compatible with the crop regarding its water and nutrient use as well as the time crops are most susceptible to pest damage. Cover crops have been used in viticulture to de-vigore grape vines (Wheeler and Pickering 2003) in order to manipulate vine growth to achieve quality grape production (Landis et al. 2000). Also, water penetration of the soil may be improved because the rooting system of the added plants may improve water infiltration and thereby reduce run-off (Bugg and Van Horn 1998).

MATERIAL AND METHODS

The experiment was proceeded in Lednice, South Moravia Region, Czech Republic, during the year 2014. Lednice belongs to corn agriculture production type and altitude is between 160 m to 200 m a.s.l.. Average year temperature is 9.2 °C and average year precipitation is 479.7 mm. Average month temperatures, precipitation and air humidity during the months of experiment shows (Table1).

Flowering plants mixture GreenMix multi (Biocont Laboratory), designed for greening of vineyards and orchards, was used in this experiment. Assumed duration of vegetation is 3–8 years. Term of sowing is from March to April, or in September. The mixture consist of annual species *Medicago lupulina, Trifolium incarnatum, Fagopyrum esculentum, Phacelia tanacetifolia, Leucosinapis alba, Malva verticilata*; biennial species *Daucus carota*; and perennial species *Onobrychis vicifolia, Trifolium repens, Coronilla varia, Lotus corniculatus, Plantago lanceolata, Festuca rubra rubra, Festuca rubra commutata, Festuca ovina, Poa pratensis*.

Sowing took a place in experimental organic field of Faculty of horticulture in Lednice on 3 April 2014 with sowing machine Demeter Classic 3000 in seeding amount of 10 kg/ha. There were two variants of flowering plant strips, with and without irrigation. Strips were 3 m wide and 63 m long. The distance between both stripes was 25 m. Evaluation of flowering period and phytosociological survey were evaluated in marked rectangle blocks large 3 x 2 m with 3 repetitions for every variants. Irrigation was running in amount 10 mm in one day per week during the weeks without precipitation. First mulching (0.15 m high) was proceeded on 28 July 2014 and second on 14 November 2014 (end of experiment).

Table 1 Weather conditions from April 2014 till November 2014

Month	Average day temperature [°C]	Relative air humidity [%]	Precipitation [mm]
April 2014	11.6	71	20.6
May 2014	14.6	71	46.2
Jun 2014	18.8	58	31.4
July 2014	21.3	70	69.9
August 2014	17.9	80	146
September 2014	15.4	85	166
October 2014	11	88	30.1
November 2014	7.4	87	25.2

Phytosociological survey

Phytosociological surveys were took 4 times for every variants. The areas that species covered were estimated in percentage for every species. Results were processed by ordinal analysis in program CANOCO 4.5. The factors were term (time) and impact of irrigation. Data were logarithmically transformed. Due to estimated length of gradient (1.343 SDU) by detrended correspondence analysis (DCA), redundancy analysis (RDA) based on linear respond model was sequentially used. By testing signification via Monte-Carlo test were counted 999 permutations ($p < 0.05$). Ordinal diagrams were created in program CONODRAW 4.0. (Ter Braak and Šmilauer 2002).

Continuance of blooming period was estimated from same rectangle blocks that were used for phytosociological survey in 3 repetitions for both variant. Estimation took a place every week according to classification after Kuťková, 2012 showed in (Table 2).

Table 2 Classification of blooming phases

No.	Description of phase
1	Beginning of flowerage; visible flowering terminal flower
2	Start of bloom; 30% of blooming flowers on the plant
3	Full blooming; 30–100% of blooming flowers on the plant
4	End of bloom; 30% of flower is past blossom
5	Taxon is past blossom

RESULTS AND DISCUSSION

Phytosociological survey

Impact of term of evaluation and irrigation of flowering plant strips is expressed by diagram (Figure 1). Level of significance level is alfa = 0.002. Taxons are sorted in 5 groups.

First group of taxons has higher affect or larger coverage in variant with irrigation: *Amaranthus* sp., *Artemisia vulgaris, Leucosinapis alba, Onobrychis viciifolia, Solanum nigrum*.

Second group of taxons has higher affect or larger coverage in variant without irrigation: *Malva verticilata, Polygonum aviculare, Rubus fruticosus, Veronica persica, Vicia villosa*.

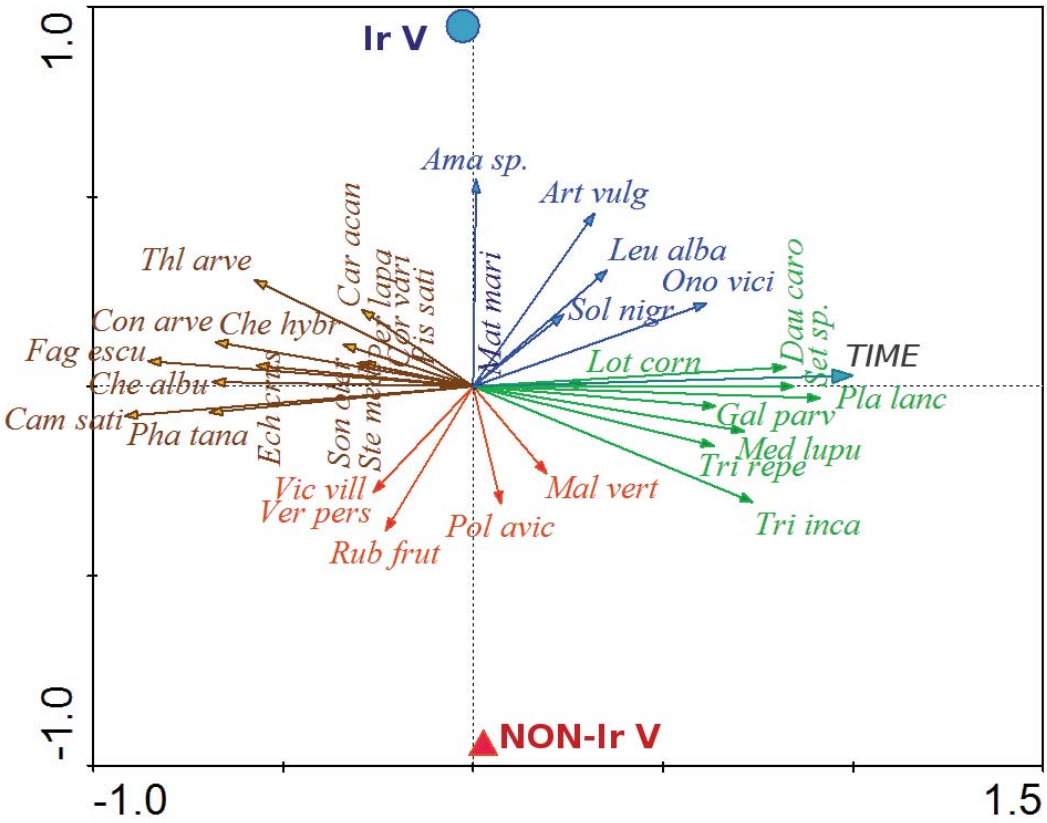
Third group of taxons has affect or larger coverage at the beginning of experiment (earlier terms of evaluation) and with time its affect and coverage decreased: *Phacelia tanacetifolia,*

Fagopyrum esculentum, Camelina sativa, Coronila varia, Thlaspsi arvense, Chenopodium album, Chenopodium hybridum, Convolvulus arvensis, Carduus acanthoideas, Echinochloa crus galli, Pisum sativum, Persicaria laphatifolia, Stellaria media, Sonchus oleraceus.

Fourth group of taxons has higher affect or coverage at the later terms of evaluation and with time its affect and coverage increased: *Daucus carota, Galinsoga parviflora, Lotus corniculatus, Medicago lupulina, Plantago lanceolata, Setaria* sp., *Trifolium repens, Trifolium incarnatum*.

Fifth group of taxons was influenced by other factors that were not included in analyses: *Matricaria maritima*.

Figure 1 Phytosociological survey of taxons non-irrigated and irrigated variant in time



Legend: NON-Ir V = non-irrigated, IR V = irrigated variant

Flowering period

The correct timing of floral provision is important to avoid or minimise negative effects such as increased parasitism or hyperparasitism rates, or increased herbivory. Synchronisation between the flowering period and when the natural enemies are present and require the resource is important (Fiedler et al. 2008). The results of experiment can help to set optimal mixture of plants due to local climate conditions and grown crops.

Most of the taxons were in full bloom phase approximately from 36th calendar week till 43th calendar week. Period of full bloom in duration of 10 to 12 weeks had on both variants species *Leucosinapis alba* and *Malva verticilata*, on non-irrigated variant further *Trifolium incarnatum* and *Fagopyrum esculentum* (Table 3). Those two taxons were in bloom 8 to 9 weeks on irrigated variant thus 2–3 weeks shorter. Taxons *Medicago lupulina* (9 weeks), *Lotus corniculatus* (1 week) and *Camelina sativa* (1 week) had same bloom period on both variants. Except the *Onobrychys vinciifolia*, that had longer bloom period on irrigated variant (6 weeks) than on the non-irrigated variant (5 weeks), had all other taxons longer period on non-irrigated variant: *Trifolium repens* (IrV – 0, NonIrV – 2 weeks), *Phacelia tanacetifolia* (IrV – 1, NonIrV – 2 weeks), *Plantago lanceolata* (IrV – 0, NonIrV – 2 weeks). Development of flowering period was similar on both variants, but the total sum of weeks of full bloom phase is higher on non-irrigated variant (Table 3).

Even though both variants were on the same locality, with the same plant species and soil conditions the effect of irrigation on development of species and weeds was confirmed.

Plants than could be effectively used in localities with higher precipitation from tested mixture could be taxons that had higher affect and larger coverage in irrigated variant (*Leucosinapis alba, Onobrychis viciifolia).* On the other hand the selection of plant species with lower water requirements can effectively minimise water competition.

Table 3 Results of blooming evaluation of chosen species from 23th till 43th calendar week

Calendar week /taxon	IrV	Non-Ir V	IrV	Non-Ir V	IrV	Non-Ir V	IrV	Non- Ir V	IrV	Non-Ir V
	Trifolium incarnatum		*Fagopyrum esculentum*		*Medicago lupulina*		*Onobrychis vicifolia*		*Trifolium repens*	
23			▮	▮						
24			▮	▮			▲			
25			▮	▮			▲	▲		
26	▮	▮	▮	▼	▲	▮	▼	▮		
27	▮	▼	▼	▼	▲	▼	▮	▮		
28	▮	▮	▮	▼	▲	▼	▼	▼		
29	▼	▮	▼	▼	▲	▮	▼	▼		
30	▼	▼	▼	▼	▲	▼	▼	▼		
31	▼	▮		▮	▲	▮				
32	▲	▮	▲	▲	▮	▮				
33	▮	▮	▼	▮	▮	▼				
34	▮	▮	▼	▼	▼	▼				
35	▮	▮	▼	▮	▮	▮				
36	▮	▮	▮	▮	▮	▼				
37	▮	▮	▮	▮	▮	▮	▲			
38	▮	▼	▮	▮	▮	▮	▲	▲		
39	▼	▼	▼	▮	▮	▼	▮	▲		
40	▼	▼	▼	▼	▮	▼	▮	▲		
41	▼	▼	▼	▼	▮	▼	▮	▮		▲
42	▼	▼	▼	▼	▼	▮	▮	▮		▮
43	▼	▼	▼	▼	▼	▮	▮	▮		▮

Legend: ▲ *beginning of bloom,* ▮ *full bloom,* ▼ *end of blooming; NON-Ir V = non-irrigated, IR V = irrigated variant*

CONCLUSION

Results showed that proper mixture of flowering plants can offer full bloom period from 36th to 43th calendar week, thus ensure ecosystem service (e.g. attractiveness for beneficial insects and pollinators). Irrigation had negative effect on length of blooming period for most of taxons, so introduced non-production vegetation should be chosen with regard to local soil and weather conditions to achieve maximum ecosystem service from flowering plants strips.

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THE IMPACT OF LEACHATE RECIRULATION DURING AEROBIC BIOSTABILISATION OF UNDERSIZE FRACTION ON THE PROPERTIES OF STABILISATE PRODUCED

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Abstract: The mixed municipal solid waste collected from households is sent for processing in the mechanical-biological treatment plants (MBT). The aim of the study was to evaluate the effect of recirculation of leachate formed in the process of aerobic biostabilisation of undersize fraction (produced from mixed municipal solid waste in MBT process) on selected parameters of stabilised waste (end product of the process). Two variants of the system performance were analysed that is with and without recirculation of leachate into a bioreactor for which 12 and 10 test replicas were performed respectively. The analyses focused on selected technological properties of waste (undersize fraction and stabilised waste) and included morphological composition, density, dry mass, total organic carbon, loss on ignition and respiration activity AT4 (Atmungsaktivität nach 4 Tagen - eng. Respiratory activity after 4 days). The research was conducted in the waste treatment plant in Cracow (Poland) in the period from December 2016 to April 2017. The aerobic biostabilisation process on the MBT system should be based on leachate recirculation technology due to the fact that in this series of tests 83% of the samples achieved all the desired parameters of stabilised waste whereas in the second variant only 30%.

Key Words: mechanical-biological treatment, municipal solid waste, aerobic biostabilisation, stabilised waste

INTRODUCTION

According to the act on the maintenance of cleanliness and order in the municipalities, every municipality is obliged to ensure order and cleanliness on its premises, establish the necessary conditions for its maintenance, embrace all property owners with the municipal waste management system and ensure the construction, maintenance and operation, of its own or common with other municipalities, of the regional municipal waste treatment plant (Journal of Law 2017, item 1289), most often of the mechanical-biological treatment (MBT) plant. In these plants, fractionation such as paper, metal, plastics and glass takes place and which then are recycled and reused (Dias et al. 2014). In MBT plants an oversize fraction exceeding 80 mm (which can be used for example in cement plants as an alternative fuel) is separated on drum or vibratory screens with a mesh size over 80 mm and the undersize fraction below 80 mm is directed to the biological part of the MBT plant in order to be subjected to biodrying, aerobic biostabilisation or methane fermentation (Dębicka et al. 2017). According to Dębicka et al. (2013) biological drying (biodrying) is a relatively new and still unrecognized method of waste management. Biodrying contributes to the reduction of moisture content in waste and to the disappearance of biological degradation resulting in stable and usable fuel (Domińczyk et al. 2012, Flamme 2006, Sugni et al. 2005, Velis et al. 2009). Anaerobic biostabilisation is the process of biological disposal of waste under anaerobic conditions resulting in the production of biogas and digestate, which can then be recovered or disposed of by storage (Jędrczak and Szpadt 2008, Sikora and Mruk 2016). Aerobic stabilisation is the biological waste disposal process (D8) conducted under aerobic conditions that produces two new wastes - stabilised waste (for landfilling) and compost (for land reclamations). The MBT process is particularly popular in Europe (Adani et al. 2004, Dziedzic et al. 2015), and one of its most important goals is to reduce the amount

of waste deposited in landfills (Abeliotis et al. 2012, Grzesik and Malinowski 2016). MBP plants can also be considered as an alternative to thermal conversion of waste (Soboniak and Bień 2015).

The MBT method does not fully destroy the biodegradable waste (Voberková et al. 2017, Gliniak et al. 2017), but if conducted properly it can obtain the degree of decomposition of biodegradable matter to reach the parameters specified in the Regulation of the Minister of the Environment (Journal of Law 2012, item 1052, Żygadło and Dębicka 2014). The provisions of the regulation are consistent with the provisions of the European Union Directive 1999/31/WE and the European Council Regulation 2008/980/WE (Directive 1999, 2008). In pursuance of the mentioned law regulation regarding the stabilised waste designed for safe landfilling, after the biological step one of the following criteria should be filled (Journal of Law 2012, item 1052):

- The loss of ignition (LOI) of the stabilised waste should be less than 35% related to the dry mass, and the amount of total carbon organic content (TOC) should be less than 20% in the dry mass, or
- The indicator ΔS of the LOI difference based on comparing the waste before and after the biological treatment should be greater than 40% d.m, or
- Respiration Activity AT4 should be less than 10 mg O_2/g d.m.

The share of the undersize fraction obtained from mixed municipal waste depends on the origin of the waste and the season. During spring and summer, the percentage of undersize fraction is lower, while in autumn and winter higher. The average of 550 kg of the undersize fraction (Malinowski 2012) is obtained from 1000 kg of mixed municipal solid waste collected from the countryside. In the aspect of biological processing of the undersize fraction, the share of the biodegradable fraction is crucial. The results of the study carried out by Baran et al. (2016) show that the largest amount of biodegradable waste is produced in the summer and autumn which translates into the rate of biostabilisation of waste.

The aim of the study was to evaluate the effect of leachate recirculation formed in the process of aerobic biostabilisation of undersize fraction (produced from mixed municipal solid waste in MBT plant) on selected parameters of stabilised waste in order to specify the optimum model of functioning of the system in the MBT plant in Krakow (Poland). The optimum process is the one where the parameters set for the produced stabilised waste are obtained in the shortest period of time. The analyses included the selected technological properties of the undersize fraction and stabilised waste that is morphological composition, density, dry mass, total organic carbon (TOC), loss on ignition (LOI) and respiration activity (AT4).

MATERIAL AND METHODS

Description of system

The MBT plant where the study was carried out consists of 4 bioreactors (made of reinforced and acid-resistant concrete). Every bioreactor is a process-independent, sealed module measuring 16 m in length, 7.35 m in width and 6 m in height, equipped with a monitoring system for process parameters, aeration, ventilation and spraying. The front of the bioreactor is closed by a sliding gate. Aeration channels functioning as air supply lines and in the interim as drain pipes as well as six biofilters and control rooms were built into the floor of every bioreactor.

Process leachate is drained by aeration and drainage systems to one of the three underground hermetic septic tanks, and then, after purification on filters it can be pumped into the sprinkler system located under the ceiling of every bioreactor.

The process of biological treatment of waste in bioreactors takes place using active aeration. Process air, after passing through the waste bed, is fed into a common for all bioreactors channel through which, by water scrubber mounted on their roof, is directed to the biofilter system located outside the hall. Biofilters have a special bottom construction that allows for even distribution of process air throughout the bed and penetration through the filter material. Optimal parameters of biofilter performance allow for the neutralisation of odours of about 80–90% of the loaded charge.

Process conditions

The aerobic stabilisation process of the undersize fraction was conducted in 2 variants:

a) without moistening (leachate from the process was not used in the process) for 10 samples;

b) with moistening (closed water circuit) for 12 samples. Moistening was carried out using 100% of the leachate formed in the process to enrich the bed with microorganisms.

The mean weight of the waste loaded into the bioreactors amounted to 242.64 ± 21.29 Mg and the mean height equalled 3.03 ± 0.18 m. For every process, the temperature was monitored using two PT 100 sensors. The temperature record was automatically run every 10 minutes in TeamViewer 12 program allowing for determining the time of completion of the intensive phase and the maximum temperature reached in the bioreactor. The estimated waste processing time amounted to 28 days. During the course of the study, the process was terminated when the temperature was lower than 45 °C that is the moment when the intensive phase of the process ended.

Sampling for analyses

The laboratory sample was taken on the basis of the quartering method. Each of the following indications was run in 3 replicates in the laboratories of the Faculty of Production and Power Engineering, University of Agriculture in Krakow and Ferrocarbo. The results of all analyses (30 for non-moistened waste and 36 for moistened waste) were utilised to perform analysis of variance and Tukey's test (STATISTICA 12 package) to determine the significance of the differences between the results of the analyses.

The morphological composition of waste was analysed in 12 categories (groups) of waste (Table 1). The determination of respiration activity that is the AT4 parameter for the undersize fraction and the obtained stabilised waste was enabled by calculating the amount of CO_2 emitted by leachate and measured by OxiTop Control measuring system. The determination includes (EU-Notice number 2001/423/A, Vienna 2002): weighing a sample of about 40g and of assumed humidity, then placing the waste in a glass container of capacity of 2.5 dm^3 and a few grams of CO_2 absorber in the lid of the container and then closing it; placing the container in a thermostatic cabinet with a constant internal temperature of 20 °C and checking daily the value of negative pressure. The final value of negative pressure produced in the vessel is converted to the amount of oxygen consumed during the process in accordance with the Clapeyron equation. The other parameters were determined on the basis of the following standards:

a) PN-EN 14899:2006: Characterization of Waste - Sampling of Waste Materials;
b) PN-EN 14346:2011: Characterization of Waste - Determination of dry mass and water content;
c) PN-EN 13137:2004: Characterization of waste - Determination of total organic carbon content in waste, sludge and sediments;
d) PN-EN 15169:2011: Characterization of waste - Determination of loss on ignition in waste, sludge and sediments;
e) BN-87/9103-04: Characterization of waste - Determination of density.

RESULTS AND DISCUSSION

The duration of the process amounted, on average, to 15 ± 2 days. The differences between the average periods of non-moistened and moistened processes were not statistically significant. The volume of water (leachate) introduced into the process in tests with additional bed moistening was on average over 18.5 m^3. The mean maximum process temperature came to 60.89 ± 2.96 °C. The temperature of the treated waste for the non-moistened samples was higher and amounted to 61.25 ± 2.71 °C, while for moistened samples it was 60.55 ± 3.26 °C. However, these differences were not statistically significant.

Table 1 presents the average morphological composition of the undersize fraction and the waste processed. The conducted studies indicate that in the morphological composition of the undersize fraction, as a result of the aerobic stabilisation process, the share of fine fraction e.g. the share of waste of particles below 10mm was increased over time from 33.97 ± 3.21% to 41.21 ± 3.16%. The fine fraction predominated in the waste composition. As a result of the process, the amount of organic fraction was reduced from 19.54 ± 3.04% to 12.11 ± 2.81%. The share of the remaining waste fractions as a result of the process did not change by more than about 2%. The reduction of share of the organic fraction and the simultaneous increase of the share of the fine fraction containing mainly mineral waste indicates the correct course of the process and the high activity of microorganisms responsible for the processing of organic waste into inorganic products.

Table 1. Average morphological composition of the undersize fraction on individual days of the process

		Day of biostabilisation process				
		3	6	9	12	15
1	Plastic	5.87	6.41	6.12	6.32	5.87
2	Glass	9.81	10.54	10.41	10.34	10.47
3	Metal	2.90	3.10	2.40	2.90	2.60
4	Paper	5.22	4.92	4.84	4.74	4.51
5	Organic waste	18.67	17.24	14.42	13.29	12.11
6	Wood	5.42	5.27	4.77	4.42	4.35
7	Hazardous	1.14	1.35	0.99	0.74	1.47
8	Hygienic waste	0.48	0.48	0.49	0.63	0.52
9	Concrete waste	5.90	5.70	6.20	6.30	6.10
10	Textile waste	8.15	7.96	8.12	7.84	8.21
11	Fine fraction	34.01	35.02	38.15	40.21	41.21
12	Other categories	2.43	2.01	3.09	2.27	2.58

Table 2 shows selected physicochemical properties of the waste after the aerobic stabilisation process for the 22 analysed samples. For all bioreactors tested, the results for stabilised waste are as follows:

a) The mean density of waste came to 730 ± 70 kg/m^3. There was an increase in the density of waste in comparison to the unprocessed undersize fraction by 170 kg/m^3, for the non-moistened samples the density increased by 140 kg/m^3 whereas for moistened samples the density increased by 190 kg/m^3;
b) The mean dry mass was equal to 86.07 ± 6.16%, which means a 21.47% increase in dry mass in comparison to unsteady waste. In tests carried out without moistening, the dry mass content in the stabilised waste rose by 21.46% and with moistening by 19.82% in relation to the undersize fraction;
c) The mean organic carbon content came to 12.98 ± 3.39% d.m. which indicates a decline of 25.42% d.m. with respect to the C content in the undersize fraction, the carbon content of the non- moistened sample decreased by 25.54% d.m., while for the moistened sample the drop was by 25.32% d.m.;
d) The mean values of loss on ignition (LOI) were 20.18 ± 7.17% d.m., which indicates a decrease of 21.12% d.m., the non- moistened samples showed an average decline of 23.26% d.m., whereas for the moistened samples the mean values decreased by 19.33% d.m.;
e) The mean respiration activity AT4 amounted to 8.16 ± 5.08 mg O_2/g d.m., resulting in reduction of the parameter by 20.64 mg O_2/g d.m., for the non- moistened samples, the reduction came to 16.78 mg O_2/g d.m., while for the moistened sample the decrease was by 23.86 mg O_2/g d.m. Statistical analysis of the research results showed that the tested biostabilisation variants differed in a statistically significant manner only in the case of AT4 parameter.

In the non-moistened variant as many as 7 in 10 samples did not reach the desired AT4 parameter. The other parameters described in the MBP Regulation (Journal of Law 2012, item 1052) that is the loss on ignition as well as the organic carbon content, were achieved. In the case of closed circuit of leachate, only 1 out of 12 cases did not achieve the AT4 parameter and subsequently LOI. The total organic carbon content for all 22 samples tested reached the value expressed in MBP Regulation (Journal of Law 2012, item 1052). The obtained results of biostabilisation are parallel to those of Adani et al. (2004) and Dziedzic et al. (2015) but are considerably higher than those obtained by Baran et al. (2016) and Dębicka et al. (2014).

Table 2 Selected physical and chemical properties of the waste after the aerobic biostabiliasation process

Sample type	Density [Mg/m^3]	Dry mass [%]	TOC [% d.m.]	LOI [% d.m.]	AT4 [mg O_2/g d.m.]
Undersize fraction	0.56	64.60	38.40	41.30	28.80
Mean	0.73 ± 0.07	86.07 ± 6.16	12.98 ± 3.39	20.18 ± 7.17	8.16 ± 5.08
Mean for samples 1–10 (without moistening)	0.70 ± 0.04	88.06 ± 3.28	12.86 ± 2.86	18.04 ± 4.33	12.02 ± 3.83
Mean for samples 11–22 (with leachate being recycled)	0.75 ± 0.08	84.42 ± 7.55	13.08 ± 3.90	21.97 ± 8.66	4.94 ± 3.52

CONCLUSION

In waste processing, the organic fraction share and the organic carbon content diminished in its morphological composition which indicates the high activity of microorganisms in the analysed process and its correct course. The stabilised waste after all processes reached at least one of the three parameters that must be met by waste after the biological stabilisation process. In aerobic stabilisation without moistening, the stabilised waste reached all parameters in only 3 tests, whereas in case of leachate recirculation it was feasible to achieve stabilised waste of the desired parameters over the period of 15 ± 2 days in 83% of samples. In two cases the process was completed on the twelfth day.

ACKNOWLEDGEMENTS

This research was financed by the Ministry of Science and Higher Education of the Republic of Poland – BM 4641/WIPIE/2017.

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WATER QUALITY ANALYSIS IN THE UPPER PART OF LITAVA RIVER BASIN FOCUSED ON NITROGEN COMPOUNDS CONTAMINATION

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Abstract: Nitrogen and phosphorus belongs among essential elements and their compounds play key role in cycles of matter. However, over-enrichment with these elements in surface water is a widespread problem resulting for example in eutrophication, methemoglobinemia caused by nitrates and fish poisoning by toxic ammonium ions. The evaluation of annual development of water pollution by nitrogen compounds in the upper part of Litava basin is the main aim of this paper. Twelve sampling profiles have been determined in the interest catchment placed east of Brno – eight directly on Litava stream and four on its main tributaries. The monitoring of selected water quality indicators (total nitrogen, nitrate nitrogen and ammonium nitrogen) was performed since June 2016 to May 2017. Results have been compared with valid legislation – ČSN 75 7221 and Government order No. 401/2015 Coll., as amended and clearly confirmed influence the ammonium nitrogen pollution of water quality in Litava river. The limit specified in the Government order was exceeded in almost all profiles. This pollution is caused by point sources of pollution, especially municipality.

Key Words: water quality, Litava river, nitrogen compounds, water pollution

INTRODUCTION

Surface and ground water pollution is an issue currently being resolved by many developed countries. The European Union has made significant efforts to reduce nitrate pollution from agriculture by determination Nitrate Vulnerable Zones.

Nitrate from nonpoint sources has been identified as the main cause of groundwater degradation in Europe (Sutton et al. 2011).

Upper part of Litava river is placed in east of Brno on the border between South Moravian Region and Zlín Region. Pour point is located in front of Bučovice town. Litava, formerly known as Cézava, flows into the Svratka left tributary near Židlochovice and then continues to the Black Sea.

The catchment area has 136.83 km^2 and consists of seventeen subbasins. Main channel which have been narrowed and entrenched is 23.64 km long. The surrounding floodplain serves for agricultural production. Litava basin is mostly formed by large blocks of arable land which covers 71% of basin. The main grown crops are wheat, barley, canola and sugar beet. Arable land is threatened by water and wind erosion. The basin is overall densely populated with 22 municipalities and towns and several large areals with livestock. The average annual flow in measured profile No. 381 in Brankovice is 0.22 m^3/s.

Nitrogen compounds

Nitrogen belongs among essential elements and it occurs in surface water in several forms. Natural sources of nitrogen are decaying of vegetable and animal matter, precipitation and fixation of aerial nitrogen (Pitter 2009). 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).

Total nitrogen is expressed as summation of all its forms observed in water – ammonia, nitrite, nitrate and organic nitrogen (Pitter 1999). The most significant indicators of pollution sources and pollution itself are nitrate nitrogen and ammonia nitrogen. For these forms of occurrence are characteristic various sources of pollution. Ryšavý and Hanák (2013) in their study „Model of the quality of the Jihlava River Basin above the Dalešice dam" examine participation of particular main pollution classes on emissions of total nitrogen into water. The study has shown that 81% of total nitrogen originates from nonpoint sources of pollution (from which 70% is from agriculture) and 19% comes from point sources (from which 17% is from sewage water).

Ammonia nitrogen origins mostly from point sources of pollution: industrial or municipal. Among municipal sources outweigh sewage water where the ammonia nitrogen forms part of physiological wastes causing average production of nitrogen is 8 g/person/day. This indicator is very important from hygienic point of view since it indicates fecal contamination and insufficient quality of wastewater treatment (Pitter 2009). Ammonia nitrogen in form of ammonia salts is harmless for most of organisms even in amounts of several dozen mg/l, however gaseous ammonia is highly toxic for fish. The toxicity of ammonia is increasing with decreasing concentration of oxygen (Kopp et al. 2015).

The main sources of nitrate nitrogen are nonpoint sources of pollution (agriculture in concrete) and drains from wastewater treatment plants. Nitrates runoff from agriculturally exploited areas on which the natural and synthetic nitrate fertilizers are applied (Kopp 2015).

MATERIAL AND METHODS

Monitoring program

Twelve sampling profiles have been established in the catchment area (Figure 1), from which eight are directly on the river Litava stream (profile No. 1–8) and four are on main tributaries – Hvězdlička (A), Litenčický stream (B), Zámecký stream (C), Litávka (D).

The design of the monitoring program must locate appropriate sampling profiles taking to account objectives and the type of monitoring planned (Kunkel et al. 1987). Monitoring program is performed by above-and-below approach, where the sampling profiles are placed upstream and downstream of the treatment area section. The sampling profiles are located in places where potential deterioration of water quality standards caused by particular activity is expected (Brooks et al. 2003).

Several sampling profiles have been added during monitoring period in accordance with above-and-below approach. These additional profiles will be described further in order to be able to more exactly determine or on the contrary exclude influence of pollution source on the water quality.

Measuring process

Monitoring of the Litava water quality was being performed monthly since June 2016 to May 2017. Water sample is collected from each profile in form of single „grab" sample by hand into plastic bottle.

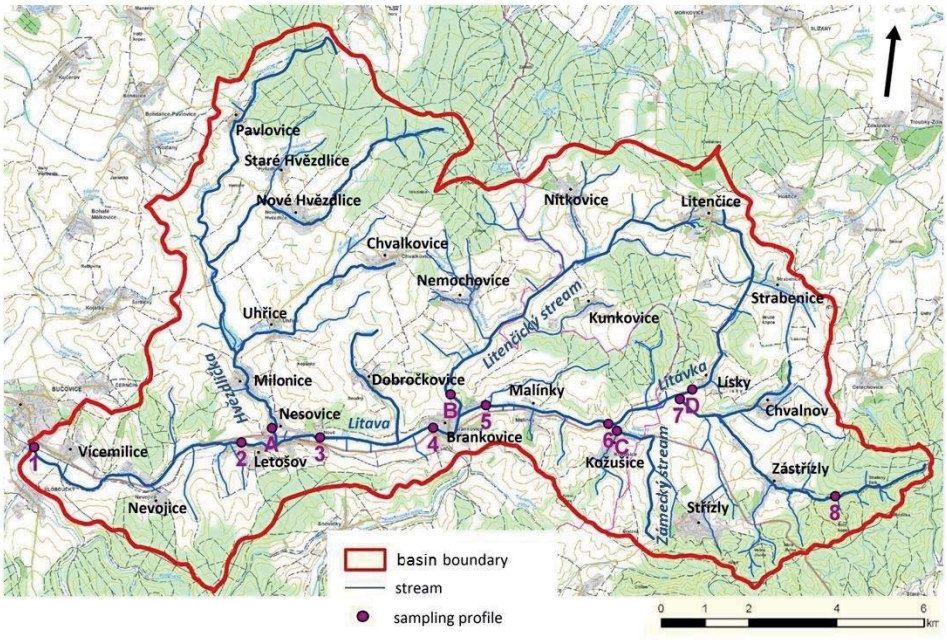
Single sample is representative of the stream discharge only at the time of sampling (Brooks et al. 2003). Samples were analysed within 24 hours in the laboratory of water management. Determination of total nitrogen, nitrate nitrogen and ammonium nitrogen is performed according to HACH LANGE Company standardized methods using spectrophotometer HACH DR 4000.

Evaluation of indicators

Results collected from profiles located directly on the river Litava stream (profile No. 1–8) were graphically demonstrated and evaluated according to the valid legislation – 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 contains permissible surface water pollution requirements specified for each indicator of water quality. Discussion includes also evaluation

of profiles on the main tributaries (profile A – D) which are hardly representable in the graphic form and therefore commented in the text only.

Figure 1 Location of the twelve sampling profiles in upper basin of Litava river (source: author, basemap: INSPIRE – cenia_t_podklad)

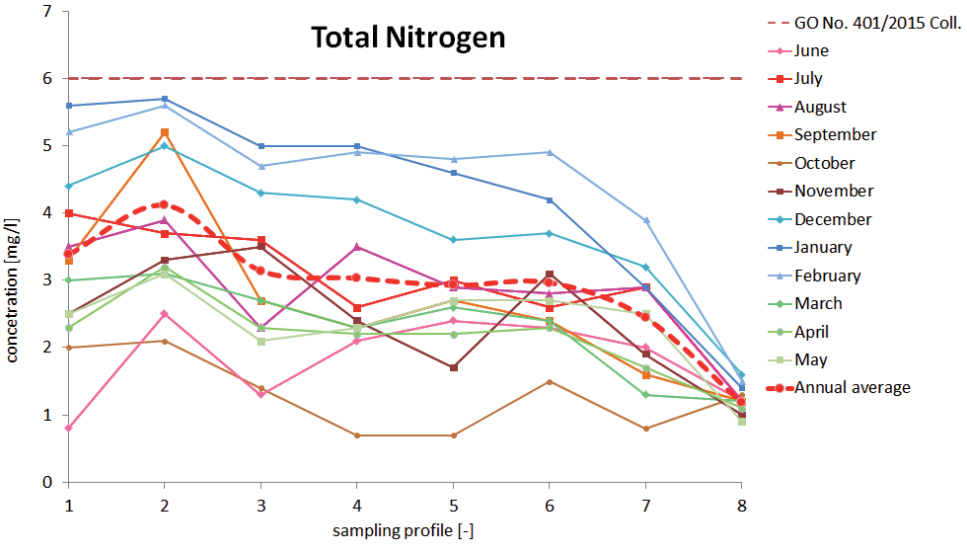


RESULTS AND DISCUSSION

Total Nitrogen

The monitoring has shown, that concentration of total nitrogen in the main stream is gradually increasing towards the pour point, but annual average of particular monitored profiles never exceeded limit specified in GO No. 401/2015 Coll. – 6 mg/l, Figure 2. Peak concentrations were recorded as expected in months off the vegetation period (Pitter 2009). Highest values were recorded in profile No. 2 with maximum of 5.7 mg/l. These results are caused by Hvězdlička tributary which collects water in largest partial river basin 4-15-03-043 with total area of 38 km^2. Within the framework of this research were measured also concentrations close to the mouth of river Hvězdlička in profile A which reached annual average of 5.5 mg/l with peak value 7.4 mg/l recorded in December (not shown in the graph). Over 68% of the basin comprises of arable lands and four villages without wastewater treatment plants are located here (Milonice, Uhřice, Hvězdlice a Pavlovice).

Figure 2 Total nitrogen values in profile on Litava river (source: author)



For control purposes was also measured water quality near mouth of Pohraniční stream above the profile No. 5. This relatively small basin (2.2 km^2) is formed mostly by arable lands (72% of the surface) and there is no large area with livestock or residence to be found. The average concentration of total nitrogen calculated from 3 control measurements was 1.9 mg/l. Average value of measurements performed in the basin above profile No. 8 at the same time as in Pohraniční stream was 1.03 mg/l. In the basin above profile No. 8 was–average values of measurements performed at the same time in Pohraniční stream 1.03 mg/l. Comparison of water quality in control basin, basin above the profile No. 8 and basin Hvězdlička showed, that pollution by total nitrogen is caused mostly by draining of sewage water and that the influence of nitrogenous fertilizers from agricultural land on surface water quality can be excluded.

Highest values were regularly recorded in profile C – Zámecký stream between profiles No. 6 and 7 with annual average of 7.2 mg/l (not shown in the graph). Zámecký stream basin is formed by arable lands and Kožušice village located near mouth into the Litava river. Experiment based on measuring of total nitrogen concentrations above and under the village exposed that higher concentrations on the profile No. 6 situated downstream the Litava river are caused by point pollution, consequently by the village. The average concentrations of total nitrogen during monitoring period were 1.8 mg/l above and 5.8 mg/l under the village.

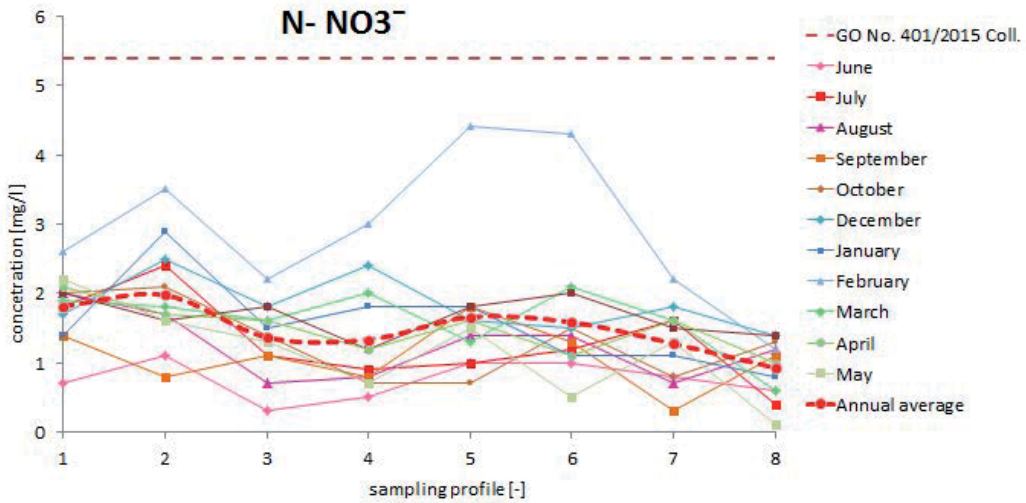
Kožušice is equipped with combined sewerage directing municipal wastewater directly into watercourse (DPWSS 2017). In this case the origin of nitrogen is in wastewater; but the impact on the profile No. 6 lying downstream is minimal considering low flow ratio between Litava river and Zámecký stream.

Nitrate Nitrogen

Recorded values of nitrate nitrogen on the main stream are rather balanced throughout the whole monitoring period and annual averages fulfill the limit specified in GO No. 401/2015 Coll. – 5.4 mg/l, Figure 3. Highest concentrations with annual average of nitrate nitrogen 1.9 mg/l were recorded in profile No. 2 which is influenced by the biggest Litava tributary Hvězdlička stream. Control measurements were performed also near mouth of Pohraniční potok where the annual average reached 1.4 mg/l.

In this case, the expectations were met again as the highest values of nitrate nitrogen concentrations were being recorded in main stream predominantly during winter months (Kopp 2015).

Figure 3 Nitrate nitrogen values in profile on Litava river (source: author)

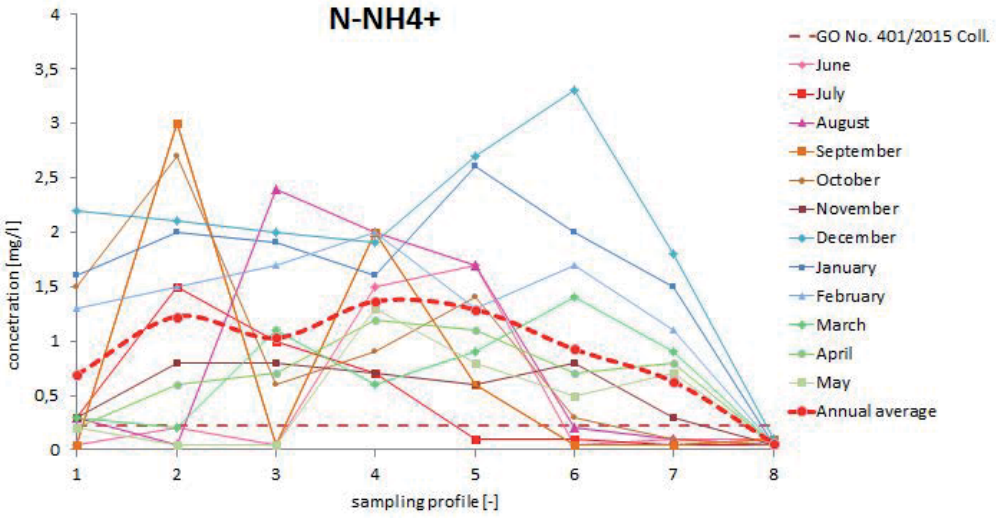


Ammonia nitrogen

Only averages of profile No. has met tight limit specified in GO No. 401/2015 Coll. – 0.23 mg/l, Figure 4. The trend of ammonia nitrogen concentrations throughout the whole main stream in not clearly comprehensible, however the significant increase of concentrations between profiles No. 8 and 4 could be probably ascribed to municipalities – villages without sewerage and wastewater treatment plants and livestock areas.

From the profiles on the tributaries only the profile D has fulfilled the limit. Highest concentrations were recorded in profile C – Zámecký stream, placed 50 m downstream Kožušice village, where the annual average value is 12.7 mg/l (not shown in the graph). Since the ammonia nitrogen is generally considered as "indicator of fecal contamination", it is obvious that pollution has anthropogenic origin particularly in sewage water. High concentrations of ammonia nitrogen from profile C slightly influence downstream profile No. 6.

Figure 4 Amonnia nitrogen values in profile on Litava river (source: author)



Czech national standard 75 7221

Surface water 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). The class is assessed for each indicator by comparison of its calculated characteristic value with adequate limit value defined for each class. Characteristic value is a number expressing the probability of not exceeding 90% and it is determined based on values collected for the whole monitoring period. In case of this research where 12 values are evaluated, the characteristic value is the one next to the last highest value.

Indicators are classified into 5 groups. Ammonia nitrogen and nitrate nitrogen belong into group „General, physical and chemical indicators" and values for total nitrogen are not listed in this standard. Table 1 contains classification of monitored profiles within the scope of physical and chemical indicators. Most of profiles are classified into third class. The most polluted profile C – Zámecký stream is classified into last fifth class.

Table 1 Classification of monitored profiles according to ČSN 75 7221 (units: mg/l)

Classification	Profile	$N\text{-}NH_4^+$	$N\text{-}NO_3$
I.	8	< 0.3	< 3
II.	D	< 0.7	< 6
III.	1, 3, 4, 5, 6, 7, B	< 2	< 10
IV.	2, A	< 4	< 13
V.	C	≥ 4	≥ 13

Based on flows and concentrations measured in pour point, the annual amount of nitrogen released of the river basin has been calculated. The resulting value 33.3 t/TN/year i.e. 2.9 kg/ha is less problematic in comparison with water quality in Jihlava river basin where the annual inflow of nitrogen into Dalešice dam amounts to 1 080 t/TN/year i.e. 9.29 kg/ha (Ryšavý and Hanák 2013).

In Nitrate Vulnerable Zones is located 65% area of Litava river basin, where farmers have to follow mandatory rules to reduce nitrate nitrogen loss from agriculture, for example: using and storing nitrogen fertilizers, crop rotation and anti-erosion measures. Results show that concentration of nitrate nitrogen in Litava river is satisfactory, which means that management

of agricultural land meets Cross Compliance and significant influence of nitrogenous fertilizers from agricultural land on surface water quality can be excluded. Furthermore, results supplemented with values measured in control profiles clearly imply, that increasing concentrations of total nitrogen are significantly supported by discharging pre-treatment or non-treatment wastewater from small municipalities, which is also confirmed by extremely high concentrations of ammonia nitrogen in almost whole upper Litava river. Only about 34% of interest basin inhabitants are connected to wastewater treatment plants, other are using cesspits, septic tanks or they are discharging wastewater into municipal sewerage leading directly into watercourse. The only permanent solution is support encouragement of wastewater treatment plants building or stricter enforcement of environmental legislation focus on illegal outset.

CONCLUSION

These results are only partial output of the presented project focused on hydrological conditions in the upper part of the basin Litava. Presented data confirmed that the biggest impact on water quality deterioration in upper part of Litava basin have point sources of pollution, especially wastewater from municipalities.

About 66% of basin inhabitants are not connected to the sewage network leading to wastewater treatment plants and discharge pre-treatment or non-treatment wastewater directly into the surface water or ground water. This is the reason why high concentrations of ammonia nitrogen which indicates presence of fecal contamination overcoming limits defined by GO No. 401/2015 Coll. have been recorded in the main stream. However critical values of ammonia nitrogen have been measured in all profiles, main stream and tributaries except only profiles No. 8 and D, which fulfilled limits for all three monitored water quality indicators.

ACKNOWLEDGEMENTS

This article was supported by the grant IGA MENDELU no. IP_9/2015 „Analysis of the hydrological conditions in the upper part of the basin Litava focused on the problems of erosion and contamination of surface waters“.

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SUITABILITY OF DENITRIFYING WOODCHIP BIOREACTOR OUTFLOWS FOR USE IN IRRIGATION

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Abstract: Denitrifying bioreactors are an innovative technology aimed at lowering high nitrate concentrations in agricultural runoff *in situ*. The most important component is a biodegradable filtration medium that serves as an organic carbon source for denitrifying bacteria, which reduces nitrates to nitrogen gases. However, undesirable excessive leaching of organic compounds from such bioreactor fillings can occur (especially in the start-up phase) with an adverse effect on the sensitive aquatic environment. The aim of this paper is to assess the possibility of using organics-rich water for irrigation. Static leaching tests and dynamic column tests were performed with various denitrifying bioreactor fill media to evaluate the leachability of organic substances (via the determination of chemical oxygen demand – COD, biochemical oxygen demand – BOD, and total organic carbon – TOC). The toxicities of the leachates were assessed via two terrestrial plant bioassays (*Sinapis alba* and *Raphanus sativus*). The tests with *Sinapis alba* indicate that some wood species (oak and acacia) exhibit higher toxicity, and that pretreatment by drying has a negative effect. A correlation was found between toxicity to *Sinapis alba* and COD, BOD, and TOC. As regards the dynamic tests, the concentration of organic compounds decreased with operation time, while toxicity to *Sinapis alba* increased. No toxic effect on *Raphanus sativus* was observed, and the toxic effect on *Sinapis alba* was slight. The results suggest that drained area irrigation using outflows from denitrifying bioreactors could be possible; however, such a decision would require more complex research involving more plant species.

Key Words: denitrifying bioreactor, wood chips, irrigation, ecotoxicity, terrestrial plants

INTRODUCTION

The contamination of the aquatic environment with nitrates has become a global problem in the last few decades due to its ability to cause eutrophication, toxic algal blooms, hypoxia and habitat deterioration (Galloway et al. 2003). It often has anthropogenic sources, with agriculture making a particularly significant contribution to the problem. The prevention of the pollution of water bodies in this way is covered by the European Directive concerning the protection of waters against nitrate pollution from agricultural sources (Council of the European Communities 1991).

Denitrifying bioreactors are a relatively simple treatment technology for the removal of nitrates from agricultural outflows. The first studies concerning this concept came from Canada and New Zealand, later followed by work from the USA (Christianson and Schipper 2016). Denitrifying bioreactors operate by causing nitrate-rich water to flow through an organic material rich in bioavailable carbon. This promotes heterotrophic denitrification, a process which converts nitrates (NO_3^-) into N-gases (Schipper et al. 2010).

Although denitrifying bioreactors have been used for a relatively long time, there are still a few unsolved problems, especially the excessive leaching of organic compounds during the start-up phase (Cameron and Schipper 2010). The release of organic substances can cause dissolved oxygen depletion in receiving waters and adversely affect biota (Schipper et al. 2010). Svensson et al. (2014a) examined dissolved organic carbon (DOC), biochemical oxygen demand (BOD), pH, colour, phenols, tannins and lignin in sawdust leachates of oak, pine, maple, and beech. Statistically significant

differences among the tree species were found. These results indicate the need to consider wood species when assessing potential environmental effects.

Malá et al. (2016) focused on the excessive leaching in the start-up phase. They performed column leachability tests with different types of wood chips and shavings. The leachates from most of the materials showed high chemical oxygen demand (COD) and BOD at the beginning of the tests, with a distinct decrease after nine weeks. Some studies point to the fact that wood leachates (e.g. denitrifying bioreactor outflow) can be toxic to aquatic organisms.

Leachate composition depends on the structure as well as the physical and chemical properties of the wood species. Trees are known to contain water-soluble phenolic compounds and mostly hydrolysable tannins or ellagitannins, such as esters of gallic acid. Some of the organic compounds present in wood leachate, like tannins, lignins, phenols, tropolones, and resin acids, can contribute to leachate toxicity (Svensson et al. 2014b, Samis et al. 1999). Phenols and resin acids such as isopimaric (IA) and dehydroabietic (DHAA) acids represent the greatest risk to aquatic life. IA is the most toxic, but also the rarest of the group of acutely toxic resin acids. DHAA is one of the least toxic resin acids, but is often mentioned in pulp & paper toxicology literature because it is the most soluble resin acid and can be reduced to retene, which is toxic to aquatic organisms. (Makris and Banerjee 2002)

Rex et al. (2016) assessed six types of wood chips (aspen, lodgepole pine, hybrid white spruce, black spruce, and two mixtures). All of the studied wood chips produced leachate that was toxic to *Vibrio fischeri* in Microtox™. Aspen chips produced the most acidic leachate, with higher organic, phenolic and ammonia concentrations compared to the coniferous and mixed samples. Resin acid concentrations for IA and DHAA were lowest in aspen, however. This indicates that either the high organic component concentration or the combination of organic compounds and resin acids is responsible for the toxicity response. Libralato et al. (2007) showed that leachates from wood species were more toxic to two saltwater organisms after 24 h of leaching than after 72 h, suggesting that whatever the components responsible for toxicity were, they were short lived. Libralato et al. (2007) thought that the toxic effect is caused by naturally occurring extractives, including aldehydes, phenols, terpinene, camphene and pinene.

TOC and COD provide relevant information about the total amount of organic compounds, but they are not informative with regard to organic compound distribution (Svensson et al. 2014b). However, a correlation between organics concentration and toxicity can be found. For example, Kannepalli et al. (2016), who focused on the leachate quality of mulch from wood materials (such as whole trees, tree trunks, and tree stumps), observed a significant negative correlation between the stage delay concentrations of zebrafish (*Danio rerio*) embryos and COD. The negative slope for this relationship indicates that with increased COD concentration in a sample, the volume causing developmental delay decreased (higher toxicity). However, Libralato et al. (2007), who investigated the potential toxic effects of wood leachates (oak, Norway spruce and three tropical species) on two saltwater organisms (the brine shrimp *Artemia franciscana*, and the embryos of the oyster *Crassostrea gigas*), did not find any significant correlation ($p < 0.05$) between physical and chemical parameters (dissolved oxygen, pH, COD) and ecotoxicological data.

Even if the most suitable bioreactor fill medium is selected and operating conditions are optimized, the leaching of organic compounds can still be too high. One possible way to protect a sensitive aquatic ecosystem of recipient is to use outflow water to irrigate a drained area in the first period of bioreactor operation. However, the effect of denitrifying bioreactor outflows on terrestrial organisms has been studied even more rarely than aquatic ecotoxicity. It is well known that tannins are toxic to microorganisms and ruminant animals; however, no information has been found about the toxic effect of tannins on plants, although they are not expected to have toxic effects on seeds (Svensson et al. 2014b).

For example, Svensson *et al.* (2014b) observed that oak wood leachate had no effect on *Lactuca sativa* (germination test). Feldmane (2010) investigated the influence of woodchip mulch on the growth and first yield of sour cherries. He measured the tree height, sum of shoot length, canopy volume and yield. The results suggest that the use of woodchip mulch tended to decrease

growth in the first two growing years, though it significantly advanced growth in the third year. However, the trees treated with woodchip mulch had a lower yield than those left untreated.

MATERIAL AND METHODS

Static leaching tests and dynamic column tests were performed to evaluate the leachability of organic substances from several wood materials that can be used as denitrifying bioreactor fill media. Besides the types of organic substances present, the leachates were assessed for toxicity to terrestrial plants.

Static leaching tests

Static leaching tests were conducted with chips fabricated (size fraction 1.4–22.4 mm) from six wood materials, these being a mixture of pine and larch bark, and poplar, beech, spruce, oak, and acacia wood. The materials were tested in their original state, with dry matter content ranging between 80 and 90%. The beech and spruce were also pretreated by drying (6 h, 105 °C).

The tests consisted of 24 hours of leaching in a rotating shaker (5 rpm). The leaching was carried out using deionized water under laboratory conditions with a solid to water ratio of 1/10.

Dynamic column tests

The dynamic column tests were conducted with two mulches, spruce and pine, each placed in two 0.3 m^3 laboratory columns. The bioreactors were filled with tap water enriched with nitrates (KNO_3). The water was dosed at 12 intervals per day and the hydraulic retention time was approx. 1 day. The length of the dynamic test was 4 weeks and samples for chemical analyses were taken on the 4^{th} and 28^{th} day of operation. The water temperature ranged from 13.0 to 17.0 °C, and the concentration of NO_3^- – N was 26.3 mg/l at the start (4^{th} day) and 20.5 mg/l at the end (28^{th} day).

Chemical properties

Organic substances were determined in the samples, which were not filtered, especially COD, BOD, and total organic carbon (TOC). The laboratory analyses were performed as follows: COD – semi-micro method with potassium dichromate and photometric evaluation; BOD – standard dilution method; TOC – purging method using Hach TOC cuvette tests.

Ecotoxicological bioassays

Two ecotoxicological bioassays were carried out – an acute bioassay with the seeds of *Sinapis alba* (white mustard), and a long-term bioassay with *Raphanus sativus* plants (cultivated radish). The *Sinapis alba* bioassay was performed as a 72-h test according to methodology set out by the Czech Ministry of the Environment. (MŽP 2007) The percentage of root growth inhibition was calculated. The *Raphanus sativus* bioassay was performed as a modified test according to Test No. 208 (OECD 2006). The test plants were grown in twenty 200 mL plastic pots filled with fertilized commercial potting soil under laboratory conditions – 3 plants in each pot. In the case of the static tests, fresh leachates were prepared every week, and the length of the test was 5 weeks. For the dynamic tests, the outflow from the columns was collected periodically and the length of the test was 4 weeks. The germinability and external appearance of the plants were assessed during the test, and the dry shoot weight was measured at the end. The results were compared with those for the control plants, which were grown under the same conditions but without the application of leachate.

Statistical evaluation

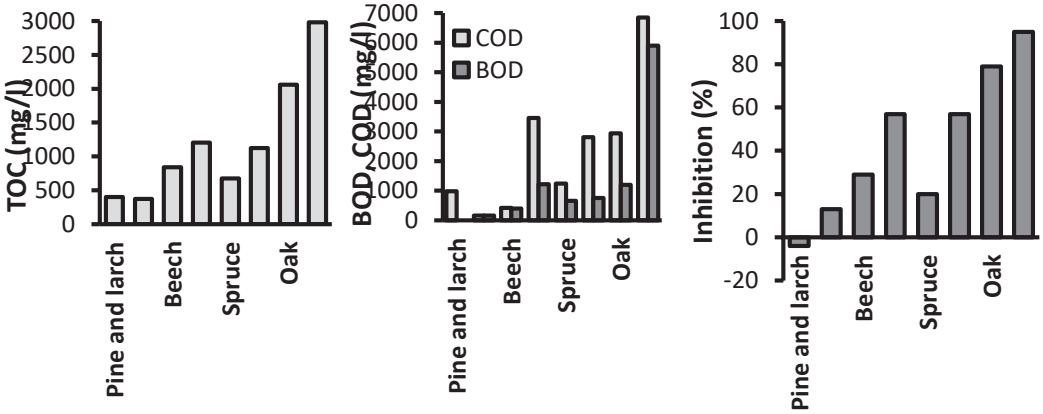
For the determination of the relationship between toxicity and concentrations of organics, *Sinapis alba* inhibition was converted to probits for linearization, and then linear regression was applied (only positive inhibition values were used). For the detection of differences among *Raphanus sativus* dry shoot weight means, data was statistically evaluated by one-way analysis of variance (ANOVA) with Dunnett's post–hoc test ($P < 0.05$).

RESULTS AND DISCUSSION

Static leaching tests

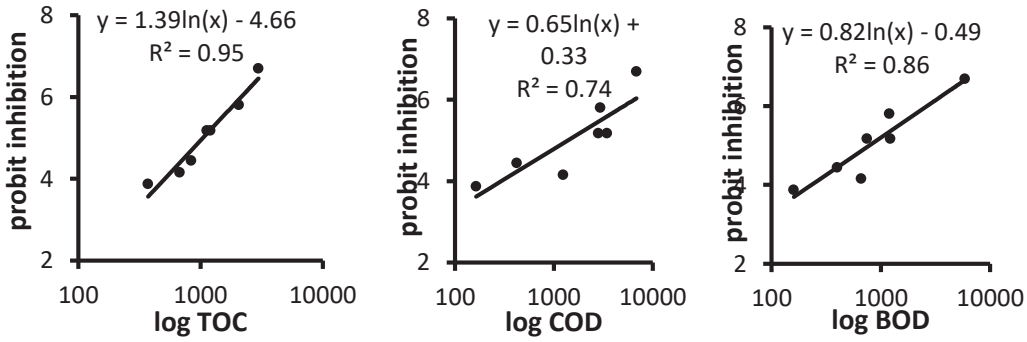
In the case of static leaching tests, TOC, BOD, COD and toxicity to *Sinapis alba* vary according to the wood species used and the application of pretreatment (Figure 1). The results confirm the statement by Svensson et al. (2014a), who determined that there was a difference between the leachates obtained from various wood species. Higher leachability of organic compounds and toxicity is shown in the case of pretreated (dried) materials in comparison with those left untreated. The levels of toxicity to *Sinapis alba* ranged from nontoxicity (lower than 30% inhibition – the pine and larch mixture, and poplar, beech, and spruce) to toxicity (higher than 50% inhibition – non-treated oak and acacia, and dried beech and spruce). However, the static leaching tests represent maximum leachability, not the real situation.

Figure 1 Results of static leaching tests: TOC, BOD, COD, and toxicity to Sinapis alba (the BOD of pine and larch was not determined)



The results suggest the existence of a relationship between toxicity to *Sinapis alba* and TOC, COD, and BOD (Figure 2). A positive linear relationship was found between toxicity and all measured parameters – TOC (correlation coefficient r = 0.97), COD (r = 0.86), and BOD (r = 0.93). Despite the fact that the studied samples were from different types of wood with varied leachability and toxicity, the data showed a strong correlation, which was found to be significant at $p < 0.05$. The results correspond with those of Kannepalli et al. (2016), who found a correlation between COD and toxicity, and also correlations between COD and BOD and potentially toxic phenolic compounds. However, there may be differences in toxic effect depending on the tested organism species, as stated by Libralato et al. (2007).

Figure 2 Relation between toxicity to Sinapis alba and TOC, COD, and BOD

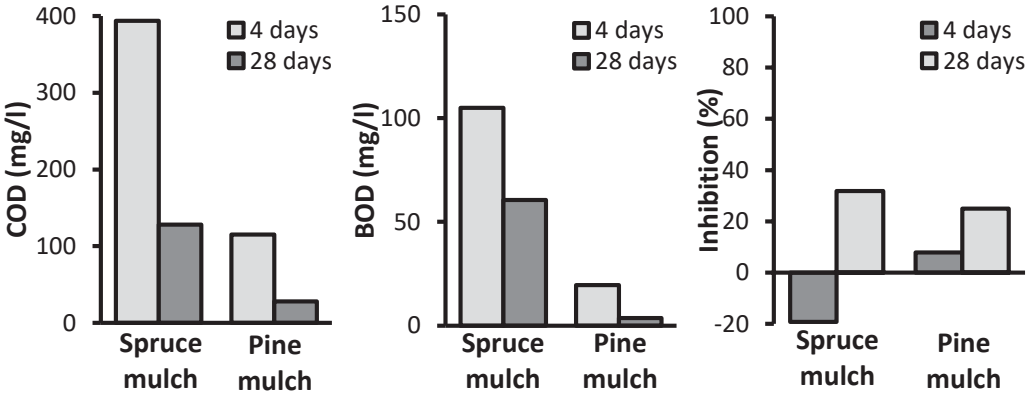


Dynamic column tests

The results of the dynamic column tests, where the data from the 4th and 28th day of operation were compared, are shown in Figure 3. These tests simulate the start-up phase of a denitrifying bioreactor. While organic compounds decreased with time of operation (which corresponds with the findings of Cameron and Schipper (2010) and Malá et al. (2016)), toxicity to *Sinapis alba*

slightly rose. Inhibition increased from -19 to 32% and from 8 to 25% in the case of spruce and pine mulch, respectively. However, although some changes in toxicity were observed, three of the samples were non-toxic; only spruce mulch leachate slightly exceeded the limit for non-toxic materials (30% inhibition) after 28 days. It indicates potential changes occur in the quality of leachates over time, leading to the leaching of lower concentrations of more toxic organic compounds. This topic requires more detailed research with more materials, longer time periods and more extensive sampling.

Figure 3 Results of dynamic column tests: Values for COD, BOD and toxicity to Sinapis alba (average values)



The long-term effect (4/5 weeks) of irrigation on plant growth was investigated with *Raphanus sativus* seeds, which were watered with fresh static leachates or column outflows. In both cases, no significant difference was found between these and the control seeds ($p < 0.05$), and there were no visual differences during growth. The inhibition values are shown in Table 1. These results confirmed the research of Svensson et al. (2014b), who did not find any effect of oak wood leachate on *Lactuca sativa* in a similar test.

Table 1 Toxicity of the leachates to Raphanus sativus

Static tests		Dynamic tests	
Material	Inhibition	Material	Inhibition
Pine and larch	-8.8%	Spruce mulch	4.7%
Poplar	-5.1%	Pine mulch	7.9%

CONCLUSION

Even though static leaching tests do not simulate a real situation, they are a usable method for the comparison of various materials. On the other hand, dynamic column tests provide a simulation of real denitrifying bioreactor processes, although under more complex conditions.

The static leaching tests indicated the toxicity of some wood species leachates (oak and acacia) to *Sinapis alba* and the negative effect of pretreatment of the wood by drying. The leachates of pine and larch, poplar, beech, and spruce were non-toxic. A significant relationship between toxicity to *Sinapis alba* and TOC, COD, and BOD was found. These results pointed out the importance of wood species selection and the state of the material for the use of woodchips in denitrifying bioreactors.

Despite the fact that the concentration of organic compounds released during dynamic tests decreased with operation time, toxicity to *Sinapis alba* slightly increased. This indicates that long-term leaching can cause the release of more complex and stable organics that may show higher toxicity.

The lack of any observed toxic effect on *Raphanus sativus* suggests drained areas could be irrigated using outflows from denitrifying bioreactors. However, although the *Sinapis alba* bioassays performed on the outflows indicate a slight toxic effect, the evaluation of the possibility of irrigation using such outflows requires more complex research with more plant species and stages of growth.

ACKNOWLEDGEMENTS

Funding was provided for this work by the Brno University of Technology Junior Specific Research Project FAST-J-16-3014 Filtration materials useable for denitrifying bioreactors.

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GEOCHEMICAL MODEL OF LEACHING FIELD GROUNDWATERS AT THE STRÁŽ URANIUM DEPOSIT

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Abstract: The groundwater in the vicinity of the Stráž uranium deposit was vastly affected by chemical uranium extraction. After the mining ceased, more than 250 million m^3 of acid solutions, containing mainly sulphuric acid, were left in the bedrock. The paper suggests a means of applying geochemical modelling in the prediction of groundwater chemistry development and the optimization of remediation. The Geochemist's Workbench programme was used to create a geochemical model based on field data gained from the Stráž deposit. Two waters were picked, both with low pH (1.1 and 3.6), and concentrations of main ions were used. The compositions were charge balanced and then gradual mixing was modelled. Synthetic waters with the same composition were prepared in a laboratory and mixed in mixing fractions 0, 0.25, 0.5, 0.75, 0.95, 0.99 and 1. Redox potential, pH, and electrical conductivity were measured and compared with the geochemical model. Both of the chosen waters were undersaturated with respect to minerals, and the ionic composition of the prepared waters and during mixing was driven only by dilution. Within the comparison of the modelled and synthetic water parameters, almost identical results were obtained for pH, while redox potential and electrical conductivity were different. The difference in the redox potentials was probably caused by the presence of oxygen in the case of the synthetic water. The electrical conductivities values were similar; however, the model values were lower with higher pH. It is thus necessary to correct the model before its application to extreme waters, such as leaching field groundwaters. The presented results may be of use in efforts to improve the modelling of very acid groundwaters and help in understanding changes in parameters which affect mineral precipitation and the dissolution of uranium compounds.

Key Words: Bohemian Cretaceous Basin, geochemistry, groundwater, mining, *in situ* leaching

INTRODUCTION

The Stráž deposit, situated near the village of Stráž pod Ralskem, is one of several sandstone-type uranium deposits in the Stráž Tectonic Block in the northwest part of the Bohemian Cretaceous Basin. The uranium is typically bounded to Cenomanian sediments around 150 to 300 m below the ground (Kafka et al. 2003).

Because of the low content of uranium and disseminated character of the Stráž deposit, the uranium was extracted by a chemical method termed *in-situ* leaching (ISL), whereby uranium ore is extracted by a liquid medium for selective extraction (lixiviant), e.g. sulphuric acid in the case of acid leaching. The method is based on the pumping of lixiviant into the orebody via injection boreholes, from which it flows through the permeable orebody, dissolving the ore, before being extracted via extraction boreholes. A confined aquifer is required to prevent contamination from spreading (Mudd 1998).

There are two aquifers in the area – an unconfined aquifer in Turonian sandstones and an underlying confined aquifer in Cenomanian sandstones, which are separated by a Lower Turonian aquitard of claystones and marlstones. The chemical composition of groundwater in the Turonian aquifer is controlled by recent precipitation, water-rock interaction and local fertiliser use. It does not naturally contain uranium and serves as a water source of regional importance. In the case of the Cenomanian aquifer, the chemical composition of groundwater is mainly determined by water-rock interaction, and partially by the infiltration of precipitation, interaction with geogenic

gases (such as CO_2), and the diffusion of fossil Na–Cl–SO_4 brines from the Permo-Carboniferous basement (Pačes et al. 2008). In contrast to the Turonian aquifer, the Cenomanian aquifer has never been used as part of the public water supply due to its uranium content (Kafka et al. 2003).

The natural chemical composition of Stráž deposit groundwater was vastly affected by the aforementioned uranium extraction via ISL, which was performed from the 1960s. Besides sulphuric acid, other chemicals were used, such as nitric acid, ammonia, and hydrofluoric acid. In 1996 the mining ceased and afterwards the remediation of the aquifer started. More than 250 million m^3 of acid solutions were left underground after mining stopped. The solutions are very low in pH (approaching 1) but high in dissolved solids and enriched with sulphates, heavy metals and radioactive elements. The aim of remediation is to prevent contamination from spreading and reduce it to acceptable values. The groundwater flow regime is maintained by pumping out the residual chemical solutions in a controlled manner and, after their partial treatment, pumping them back underground (DIAMO 2010).

The period of time necessary to complete the remediation and future development of the groundwater system are not completely clear. The situation is complicated by the extreme composition of the chemical solutions used. The main problem is the reprecipitation of minerals during the mixing of the solutions and natural groundwater, which could cause borehole clogging. Despite the fact that uranium was only recovered from Cenomanian sediments, the overlaying Turonian aquifer was contaminated too. The flow of groundwater through the Lower Turonian aquitard is enabled via insufficiently sealed boreholes, as well as by natural transport pathways, e.g. cracks created by Saxonian folding and connected to neovolcanic activity (Kozáková and Pokorný 2009). The flow between the aquifers cannot be completely eliminated.

Uranium is a toxic heavy metal. In the water environment it is mostly present as the tetravalent cation U^{4+} or hexavalent uranyl cation UO_2^{2+}. The tetravalent cation is nearly insoluble in natural water, although it can be transported in the form of a complex compound which can be created in a highly acid or alkaline environment. The uranyl cation is very stable and soluble and can create transportable complex compounds (Pluskal 1971). The oxidation state is dependent on oxidation–reduction potential, pH, the total concentration of uranium, the presence of other substances and the partial pressure of CO_2 (Zachara et al. 2013).

On the basis of the model it is possible to simulate the interactions of chemical solutions with other types of water, and their interaction with surrounding sediments. Owing to these simulations, it is possible to examine various system development scenarios, which is of help when choosing the most effective remediation method.

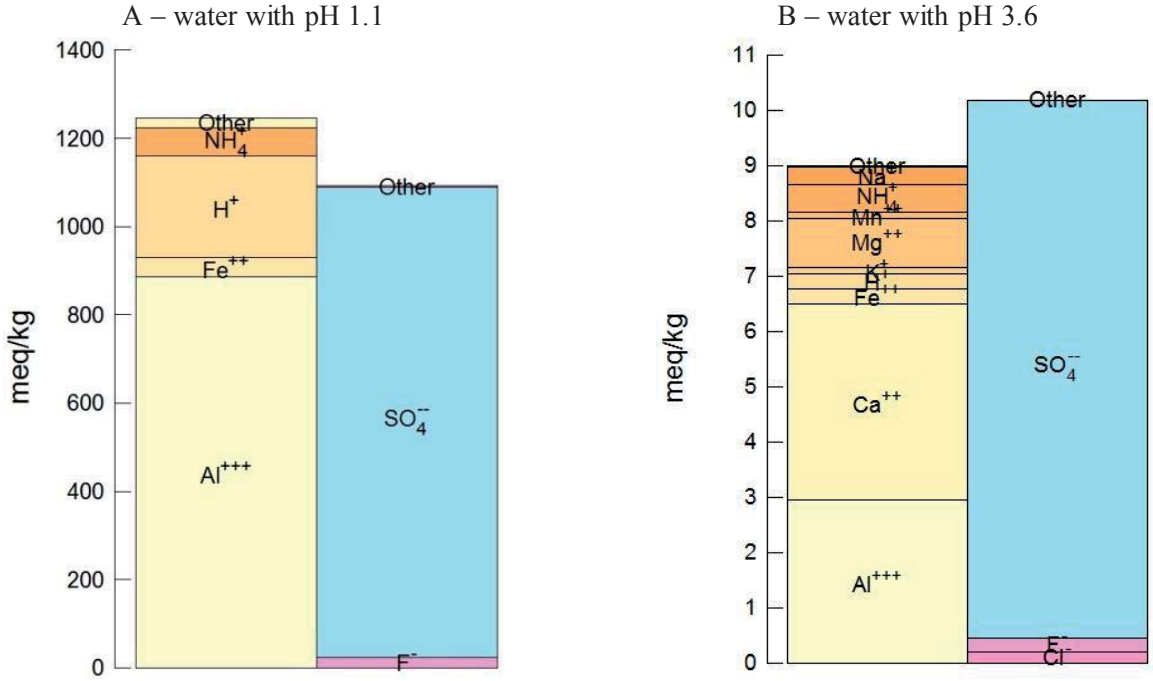
The scope of this study is a preparation of geochemical model describes mixing of two leaching field groundwaters from Stráž deposit with different physico-chemical properties. The model output was verified by laboratory experiments performed with synthetic waters. The selection of used waters was adapted in order to simplify the experiment for the purpose of model verification. Both waters are acid enough to suppress precipitation and keep ions in their dissolved form during mixing, which simplified the experiment. The published research is the beginning of a set of experiments which will be covered in future articles.

MATERIAL AND METHODS

The chemical analyses of groundwaters influenced by *in-situ* leaching were based on measurements taken from real extraction boreholes provided by DIAMO, s. p. Two waters were chosen: water A had a higher concentration of acids, with a pH of 1.1 (it was very acidic water highly affected by ISL), while water B was more alkaline, with a pH of 3.6 (it was less acidic water from the edge of the leaching field). They also exhibited dissimilarity in terms of dissolved solids concentration, which was extremely higher in the water with lower pH. Only the main physical parameters and chemical components of the waters were used for modelling. Uranium content was not considered, for instance, although the uranium concentration in waters A and B was around 70 mg/l and 1 mg/l, respectively. Nitrogen compounds present in the analyses (NH_4^+ and NO_3^-) were recalculated according to their molar weight to NH_4^+.

The chosen chemical analyses were ionic charge imbalanced; the charge balance error was 16.35% and 8.072% in the case of water A and B, respectively. The original ionic compositions are shown in Figure 1.

Figure 1 Bar Charts of the original composition of the chosen waters



The geochemical model was prepared in Geochemist's Workbench (version 8.0), which is a collection of geochemical programs that employ numerical methods and use thermodynamic databases (Zhu and Anderson 2002). For this model, the thermo.dat database (Aqueous Solutions LLC ©2011–2017) was used and the set temperature was 25 °C. The modelled waters were prepared via ionic charge balancing by sulphate ions. Anoxic conditions were set for the modelling. The conversion of nitrates into molecular nitrogen during processes was not taken into consideration, and the precipitation of hematite was suppressed.

Laboratory experiments were performed with 2 litres of synthetic waters with the same composition as charge balanced waters. The pH values were adjusted by 0.1 M NaOH. The temperature, pH, electrical conductivity and redox potential (Eh) were periodically measured via a WTW ProfiLine pH/Cond 3320 multi-parameter meter. The redox potentials were converted to standard hydrogen electrode values.

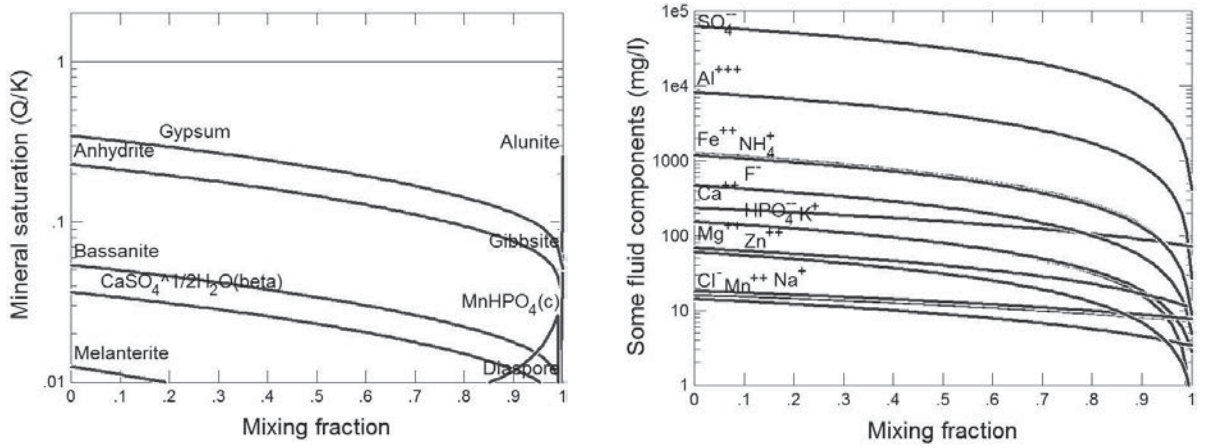
After stabilisation of the measured physical parameters, the synthetic waters were mixed in 100 ml beakers in mixing fractions 0, 0.25, 0.5, 0.75, 0.95, 0.99 and 1 (the ratio of water B with pH 3.6 to water A with pH 1.1). Stabilised physical parameters were used for the comparison of the laboratory test results with the model's output. All waters were kept contact with the atmosphere while stabilisation was taking place.

RESULTS AND DISCUSSION

Geochemical model

The geochemical model of gradual mixing shows saturation of minerals (Figure 2A). All minerals in the database have saturation below 1, what means that none of them would precipitate. The higher mineral saturations are reached by gypsum, anhydrite and alunite, although the magnitude of this parameter is often of little importance because it is dependent on the formula of the mineral (Bethke 1996, Zhu and Anderson 2002). Due to the absence of precipitation, all the concentrations of the components in the fluid (Figure 2B) should be driven only by dilution, which is suggested by the linear functions of ion concentrations.

Figure 2 Progress of mineral saturation and concentration of ionic components in fluid during gradual mixing

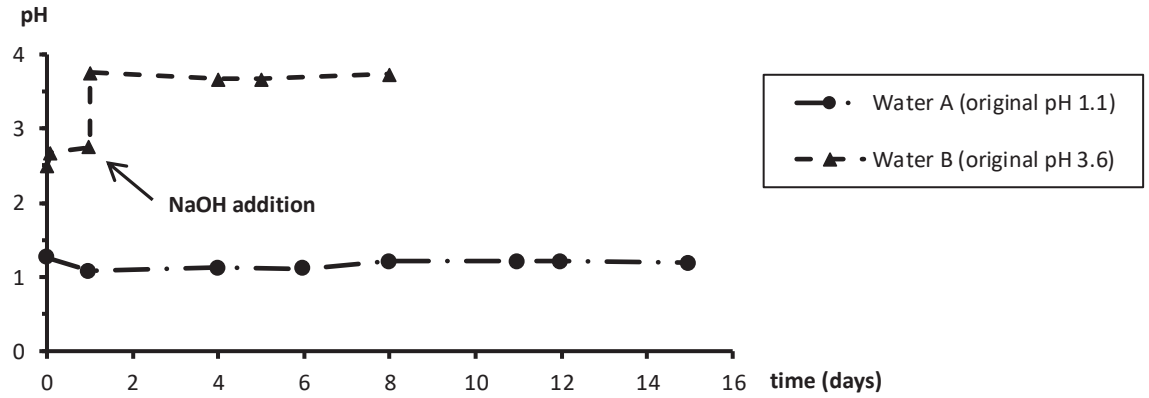


Synthetic waters

The synthetic waters were prepared under laboratory conditions and monitored for at least one week before their use in the simulation of mixing. The average pH values were 1.16 and 2.71, average electrical conductivities were 58 228 µS/cm and 1 497 µS/cm, and average redox potentials were 359 mV and 435 mV in the cases of waters A and B, respectively. The progression of the pH values of the waters from the time of preparation is shown in Figure 3. The averages were calculated using all values, with the exception of the day of preparation (time 0 days). The coefficients of variance (CV) for the data used for calculating to averages were a maximum of 4.7%, 1.3%, and 0.8% in the case of pH, electrical conductivity, and redox potential, respectively.

The pH of water B was adjusted by the addition of NaOH. The average pH value after the adjustment was 3.69 (CV 1.0%), average electrical conductivity was 1 010 µS/cm (CV 2.1%), and average redox potential was 360 mV (CV 3.3%).

Figure 3 Progression of the pH values of the synthetic waters from their preparation



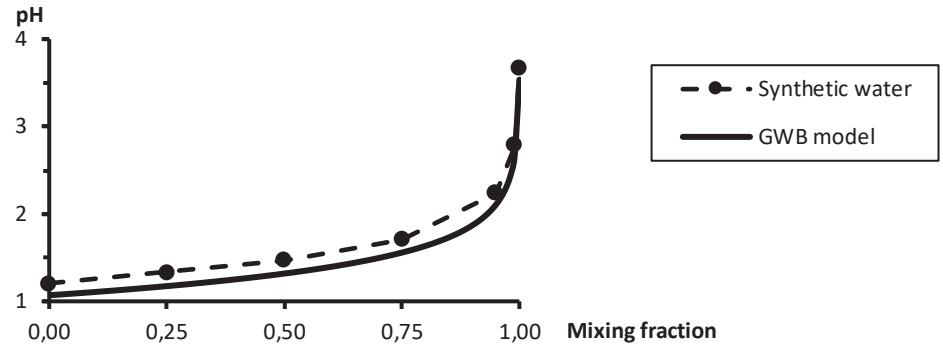
The stabilisation of the prepared synthetic waters and the mixed waters was reached after several hours. The stabilisation was so rapid because the systems were not driven by carbonate equilibria reactions (Stumm and Morgan 1996). The highly acid environment of leaching field groundwaters suppresses the precipitation of minerals (for example goethite) caused by the dissolving of oxygen from the atmosphere. The influence of oxygen could become evident over a longer period of time, but its investigation was not an objective of this experiment.

Comparison of geochemical model output and laboratory experiment results

Figures 4 and 5 show the progression of the monitored physical parameters during the mixing of water in different mixing fractions. The modelled and synthetic waters were prepared with the same composition, and the physical parameters were calculated or measured in the case of the synthetic or modelled waters, respectively. The results represent the parameters after stabilisation.

The pH values (Figure 4) show a slight difference between the modelled and synthetic waters during mixing. The difference is probably caused by the different values of the initial waters.

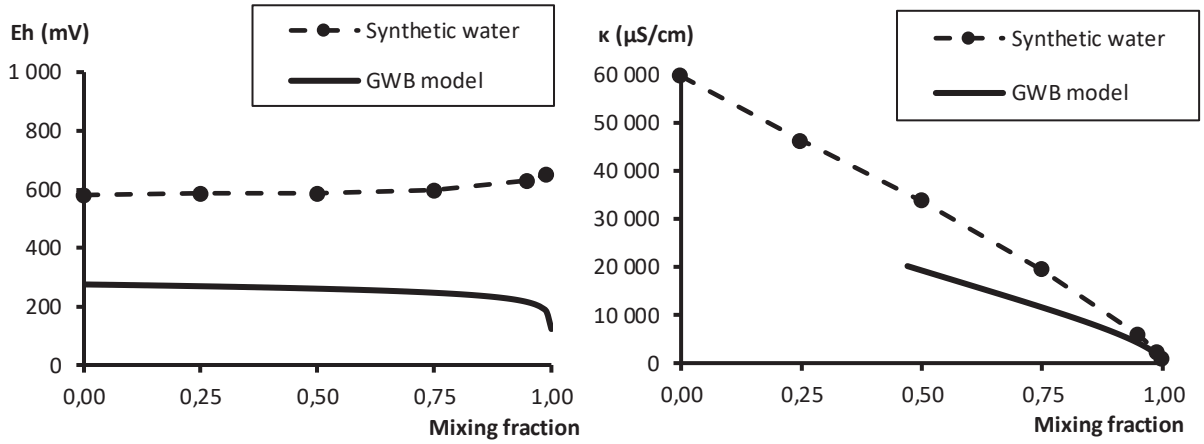
Figure 4 Results of the laboratory and modelled mixing of waters – pH



The redox potentials (Figure 5A) show a different situation. The values obtained for the synthetic water are more than twice as high, and they are increasing in contrast to the decreasing values of the model. The difference between the redox potentials is probably caused by the presence of oxygen in the case of the synthetic water, which should not have an influence on mineral precipitation or redox reactions in very acid waters, and so does not affect pH.

The electrical conductivities (Figure 5B) are similar (both decreasing); however, the model values are lower, and the difference is higher with lower water pH. This indicates that the model is applicable to the modelling of waters with low electrical conductivity (and low mineralisation), e.g. natural waters. Correction of the model is necessary before it can be applied to extreme waters, such as leaching field groundwater. The model only shows values up to 20 000 µS/cm, depending on the type and version of the programme used.

Figure 5 Results of the laboratory and modelled mixing of waters – redox potential and electrical conductivity



The slight differences between the modelled and synthetic waters could be caused by the temperature, because it was 23.2 °C on average throughout the laboratory experiment (CV 1.7%) while the model temperature was set at 25 °C.

CONCLUSION

On the basis of the geochemical model, both of the chosen waters were mineral undersaturated, and the ionic composition of the prepared waters and during mixing was driven only by dilution. The systems were not driven by carbonate equilibria reactions and redox reactions because of the extremely acid environment.

Within the comparison of the modelled and synthetic water parameters, almost the same results were obtained for pH in both cases, though redox potential and electrical conductivity are different.

The difference between the redox potentials was quite high and was probably caused by the presence of oxygen in the case of the synthetic water. The electrical conductivity values were similar; however, the model values were lower with higher pH. Correction of the model is necessary before its application to extreme waters, such as leaching field groundwaters.

Geochemical modelling is a helpful tool for Stráž deposit remediation because the processes which are taking place underground are difficult to monitor *in situ*. These results may be of use in efforts to improve the modelling of very acid groundwaters and help in understanding changes in parameters which affect mineral precipitation and the dissolution of uranium compounds.

ACKNOWLEDGEMENTS

The research was financially supported by the Rector's Programme provided by the Grant Agency of Masaryk University; project MUNI/C/1695/2016.

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EVALUATION OF THE ONSET OF PHENOLOGICAL PHASES OF SPRING BARLEY

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Abstract: Detailed analysis of the phenological data of the Czech Hydrometeorological Institute was made in this paper. The analysis was carried out for spring barley (*Hordeum vulgare* L.) for Poděbrady locality (177 m a.s.l.). This locality is situated in Central Europe, Czech Republic, Central Bohemia. These phenological phases were analysed: emergence, tillering, the beginning of leaf sheath elongation, first node, second node, swelling of the sheath of the last leaf, heading, milky ripeness, yellow ripeness and full ripeness. Processes of sowing and harvest were also analysed. It was found out that growing season has been prolonged by average of 9 days. Harvest was on average delayed by 11 days in the period 1991–2010 (according to the period 1931–1960). Analysed data show great variability in onset of phenological phases and implementation of processes of sowing and harvest. The weather has significant influence on development of spring barley and onset of individual phenological phase in given year. The greatest variability of all data was found out at sowing. Sowing of spring barley usually is going on from the 56th day of the year to the 114th day of the year.

Key Words: Growing season, Czech Republic, Phenological observations, Czech Hydrometeorological Institute

INTRODUCTION

Phenology is a science of the time course of periodic repetitive manifestations of plants and animals. Climatological and phenological data are in a close relationship, therefore phenology is perceived as an auxiliary science of climatology (Sobíšek 1993).

Data from phenological observations can be used for: allergen monitoring (Stehnová et al. 2017), erosion risk assessment of the crop (Stehnová and Středová 2016), prediction of pathogens (Středa et al. 2013), application of preparations to plant protection (Krédl et al. 2012), optimization of irrigations during critical phases of crops growth and monitoring of occurrence of dry seasons (Kohut et al. 2014) and in yield simulation models and to quantify the impacts of climate change (Škvarenina et al. 2009).

The plant is one of the most accurate and sensitive indicators of climate change because of 'its sensitivity to temperature ratios (Chmielewski et al. 2004, Tao et al. 2016). Many scientists perceive phenological observation as an indicator of climate change and perceive phenology as a means to monitor climate change. Long-term trends are determined from long-term data sets. Research shows that long-term trends are specific for each crop in phenology (Rezaei et al. 2017). The work Šiška and Takáč (2008) shows that growing season can be extended by up to 21 days by 2020 and up to one month by 2050 in the future. Extending growing season will occur due to increasing air temperature.

Onset of phenological phases is recorded in the phenological observations. Phenological phase is the externally recognizable development of organ of plant, which is usually repeated every year. Technical phases (sowing and harvest) are also monitored in phenological observations. Onset of the phenological phases is the date (calendar day in the year) during which the development of organ reached corresponding stage of phenophase (Valter 1982).

MATERIAL AND METHODS

Phenology

Analysis of phenological data of spring barley (*Hordeum vulgare* L.) was carried out in this work. Analysis of phenological data was carried out for Poděbrady locality (Central Europe, Czech Republic, Central Bohemia). This station is located at altitude of 177 m above sea level. Data was obtained from direct observation of the Czech Hydrometeorological Insitute (CHMI).

Analysis of average values of onset of phenological phases and processes of sowing and harvest was carried out for the period 1931–1960 and 1991–2010. Data for the period 1931–1960 was obtained from the publication Agroclimatic conditions of ČSSR (Kurpelová et al. 1975). Closest phenological station with similar altitude was selected in this book.

Furthermore, detailed analysis of phenological data was carried out for decades 1991–2000 and 2001–2010. Average values have been evaluated for these decades. The paper deals with changes in the onset of selected phenological phases in the mentioned periods. Length of the growing season (GS) is also addressed in this paper. GS is understood as a period between sowing and harvest.

Analysis was performed for phenological phases: emergence (EM), tillering (TI), the beginning of leaf sheath elongation (BLSE), first node (FN), second node (SN), swelling of the sheath of the last leaf (SSLL), heading (HE), milky ripeness (MR), yellow ripeness (YR) and full ripeness (FR). Processes of sowing (SD) and harvest (HA) were also analysed. More detailed specifications of these terms are given in Table 1.

Table 1 Monitored phenophases and processes of SD and HA and their description (Hájková et al. 2012; Valter 1982)

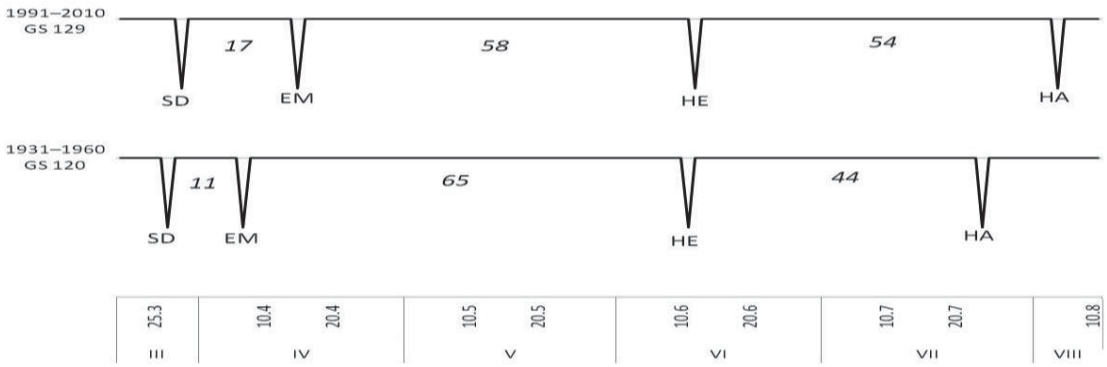
Phenological phases and processes	Abbreviation	Description
Sowing	SD	Day when the given plant was sown or planted.
Emergence	EM	The first overground parts of a plant penetrated the soil surface and the plant begins windrow. The crop is beginning to create visible rows.
Tillering	TI	A leaf apex of the first tiller could be seen in the axil of any of the lowest leaves.
The beginning of leaf sheath elongation	BLSE	Originally close leaf bases begin to markedly separate.
First node	FN	The swelling of the first node is visible just above the plant basis, hard rounded corpus could be felt inside the sheath of the lowest leaf.
Second node	SN	The description of SN is the same as for FN.
Swelling of the sheath of the last leaf	SSLL	The sheath of the last leaf helically extends as a result of the growth of a flower and at the same time the flower emerges from the sheath of the flower protrudes from the sheath of the last leaf.
Heading	HE	Precisely half of the flower protrudes from the sheath of the last leaf.
Milky ripeness	MR	All caryopses are green, soft in touch and milky coloured sap is released upon a stronger squeeze, in cereals the lowermost leaves are mortified, whereas the uppermost are still green, nodes are swollen and elastic.
Yellow ripeness	YR	The caryopsis is yellow in the central part. All leaves have fallen off and the whole stem is greenish yellow, elastic and not woody. Most leaves are mortified and lower nodes are dried and wrinkled.
Full ripeness	FR	Leaves and node are already completely dead on the plant. The nodes are brown and wrinkled. The straw has a straw colour. Grains are hard and easy to release.
Harvest	HA	The day on which the harvest of the given crop started.

RESULTS AND DISCUSSION

Analysis of phenological data

On average, the sowing was carried out two days later in the period 1991–2010 (according to the period 1931–1960). GS prolongation (by 9 days) was found out during analysed periods. Interval between phenological phases of SD and EM was prolonged by 6 days in the period 1991–2010. Interval between EM and HE was shortening of 7 days in this period. Interval between phenological phases HE–HA was prolonged on average by 10 days in the period 1991–2010. On average harvest occurs 4th August in the period 1991–2010 (see Figure 1). This shows the delay of harvest by 11days (according to the period 1931–1960). Shift of the harvest in the period 1991–2010 can have many reasons for example cultivated spring barley variety and climatic conditions. Climate change can affect the length of the GS.

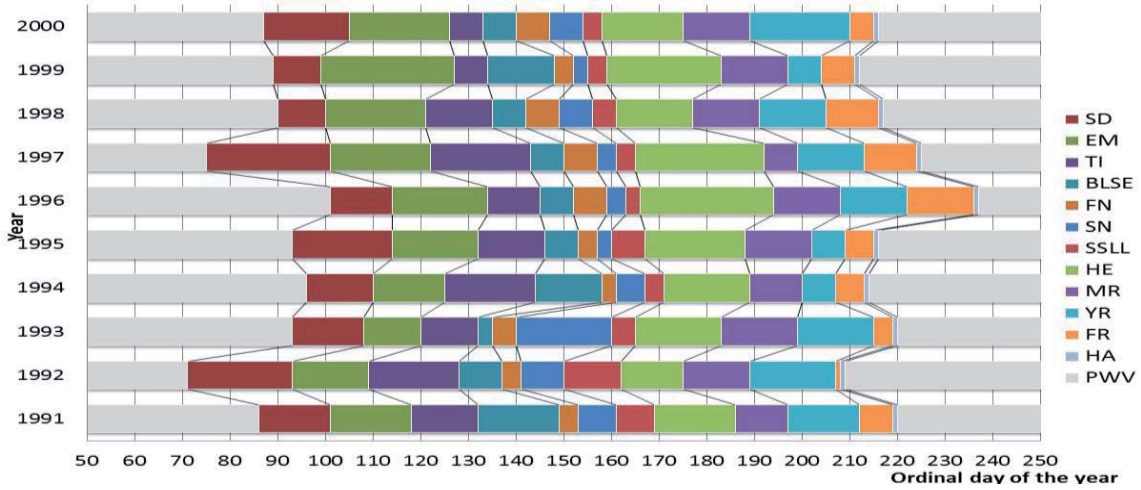
Figure 1 Average values for the period 1931–1960 and 1991–2010



Legend: SD – Sowing, EM – Emergence, HE – Heading, HA – Harvest

In decade 1991–2000, longest GS was found out in year 1997. Sowing was done 75th day of the year and harvest was carried out 224th day of the year (see in Figure 2). The shortest GS was found out in 1994 in this decade. GS was 117 days in this year. Sowing of spring barley was carried out at the earliest in 1992 (71st day of the year). Sowing was carried out at the latest in 1996 year (101st day of the year).

Figure 2 Onset of phenological phases for decades 1991–2000



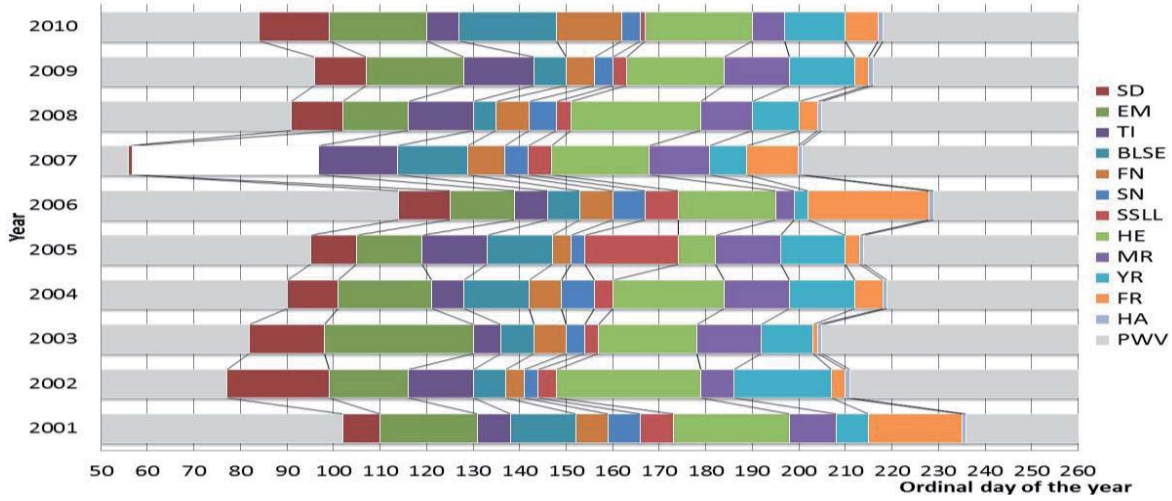
Legend: PWV: Period without vegetation, SD – Sowing, EM – Emergence, TI – Tillering, BLSE – The beginning of leaf sheath elongation, FN – First node, SN – Second node, SSLL – Swelling of the sheath of the last leaf, HE – Heading, MR – Milky ripeness, YR – Yellow ripeness, FR – Full ripeness, HA – Harvest.

Shortest interval between onsets of individual phenological phases was found out in the phenophases: BLSE–FN, FN–SN a SN–SSLL. On average the interval between these phases are: BLSE–FN 5 days, FN–SN 7 days and SN–SSLL 6 days. The longest period between onsets

of individual phenological phases was found out in the phenophases SSLL–HE (20 days). The second longest period was found out between phenological phases EM–TI (19 days).

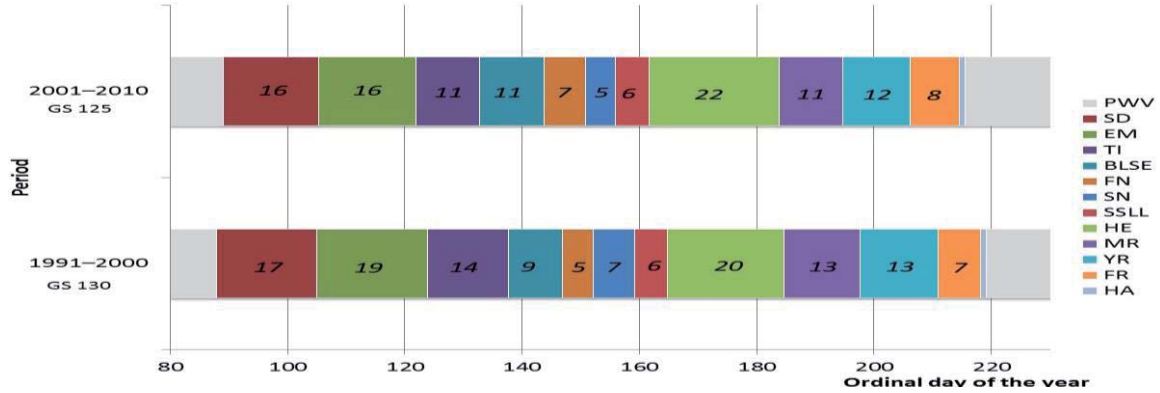
In period 2001–2010 the longest GS was found out in year 2007 with length 146 days. The earliest sowing was in year 2007. Sowing was carried out on the 56th day of the year. The shortest GS was found out in 2008, where length of GS was 113 days. Sowing was carried out at the latest in year 2006 (on the 114th day of the year). Shortest interval between onsets of phenological phases was found out in phenophases BLSE, FN, SN and SSLL as in the previous decade. Mean values between phenological phases were BLSE–FN 7 days, FN–SN 5 days and SN–SSLL 6 days. Longest time between onsets of phenological phases was found out between phenophases SSLL–HE (on average 22 days) like in the previous period.

Figure 3 The onset of phenological phases for decades 2001–2010



Legend: PWV – Period without vegetation, SD – Sowing, EM – Emergence, TI – Tillering, BLSE – The beginning of leaf sheath elongation, FN – First node, SN – Second node, SSLL – Swelling of the sheath of the last leaf, HE – Heading, MR – Milky ripeness, YR – Yellow ripeness, FR – Full ripeness, HA – Harvest. The white box on the chart shows the missing data.

Figure 4 Average values of the phenological phases for decades 1991–2000 and 2001–2010



Legend: PWV – Period without vegetation, SD – Sowing, EM – Emergence, TI – Tillering, BLSE – The beginning of leaf sheath elongation, FN – First node, SN – Second node, SSLL – Swelling of the sheath of the last leaf, HE – Heading, MR – Milky ripeness, YR – Yellow ripeness, FR – Full ripeness, HA – Harvest, GS – Growing season.

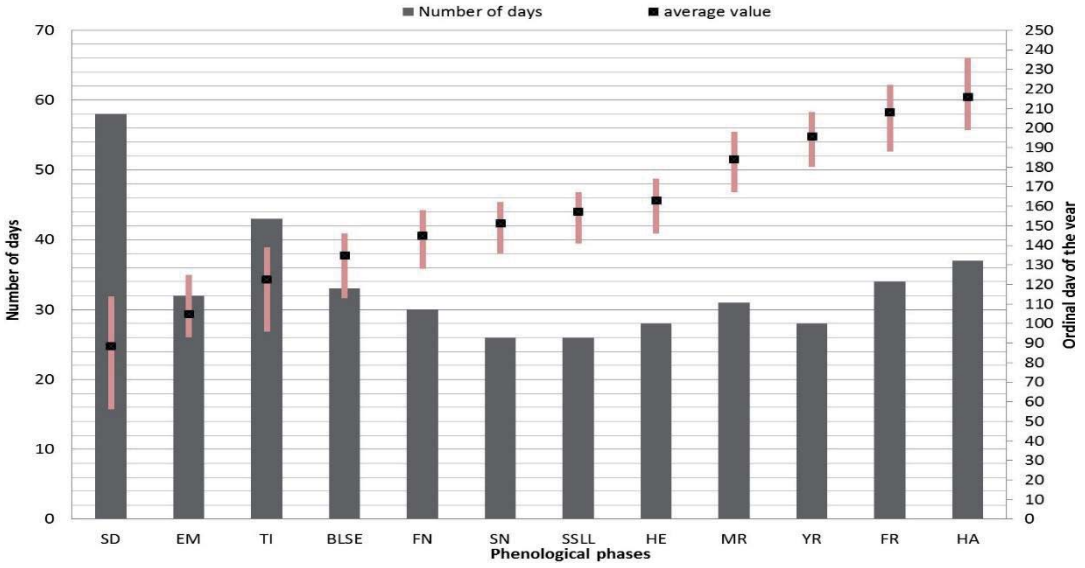
Figure 4 shows the average values of onset of phenological phases and processes of sowing and harvest of spring barley for two decades. When comparing the two decades, it was found out that GS was shorted by 5 days in the period 2001–2010. Phenophase EM occurs in both decades (on average) in same date (105th day of the year). Phenophase MR occurs in both decades (on average) in same date (184th day of the year) also. The most significant shift in the phenological phases was observed in phenophase BLSE. Phenophase BLSE occurs in average 5 days earlier in the decade 2001–2010 (according to decade 2001–2010). The longest interval between phenophases was observed between the HE and MR of 20 days (1991–2000) and 22 days (2001–2010) in both decades.

Shortest interval between phenophases was found out in decade 1991–2000 between phenophases FN and SN (5 days). Shortest interval between phenological phases was found out in length 5 days in the period 2001–2010 and between phenophases SN and SSLL.

Analysed data show great variability within onset of phenological phases and implementation of processes of sowing and harvest. Weather in the given year has a significant influential on the development of spring barley and onset of individual phenological phases. The greatest variability of onset of phenological phases was found out in phenophase of TI. This phenophase appears from 96th days to 139th days of the year (range of onset of phenophase is 43 days).

The phenological phases onset in the period 1991–2010 as follows: EM from 93rd to 125th day of the year, TI from 96th to 139th day of the year, BLSE from 113th to 146th day of the year, FN from 128th to 158th day of the year, SN from 136th to 162nd day of the year, SSLL from 141st to 167th day of the year, HE from 146th to 174th day of the year, MR from 167th to 198th day of the year, YR from 180th to 208th day of the year and FR from 188th to 222nd day of the year (see Figure 5). Processes of sowing and harvest were performed in the following terms: SD from 56th to 114th day of the year and HA from 199th to 236th day of the year.

Figure 5 Variability of the onset of phenological phases and processes of SD and HA for the period 1991–2010



CONCLUSION

We have long-term series of phenological observations currently (since 1923). These data can be used in many scientific areas (for example in medicine, agriculture, climatology etc.). Analysed data show great variability within onset of phenological phases and implementation of processes sowing and harvest. On average GS is prolonged by 9 days in long-term period 1991–2010 when compared to the period 1931–1960. On average the harvest was delayed by 11 days in the period 1991–2010 (when compared to the period 1931–1960). Shift of harvest can have different reasons for example cultivated variety of spring barley and climatic conditions. The shortest intervals between onsets of individual phenophases were found out at phenophases BLSE, FN, SN and SSLL. The longest interval between onsets of phenological phases was found out between SSLL and HE.

ACKNOWLEDGEMENTS

The research was financially supported by the IGA – Internal Grant Agency Faculty of Agronomy MENDELU No. TP1/2016 "New trend in the cultivation and use of milk thistle (*Silybum marianum* L.) in agriculture."

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PHENOLOGICAL PHASES AND THEIR POSSIBLE INFLUENCE ON SOIL EROSION AT MAIZE

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Abstract: The contribution consists of two parts. The first part deals with evaluation of data from phenological observations of the Czech Hydrometeorological Institute (CHMI). The calculation of the value of protective effect of vegetation (C factor) for years with long and short growing season was carried out in the second part. The paper deals with the onsets of phenological phases (emergence, heading and milky wax ripeness) and sowing and harvest processes of maize. Detailed analysis for particular years was carried out for Strážnice locality (Central Europe, Czech Republic, South Moravia) for period 1991–2012. Analysis of average values shows that the longest growing season (152 days) was in the period 1991–2012. When this data was compared with the period 1931–1960, the prolongation of growing season was proved (by 6 days). Analysis of the average values shows that prolongation of interval between phenological phases of emergence and heading was proved (from 38 days to 62 days). On the average the sowing was carried out at the earliest in the period 1931–1960. And the harvest was carried out at the latest in the period 1991–2012. The analysed data show a great variability in the onset of phenological phases. First of all the meteorological conditions are going to have significant influence on the progress of maize and the onset of phenological phases. The value of the protective effect of vegetation shows how certain crop protects the surface of soil from falling drops of rain. Higher values of C factor were detected in each year with short growing season. The percentage difference between years with a long or short growing season is 10% for 1968 and 1986 and 35% for 1994 and 2010.

Key Words: C factor, Growing season, Czech Republic, South Moravia, Phenological observations

INTRODUCTION

Phenology is a science that deals with the study of the time course of periodically recurring plant manifestations (Krška 2006). Phenological manifestations of plants are closely connected with seasonal atmospheric conditions (Roetzer et al. 2000). Phenological data is considered to be the most important and the strongest indicator of climate change (Wu et al. 2016, Estrella and Menzel 2006). According to research, the global climate change affects the onset of phenological phases (Hudson and Keatley 2010). Increase of temperature can cause a reduction in global crop production (Lobell et al. 2011), which can be a major problem in the future due to the increasing number of people in the Earth. Growing season can be prolonged by 21 days to year 2020 and by 1 month to year 2050 due to increasing temperature (Šiška and Takáč 2008). Increasing the average temperature in January and February by 1 °C has an effect on the beginning of growing season. There is an earlier onset of growing season by 7 days (Chmielewski and Rötzer 2001). Linear trend analysis of phenological data of apricot (Velkopavlovická variety) shows that phenophase of flowering occurs earlier about 2 days in every decades (Chuchma et al. 2016). Vilček et al. (2016) state that they found only a slight insignificant increase of continentality over time (based on the employed thermal continentality indices).

Growing inhabitant population of the Earth and the climate change should be lead to influencing of land use and its sustainability according to precondition of scientists (Reitsma et al. 2015). Soil erosion is seen as a threat for environment and security of foodstuff for mankind (Pimentel 2006). Soil erosion causes a significant change of soil physic-chemical properties (Haibing et al. 2017). And also the production and non-production functions of soils are threatened (Janeček 2012).

Soil erosion causes environmental and economic damages: soil degradation, loss of soil resources, damages on the agricultural fields, eutrophication of water, flooded areas (Blanco-Canqui and Lal 2010). The calculation of soil loss is carried out based on formula of long-term soil loss according to Wischmeier and Smith (1978). The value of the cover and management factor (C factor) enters into this formula (C factor dependents on vegetation cover and agronomic management). C factor is for maize ranges from 0.35 to 0.90 (Janeček et al. 2012).

MATERIAL AND METHODS

Phenology

Detailed analysis of phenological data for maize is performed in this contribution. The data were obtained from direct observations of the CHMI. Three long-term periods were analysed (1931–1960, 1961–1990 and 1991–2012). Data for the period 1931–1960 were taken from the publication Agroclimatic conditions of ČSSR (Kurpelová et al. 1975). The analysis was carried out or the Strážnice locality (Central Europe, Czech Republic, South Moravia) for the period 1991–2012. This station does not have data for the period 1931–1990 and therefore the row was extended of the nearest phenological station of Hodonín (Central Europe, Czech Republic, South Moravia). These stations are distance from themselves 18 km and are situated at similar altitude (Strážnice 177 m above sea level and Hodonín 190 m above sea level).

The paper deals with changes in the onsets selected phenological phases in the particular periods. Analysis was carried out for particular phenological phases: emergence (EM), heading (HE), milky wax ripeness (MWR), and sowing (SD) and harvest (HA) processes. More detailed specifications of these terms are stated in Table 1. The length of the growing season (GS) is also solved in the paper, where this period is understood as a interval between SD-HA.

Table 1 Monitored phenophases and processes (SD, HA) and their description (Hájková et al. 2012, Valter 1982)

Phenological phases and processes	Abbreviation	Description
Sowing	SD	Day when the given plant was sown or planted.
Emergence	EM	The first overground parts of a plant penetrated the soil surface and the plant begins windrow. The crop is beginning to create visible rows.
Heading	HE	Precisely half of the flower protrudes from the sheath of the last leaf.
Milky wax ripeness	MWR	Grain in the central part of the corn cob release a thick, pasty amyloid content after the squeeze.
Harvest	HA	The day on which the harvest of the given crop started.

Soil erosion and C factor

Furthermore, the calculation of the protective effect of vegetation (C factor) was performed on the grounds of Methodology – Protection of agricultural lands from erosion (Janeček et al. 2012). The values of C factor enter into the calculation (see Table 2) and also the percentage distribution of erosion dangerous rains (see Table 3). The term of SD and HA is very important to calculation C factor. These data were obtained from direct phenological observations.

Table 2 Value C factor for maize (Janeček et al. 2012)

Period	Soil conservation effect	C factor
1	Stubble and rough furrow.	0.70
2	From preparing the land for sowing within one month after sowing.	0.90
3	During the second month after sowing.	0.70
4	To harvest.	0.35
5	From harvest to seedbed preparation subsequent crops.	0.70

Calculation of C factor was carried out for extreme years (i.e. for years with long and short GS). These years were selected from phenological observations for period 1961–1990 and 1991–2012.

In the period 1991–2012, the year 1994 had the shortest GS (106 days) and the year 2010 had the longest GS (192 days). In the period 1961–1990, the shortest GS was in 1968 (81 days) and the longest GS in 1986 (178 days). Sample of the calculation of the C factor is stated in Table 4. And the results of the extreme years are shown in Table 5.

Table 3 The percentage distribution of erosion dangerous rains (Janeček et al. 2012)

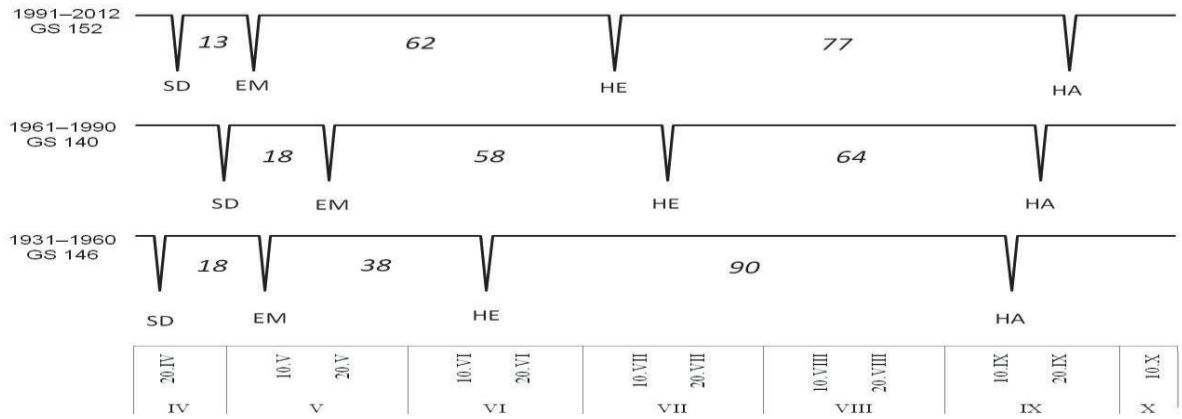
Month	IV	V	VI	VII	VIII	IX	X
% R factor	1	11	22	30	26	8	2

RESULTS AND DISCUSSION

Analysis of phenological data

Analysis of the average values of the onset of phenological phases shows that the longest GS was 152 days in the period 1991–2012 (see Figure 1). This shows to the trend of prolongation GS. The prolongation of growing season was proved (by 6 days) at comparison in two periods (1991–2012 and 1931–1960). The earliest sowing was carried out in the period 1931–1960 (19th March) and the latest in the period 1961–1990 (30th April). Also analysis shows prolongation of interval between phenological phases EM–HE. The longest interval between phenological phases HE–HA was 90 days in the period 1931–1960. In the period 1991–2012, the prolongation of interval between phenological phases HE–HA (by 13 days) at comparison with period 1961–1990 was proved. On average, the earliest harvest was carried out in the period 1931–1990 on the 12nd September (255th day in year). On the other hand the latest harvest on average was on 22nd September in the period 1991–2012 (265th day in year).

Figure 1 The average values for the period 1931–2012

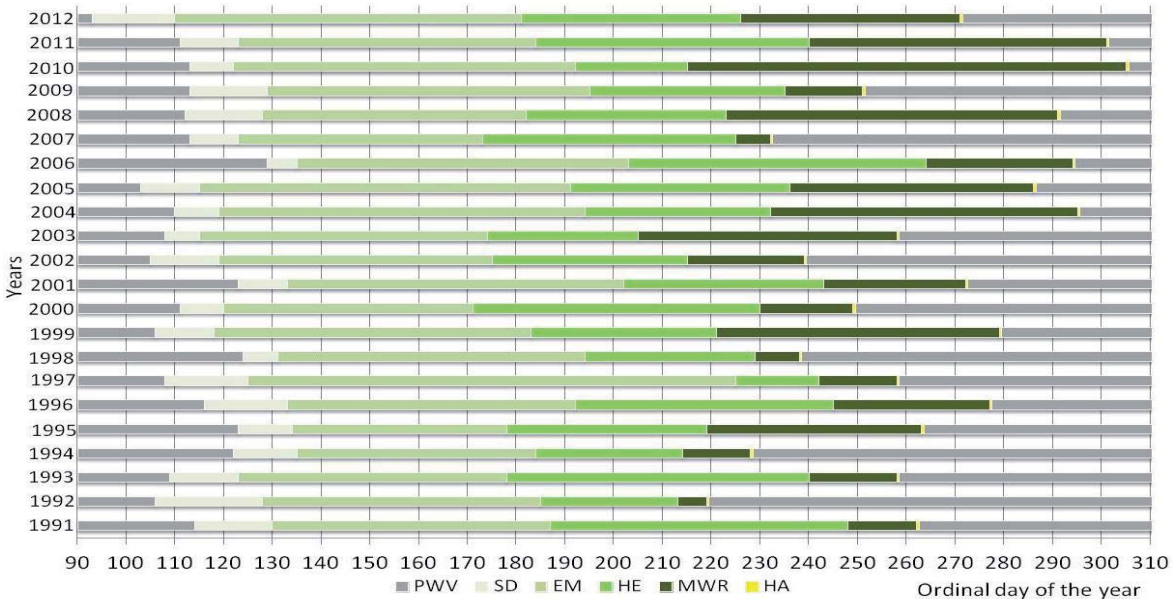


Legend: SD – Sowing, EM – Emergence, HE – Heading, HA - Harvest

Figure 2 shows the onsets of phenological phases (EM, HE, MWR) and processes (SD, HA) for particular years for maize in the period 1991–2012. At the earliest the maize was sowed in 2012 on 3rd April (93rd day in year). The sowing of maize was carried out at the latest in 2006 on 9th May. The longest GS was found out in 2010 (192 days) and the shortest in 1994 (106 days). The calculation of value of C factor was performed for these years. The harvest was carried out at the earliest in 1992 on 7th August (219th day in year) and at the latest the maize was harvested in the monitored data in 2010 on 1st November.

The longest interval between SD and EM was 22 days in 1992. The opposite was year 2006, when the interval between SD and EM was only 6 days. The interval between phenophases of EM and HE was the shortest in 1995 (44 days). The longest interval between EM and HE was found out in 1997 (10 days). The longest interval between phenological phases HE–MWR was found out in 1993 (62 days). The shortest interval was in 1997 (17 days). The longest interval between phenological phase MWR and HA was found out in 2010 (90 days) and the shortest interval between MWR and HA was 6 days in 1992.

Figure 2 The onset of phenological phases in the period 1991–2012



Legend: PWV – Period without vegetation, SD – Sowing, EM – Emergence, HE – heading, MWR – Milky was ripeness, HA – harvest

The analysed data shows a great variability in the onset of phenological phases and in implementation of sowing and harvest processes. First of all the meteorological conditions are going to have significant influence on the progress of maize and the onset of phenological phases.

C factor and Maize

Table 4 shows an example of calculation of the C factor according to Methodology – Protection of agricultural land from erosion (Janeček et al. 2012). The biggest value of C factor was found out in the period 1991–2012 in 1994. The value of C factor was 0.721 in this year. The growing season had 106 days in 1994. The reason for this high number is later sowing of maize. So erosion risk phases of plants occur in the period when the erosion risk rains occur (May, June). Another reason why this value of C factor is so high is a very early harvest. Erosion risk rain will occur with 33% probability in these months. The years with shorter growing season always have higher value of C factor. The percentage difference between years with a long or short GS is 10% for 1968 and 1986 and 35% for 1994 and 2010.

Table 4 Example calculation of C factor

Strážnice 2010	**Sowing**	**23.4.**	**Harvest**	**1.11.**
Month	**% R**	**Period**	**C**	**% R.C**
IV.	0.733	1	0.700	0.513
	0.267	2	0.900	0.240
V.	8.161	2	0.900	7.345
	2.839	3	0.700	1.987
VI.	16.867	3	0.700	11.807
	5.133	4	0.350	1.797
VII.	30.000	4	0.350	10.500
VIII.	26.000	4	0.350	9.100
IX.	8.000	4	0.350	2.800
X.	2.000	4	0.350	0.700
C factor			**0.468**	

Legend: %R.C = percentage distribution of erosion dangerous rains multiply with value C factor

One of the lowest values of C factor was found out in 2012 (0.413). The maize was sowed very soon on 3rd April (93rd day in year) in 2012. The phenological phase of emergence was started on 20th April, which is date when in another years maize was sowed. The earlier maize is sowed (taking into account the optimal meteorological and physiological conditions), the lower the value of C factor. The growth is previously involved and it protects the soil from soil erosion much better

than before. The lowest value of C factor was in 2012 when maize was sown within the survey period at the earliest. Value of C factor was 0.413. The percentage difference between 2012 and 1994 is 43%. The soil erosion on the estate would be less (by 43%) than in 1994.

This is followed by other C factor calculations for other crops such as sugar beet (Stehnová and Středová 2016), spring barley (Stehnová et al. 2016).

Table 5 Values C factor for period 1961–1990 and 1991–2012

Hodonín	1961–1990					Strážnice	1991–2012			
	1968		1986				1994		2010	
	SD	HA	SD	HA			SD	HA	SD	HA
	17. 5.	6. 8.	23. 4.	18. 10.			2. 5.	16. 7.	23. 4.	1. 11.
C factor	**0.524**		**0.471**			**C factor**	**0.721**		**0.468**	

CONCLUSION

The analysed data show a great variability in the onset of phenological phases. The essential impact on the onset of phenological phases have mainly course of weather in particular year. When comparing average values in the period 1931–1960 was found out that prolongation of growing season was proved (6-day) in the period 1991–2012.

Furthermore, the calculation of the value of protective effect of vegetation (C factor) for years with long and short growing season was carried out. The largest percentage difference was found out between 1994 and 2010 (35%). The lowest value of C factor was found out in 2012, when the sowing was performed on 3rd April (C factor was 0.413). When comparing this value with value of 1994 is percentage difference 43%. This means that soil loss in particular parcel would be smaller about 43% due to the earlier sowing.

ACKNOWLEDGEMENTS

The research was financially supported by the IGA – Internal Grant Agency Faculty of Agronomy MENDELU No. TP1/2016 "New trend in the cultivation and use of milk thistle (*Silybum marianum* L.) in agriculture."

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THE SOIL SEALING OF AGRICULTURAL LAND BY THE DEVELOPMENT OF INTRAVILAN IN THE CADASTRAL AREA OF MODŘICE

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Abstract: The article focuses on the issue of agricultural soil sealing in the Modřice cadastre (South Moravian Region). Available data and map resources were used for this study (historical and up-to-date). Data was processed using manual digitalisation in GIS software. The reduction in agricultural land is analysed in individual periods starting with the year 1824 (source of data of Stable cadastre) until present. It is also evaluated the soil sealing of individual types of land and soil types (including the price of land) according to Evaluated Soil-Ecological Units (EPEU). The soil sealing of agricultural land is connected with the development of the municipality. For the comparison of the future development, the potential (future) soil sealing of agricultural land, which is proposed within the framework of the valid Landscape-plan, is evaluated. The results show that 396 hectares of agricultural land (40% of the cadastral area) have been built since the year 1824 until the present day. In the events of this trend, a large loss of agricultural land can be expected in the future.

Key Words: Soil sealing, degradation, EPEU, price of soil, Landscape plan, Stable cadastre

INTRODUCTION

Soil and landscape are degraded in their quality (soil erosion, nutrients washing out, soil compacting, landscape homogenisation, falls in biodiversity and fragmentation of landscape structure), as well as lost completely in terms of their quantity. However, at present, soil sealing for other, non-agricultural purposes can be considered as one of the gravest degradation factors. The development of human settlements and society changes landscape structure and the functional use of the land. Due to the expansion of towns and villages including ensuing infrastructure, agricultural land including the best crop-yielding areas has declined dramatically. Agricultural soil sealing is currently one of the prime engines driving economic growth. This statement is given by Van der Heijde (2012) in relation to the Netherland's urbanisation. In the last fifty years the Netherlands have been intensely urbanised and many polycentric town regions have arisen. In their work, Hersperger and Buergi, (2008) quantified the engines of landscape changes and discovered that urbanisation was the most important force of landscape and societal change in the three periods under scrutiny (the beginning, middle and end of the 20th century). Economic and political reasons only followed.

The issue of agricultural soil sealing is both world-wide (Africa, China: sealing for quarrying, infrastructure) as well as specifically European (Netherlands, Italy, Germany: sealing for infrastructure and industry). At present we are probably experiencing what is historically the most extensive obliteration of agricultural land. It is being altered on a large scale for the most diverse purposes (commercial, transport, housing construction, etc.).

In the EU, the Member States with high sealing rates (exceeding 5% of the national territory) are Malta, the Netherlands, Belgium, Germany, and Luxembourg. Furthermore, high sealing rates exist across the EU and include all major urban agglomerations, and most of the Mediterranean coast. The latter experienced a 10% increase in soil sealing only during the 1990s (Janků et al. 2016). A European Union report (EU) asserts that between 1990 and 2000 at least 275 ha of soil were lost in the EU every day which corresponds to 1000 km^2 per year. Half of it is permanently covered

with an impenetrable layer of buildings, streets or parking lots. This unfavourable movement threatens a lack of arable land and sources of underground water availability for future generations (Agrarheute 2011).

In the Czech Republic, the conversion of farmland to urban uses (soil sealing) represents a very serious problem. On arable land in particular, there is a trend of soil loss (approximately 9100 ha/year), which means approximately 25 ha/day, or an area equal to 40 football pitches per day (Janků et al. 2016).

Since 1927, the Czech Republic has lost over 851 thousand hectares, i.e. 22.3% of agricultural soil (Charvát 2010). Historically the worst period came between the years 1976 and 1981 when 37.9 ha of soil vanished every day. This trend is perceived as extremely unfavourable and represented one of the worst developments in Europe. The intensity of soil sealing in the Czech Republic has been oscillating around 25 ha of soil per day lately (Spilková and Šefrna 2010).

Building on agricultural land irreparably destroys one of the most precious natural sources – the soil. Vast impermeable urbanised areas with zero retention of rainfall water are turning up more and more often.

Despite the existence of the agriculture land protection law (Law No. 334/1992 Coll.), land protection in the Czech Republic seems to be ineffective. Rectification of damages caused is a long-term process that is often, in the case of soil loss, irrecoverable (Podhrázská and Karásek 2014).

MATERIAL AND METHODS

The source for primary analysis of changes in the countryside and assessing the sealing of agricultural soil to the benefit of the progressing residential area were primarily digitalised maps of land use dated 1824 (stable cadastre - only residential area - built-up areas - covered), 1836–1852 (2nd military mapping), 1876–1878 (3rd military mapping), 1950 (aerial snaps), 1990 (orthophoto), 2006 (orthophoto) and 2016 (orthophoto). The selection of time horizons for analysing countryside structure were set with the intention of covering the most crucial alterations in landscape structure from the mid - 18th century up to the present. A stable cadastre was set as the starting point. The current situation at the opposite end of the time scale is depicted by a colour orthophoto dated 2016.

Another part of the analysis used the available Modřice landscape plan that stipulate the manner of using village land in future. As in the case of the stable cadastre, only the residential area was covered in the case of the landscape plan. For the purposes of ascertaining the theoretical future development of built-up areas, the coverage of built-up areas in 2100 was calculated using a method for stipulating the annual intensity of residential area expansion from the stable cadastre until 2016 (X ha/year) and applying this figure up until the year 2100.

Figure 1 Map of Stable cadastre (archivimapy.cuzk.cz) (left); Part of Lanscape plan of Modřice (right)

Characteristics of the area

Modřice is a town located south of Brno (South Moravian Region). The Modřice cadastral area (1005 ha) follows directly from the southern edge of Brno and its industrial and commercial estates. The town lies on a low, slightly sloping terrace at the Western periphery of Svratka floodplain. Modřice are located on the north-west tip of the Dyje-Svratka valley lowlands. Housing converges on the undulated plain above the Svratka River at the foothills of stretched slopes running from the ridge

of the mesoregion of the Bobrava Highlands that forms a part of the Czech Highland orography units. The eastern part lies in the lowlands of the relatively new Carpathian relief of Western Subcarpathia.

Evaluation of changes in land characteristics

To evaluate agricultural land and its productive capacity expressed in prices, in the 1970s, the Czech Republic introduced the "Land Evaluation Information System". This system contains basic data on the land defined using a 5-digit code, forming together an Evaluated Soil-Ecological Unit (EPEU). Each of the digits (or pair of digits) expresses a particular land characteristic. The system of evaluated soil-ecological units reflects all characteristics and differences in a particular agronomical area (soil, climatic and morphological conditions). Regionalization of individual EPEU and their corresponding codes is done based on a digital collection of EPEU maps, using the borderlines surrounding individual EPEU surfaces with their numerical designation (Mašát et. al. 2002).

The structure of the EPEU code is defined in the following way (A.BB.C.D):

A - climatic region code (0–9); BB - code of the main soil unit (0–78); C - combined code of slope and exposure; D - combined code of skeletality and soil depth.

Changes to these soil characteristics were used to assess the qualitative degradation of agricultural soil due to expansion of built-up areas. This assessment was divided into three time periods (second military mapping (1836–1852)–2016; 1950–2016; 2016–Landscape plan of the village).

Based on the Decree No. 441/2013 Sb the official price is fixed for every EPEU (€/m^2). By comparing lost land due to the build-up and the price of land (Decree No. 441/2013) we can determine the total price of degraded land (Podhrázská et al. 2015). This approach was applied to the assessment of economic impacts of soil sealing in the model location Modřice.

RESULTS AND DISCUSSION

Historical maps of the stable cadastre were the source for assessing the overall soil sealed by buildings (Figure 2) that comprises 13.11 ha. At the time of the second military mapping (1836–1852) built-up areas had grown to 23.53 ha. There was a further increase of built-up areas by about 12 ha at the time of the third military mapping. Between 1950 and the present, the total residential coverage grew to 408.93 ha, which corresponds to some 41% of the area cover. Total sealing of 123.29 ha has happened over only the last ten years (2006–2016). The landscape plan proposes sealing of a further approximately 40 ha of soil. The results obtained were used to ascertain the potential scope of built-up areas in 2100 when they might take up to 58% of the area in question (Figure 2).

Figure 2 Graphical representation of the development of built-up areas – Modřice

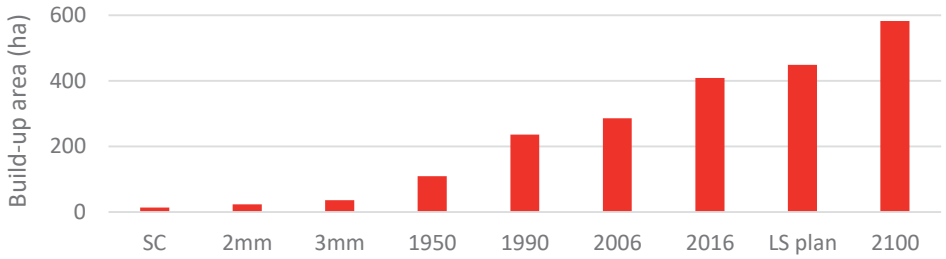
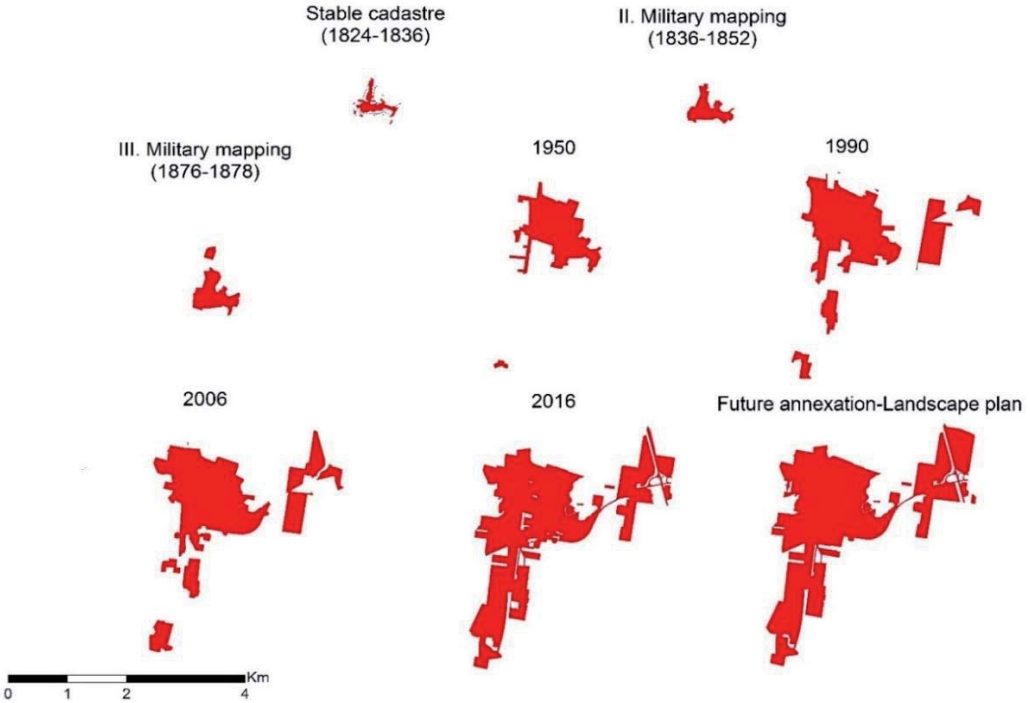


Table 1 Development of built-up areas in Modřice cadastre

Period	Build-up area (ha)	% of cadastral area
SC (Stable Cadastre)	13.11	1.3
2MM (Military mapping)	23.53	2.34
3MM (Military mapping)	35.45	3.53
1950	109.09	10.85
1990	235.91	23.47
2006	285.61	28.42
2016	408.93	40.68
LS plan (Lansdcape plan)	448.99	44.67
2100	581.97	57.91

Figure 3 Maps of built-up areas in the analysed time period



Analysis of the decrease in individual cultures showed that most sealed category in all the time periods under scrutiny was arable land (more than 80% of built-up area in all instances). The Table 2 below shows the total scope of sealed cultures in individual time periods.

Table 2 Sealing of individual cultures – Modřice

LU cathegory	LS plan–2016		2016–1950		2016–2mm	
	ha	%	ha	%	ha	%
Arable land	34.07	85.05	291.52	96.41	309.92	80.22
Pernament grassland	2.75	6.86	0	0	34.07	8.82
Vineyard	0	0	0	0	2.66	0.69
Orchard	2.73	6.82	4.45	1.47	1.5	0.39
Forest	0.51	1.27	1.61	0.54	38.17	9.88
Other area	0	0	4.78	1.58	0	0
Total	40.06	100	302.36	100	386.32	100

Qualitative analysis of agricultural land carried out, expressed by the decrease of Evaluated Soil-Ecological Units demonstrates the built-up EPEU in individual monitored periods.

Description of EPEU in Modřice cadastre

0.01.00, 2.01.00, 2.01.10 - Flat and moderately sloped Haplic Chernozems, soils with thick humus horizon, with crumb to granular structure, developed from loose carbonate substrates, deep soil

0.08.00 - Washed-off (eroded) Haplic Chernozems with cultivated substrate covering more than 50% of moderately sloped area

2.02.00, 2.02.10 - Flat and moderately sloped Luvic Cheznozems, without skelet, predominatly with favourable water regime

2.08.10, 2.08.50 - Washed-off (eroded) Haplic Chernozems with cultivated substrate, in moderately sloped and sloped terrain

2.10.00 - Flat and moderately sloped Haplic Luvisoils, with heavier bottom, skeleton-less, deep, moderately sloped

2.56.00, 2.57.00 - Flat and moderately sloped Fluvisoils, deep soil profile, slightly dry climatic region

2.61.00 - Flat and moderately sloped Phaeozems, deep soil profile, slightly dry climatic region

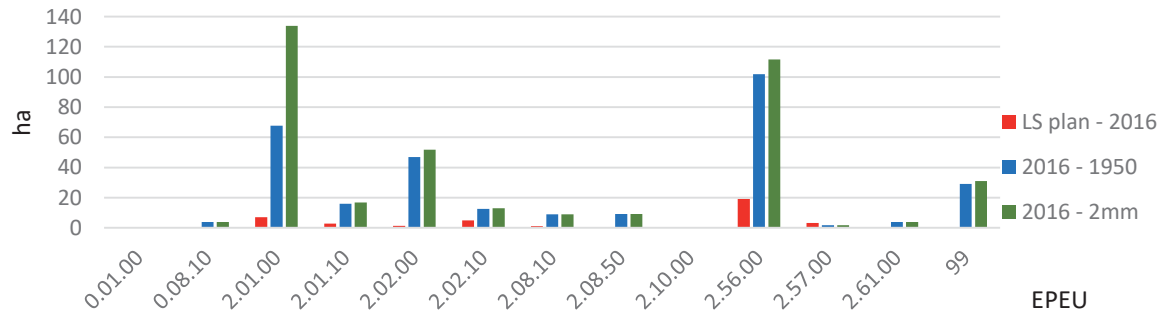
99 - no agricultural land

Table 3 Sealing of individual EPEU and total price of degraded soil – Modřice

EPEU	Price of EPEU (€/m²)	LS plan - 2016			2016–1950			2016 - 2mm		
		ha	%	Total price (€)	ha	%	Total price (€)	ha	%	Total price (€)
0.01.00	0.62	0	0	0	0.1	0	680	0.1	0	744
0.08.10	0.44	0	0	0	3.9	1.3	17 116	3.9	1	17 116
2.01.00	0.64	7	17.6	44 992	67.7	22.4	433 472	133.8	34.6	856 512
2.01.10	0.57	2.9	7.2	16 530	15.9	5.3	90 573	16.8	4.4	95 874
2.02.00	0.64	1.3	3.3	8 384	47	15.5	300 608	51.8	13.4	331 584
2.02.10	0.57	4.8	12.1	27 531	12.6	4.2	71 991	13	3.4	73 986
2.08.10	0.44	1.1	2.7	4 752	9.1	3.0	39 820	9.1	2.3	39 820
2.08.50	0.37	0.3	0.7	999	9.2	3.1	34 188	9.2	2.4	34 188
2.10.00	0.59	0	0	0	0.4	0.1	2 124	0.4	0.1	2 124
2.56.00	0.52	19.2	47.9	99 788	101.8	33.7	529 568	111.7	28.9	580 684
2.57.00	0.43	3.1	7.8	13 459	1.7	0.6	7 095	1.7	0.4	7 095
2.61.00	0.54	0	0	0	3.9	1.3	20 790	3.9	1	20 790
99	-	0.3	0.8	0	29.2	9.6	0	31.1	8	0
Total		40.1	100	216 435	302.4	100	1 548 027	386.3	100	2 060 517

The total surface of the sealed soil between 2nd military mapping and present (2016) is 386.32 ha. The total soil price of this area is 2 060 517 EUR.

Figure 4 Graphical representation of sealed Evaluated Soil-Ecological Units



CONCLUSION

The results presented above suggest the amount of soil in the Modřice cadastre that was used for agriculture or as forest had dropped considerably. This sealing of agricultural soil for the benefit of technical infrastructure, settlements and industrial zones is largely irrevocable. Built-up areas change the land hydrology thus creating an extensive impermeable zone that cannot be used agriculturally. Total agricultural land that was built on in the Modřice cadastre area from between 1836 and 1852 (2nd military mapping) until the present (2016), is about 386 ha (40% of the cadastral area).

The lands with the best soils (Chernozems) in 1st and 2nd class protection (highest priority of soil protection) are used for building. The Landscape plans calculate with the areas of the best soils for building purposes in the future. In the last 180 years approximately, 297.79 ha of 1st class soil of the agricultural land resources (most valued chernozem) in the Modřice cadastre was irrevocably sealed). Comparing the future situation (landscape plan) and presence (2016), the further development of built-up areas (about 28 ha) is anticipated (again soil in the 1st class of agricultural soil resource protection). The price of thus irrevocably lost agricultural soil was ascertained as about EURO 2 060 517.

Agricultural soil sealing by building is a world-wide problem. For a long time the drop in agricultural soil was not perceived particularly negatively given that agricultural production kept increasing courtesy of breeding new, more hardy breeds and using agricultural fertilisers and pesticides. Gradual degradation of the soil fund associated with decreasing biodiversity and the environmental balance in the agricultural landscape went hand in hand with this trend. At present, the negative impact of intensive farming on ecosystems is increasingly emphasised while promoting organic agriculture.

However, this manner of farming puts heightened pressure on the coverage of cultivated land because the yields of agricultural crops without using chemicals cannot attain the same values as intensive farming. The agricultural policy of the EU also plays a significant role in the use and protection of the soil fund. In the years of its functioning the SZP urged farmers to use modern mechanisation and new approaches, including chemical fertilisers and plant protection preparations. This policy was efficient and productivity grew. Crop yields have increased, but have remained on the same level since 2000. Consequently, the task farmers face is to produce as much foodstuff as possible from diminishing sources. At present, there is over-production in the EU but the threat of climatic extremes, including agricultural drought that may in future adversely affect our best-yielding locations, will probably cause a drop in strategic commodities production. The intensity of agricultural soil fund sealing and the devastation of agricultural soil presented in the article is another negative factor that must be taken into consideration when planning agricultural policy.

ACKNOWLEDGEMENTS

This study was supported by the Internal Grand Agency Faculty of AgriSciences MENDELU No. AF-IGA-IP-04/2017 "Degradation of agricultural land resources in selected areas in the South Moravian region".

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ANALYSIS OF STABLE AREAS IN THE LANDSCAPE - REGION HUSTOPECSKO

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Abstract: The paper presents the method and manner for analysing the locations of stable areas ("core areas") - sites without any changes to the manner of land use in the period between the first half of the 19th century until the present (in the model area of the Hustopeče region - South Moravia). Stable areas in the landscape (especially forests, meadows) are of great ecological importance - they increase biodiversity, facilitate the movement of animals. Stable areas give the landscape its typical landscape character. Areas without a change of land use – without qualitative alternation to the type of plot were identified by analysing 6 lines of historical land use development in GIS. Inputs of this analysis are historical maps, historical aerial pictures and current orthophotos. The analysis suggests some 30% of the land cover of the Hustopeče model area is without land use changes. The remaining 70% of land has undergone at least one change in the type of plot over the monitored period. The most represented area without any change in land use are arable land – 23.6%, followed by forests (3.3% of land), built-up area (1.8%). Other land use types (permanent grassland, vineyards and orchards) are represented to a negligible extent.

Key Words: stable area, land use, Hustopeče, historical maps

INTRODUCTION

Landscape and its disposition are characterised in their uniqueness based on a remarkable variety of natural and cultural conditions (Kupka 2010). Lokoč and Lokočová (2010) quote that landscape can be visualised as a jointly-created and controllable organism. It is subject to creative and destructive forces of different intensities and duration. Landscape is formed by cultural and natural processes that mutually affect one another, blend but are also independent of one another. Lipský (2000) states anthropogenic processes act very quickly and over a short space of time. These processes alter the appearance, structure and function of the landscape directly (surface mines, ploughing, planting, etc.) or indirectly (erosion, ecological succession, etc.).

Changes in landscape are built on monitoring changes in individual landscape segments, like area, dynamics and spatial configuration (Palmer 2004). Soil and its condition are often the decisive factor that determines the character of landscape, variety of vegetation cover, including fauna and which is an essential condition for the economic use and human society development (Hudec and Novák 1997). However the real face of the landscape is the intersection of current social ambitions, given technological options, historical heritage and natural influences (Klvač and Bielová 2012). Landscape is constantly changing and even the less perceived alterations may be crucial. Therefore, it is essential to perceive changes in the landscape in relation to previous and current societal trends, manner of economic use and industrial development. The state and appearance of landscape can retrospectively determine the previous economic situation, state of society or for instance the force of natural processes (Lokoč and Lokočová 2010, Boltiziar et al. 2008, Fladmark et al. 1991).

By using the landscape, man alters its disposition which is an issue covered by several authors (for instance Löw, 1995), who identify this process as the cultural landscape. Lipský and Romport

(2007) state that the cultural landscape as a dynamic system is the result of continually acting landscaping processes and factors. Natural and anthropogenic processes intermingle in cultural land settled and used by humans, of which many have a negative character and cause bigger or smaller changes to the landscape. However, changes and disturbances are natural in the landscape and form part of its development.

The cultural landscape of the Czech Republic is a very rich and varied one due to the changeable natural conditions of individual landscapes as well as the historical development that affected their appearance. The Czech landscape is a typical historical and cultural landscape that encompasses settlements that form integral parts of it (Štréblová et al. 2014). Míchal (2001) states that large towns and cities that form strong, though perhaps not necessarily extensive "townscapes" - town landscapes dotted with houses are not part of landscape. Bičík (2010) considers a cultural landscape a landscape created by man in distant history that have since undergone numerous changes in different developmental milestones. According to Lipský (2000) its development is wedded to historical events, social development, agricultural farming, settlement and the advance of crafts and later industry.

In the last few centuries the Czech landscape has undergone massive changes in the way the land is used. The archive documents preserved to date (maps) enable us to accurately and efficiently determine the manner of using a specific area in the past. Changes in land use happened at many places and several times and the landscape structure also changed several times over the last few centuries (Stejskalová et al. 2013). Maps of land use change are an essential source of information that suitably augment the basic maps of landscape use. Changes in land use can be monitored in the context of two terms in comparison or over an entire term. Maps of landscape change use in the entire period under scrutiny give comprehensive information on the most changed areas and vice versa on areas used with stability.

Society – its environmental, social and economic development needs - have a crucial influence on landscape changes (countryside). Changes in landscape are among other things currently controlled by landscape planning (Karásek et al. 2014).

Havlíček (2013) states changes in land use can be characterised in the form of several different map outputs – the number of changes to land use, type of landscape use changes, processes of land use changes, intensity of land use changes, stable used areas, trajectories of changes to land use. etc. Land used in a stable manner creates the backbone of the landscape, and testify on the long-term use of the landscape in the given region. Their spatial distribution is one of the basic indices on the use of landscape.

Stable areas in the landscape are important for biodiversity, landscape character, landscape aesthetics. They create the typical landscape character of the countryside and enhance the connection of man to nature (Fábos, Ahern, 1996).

The paper presents an analysis of these historical changes in the Hustopeče area under scrutiny. The objective is to localise places in this territory with as few historical changes (or possibly none) as possible. We call these areas for the purposes of this article "stable areas" (core areas) - from the point of view of landscape structure and landscape character.

MATERIAL AND METHODS

Characteristics of the area

The area of Hustopeče is located in the South Moravian region some 28 km south east from Brno and 25 km northwest of Břeclav. This area lies on the northern perimeter of the Pannonian biogeographic province (Culek 1996) and the Western edge of the extensive Carpathian range (Buček 2010). The model area of Hustopeče contains a total of 7 cadastral locations. The total acreage is 92.28 km^2.

Spatial grid data and vector data were used as a basis for research utilising maps (historical maps and current orthophoto maps) provided by the State Administration of Land Surveying and Cadastre and also the Military Geography and Hydrometeorological Office in Dobruška (historical aerial snaps). All data was processed and assessed using the methods of mathematical statistics. ArcGIS software methods and approaches were applied when processing data, creating mapping outputs and comparing information on the sealing of agricultural soil

Land-Use change

Comparing the changes in land use, six periods were under scrutiny (1836–1852 - 2nd military mapping, 1876–1878 - 3nd military mapping, 1950 - historical aerial photo, 1990 - historical aerial photo, 2006 - orthofoto, 2016 - present orthophoto). All analyses were carried out in GIS (geographic information systems). Land use in individual time series was assessed as well as changes in land use over the monitored period. Analysis of polygons was used for processing changes in land use and analysis of stable areas. ArcGIS 10.3 tools - *Analysis Tools → Overlay → Union* applying code for each land use category were applied too (Table 1). The code was set from the method issued by the Research Institute for Landscape and Ornamental Gardening (RILOG).

Table 1 Outline of landscape categories with the RILOG method.

Code	Culture
0	Other area
1	Arable land
2	Permanent grassland
3	Orchard
4	Vineyard
5	Forest
6	Water area
7	Built-up area

Figure 1 Examples of input data (historical map, aerial historical photo, present orthophoto) of a part of the model territory in the studied time horizons (from left the years 1839, 1950 and 2016)

Having carried out overall analysis of six input map layers (Land Use_2ndmm, Land Use_3ndmm, Land Use_1950, Land Use_1990, Land Use_2006 and Land Use_2016), a new data layer came into being; the "Hustopeče area_union" (Figure 2). This layer contains information on land use in the attribute table for individual polygons (locations in the model area) for each time series. This layer is identified by a six-digit code. The six-digit code 111111 for instance shows that at all terms there was arable land in the location. Should there be a change in land use in the course of the 6 series in the given polygon (zone) (for instance 111121 – in 2006 the site was grassed over). These areas will be identified as areas, where there was 1 change in land use. All areas were subsequently unified by the Dissolve function and exported to a separate shapefile (Hustopečsko_Stable_areas) (Figure 3).

Figure 2 Sample of attribute table in ArcGIS of the "Hustopeče area _union" layer (rows represent individual areas in the landscape - polygons, columns represent time series, numbers identify the culture - type of plot)

FID	Shape *	2mm	3mm	1950	1990	2006	2016
0	Polygon	0	1	1	1	1	1
1	Polygon	0	1	1	1	5	1
2	Polygon	0	1	1	5	5	1
3	Polygon	0	1	5	1	1	1
4	Polygon	0	1	5	1	5	1
5	Polygon	0	1	5	5	5	1
8	Polygon	1	1	1	1	1	1
9	Polygon	1	1	1	1	1	2
10	Polygon	1	1	1	1	1	4
11	Polygon	1	1	1	1	2	1

Figure 3 Sample of attribute table in ArcGIS "Hustopeče area _union" layer (rows represent individual areas in the landscape – polygons, columns represent time series, numbers identify the culture – type of plot – in this case always the same – localities free of change to the landscape structure and land use)

FID	Shape *	2mm	3mm	1950	1990	2006	2016
0	Polygon	1	1	1	1	1	1
1	Polygon	2	2	2	2	2	2
2	Polygon	4	4	4	4	4	4
3	Polygon	5	5	5	5	5	5

RESULTS AND DISCUSSION

The Hustopeče area of interest was historically used for farming mostly (arable land). This is particularly due to the terrain morphology – excellent quality soil (chernozem), warm climate, development of villages and towns. Table 2 and Figure 5 clearly show that the most extensive in all categories of land use in terms of acreage in all analysed time series is arable land. Yet the area scope of individual land use categories has changed considerably over time.

Table 2 Land Use in the Hustopeče area of interest in time series under scrutiny

Hustopeče area	2mm	3mm	1950	1990	2006	2016
Culture	ha	ha	ha	ha	ha	ha
Forest	1287.6	1219.5	1271.5	546.5	717.0	853.0
Arable land	4025.3	4497.2	5759.7	4768.8	4288.2	3729.9
Orchard	16.2	6.7	81.6	402.8	578.2	477.2
Permanent grassland	2051.1	1574.2	1160.4	203.0	444.0	474.3
Vineyard	1605.3	1697.7	518.7	944.7	801.3	1129.9
Water area	37.3	11.4	70.9	1814.5	1762.4	1788.9
Other area	0.9	0.0	5.2	3.2	3.9	7.0
Built-up area	204.9	221.8	360.6	545.1	633.8	768.2
Total	9228.6	9228.6	9228.6	9228.6	9228.6	9228.6

Figure 4 Land in the Hustopeče area of interest in time series under scrutiny

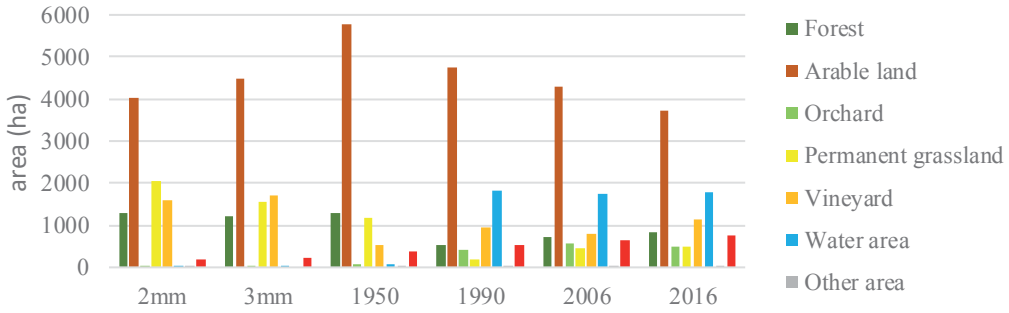


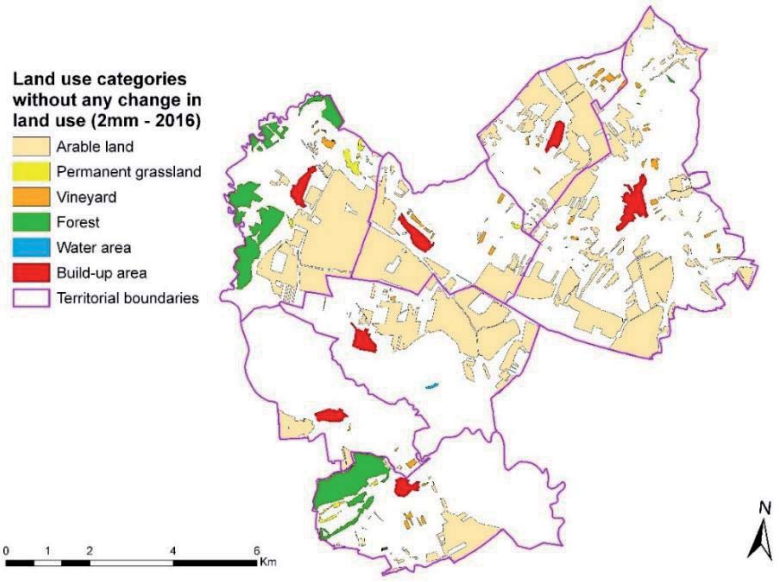
Table 3 Areas without change in land use during the first half of the 19th century to the present (2016)

Hustopeče (study area)	Areas without any change in landuse in all time periods (2mm–2016)		Areas with maximum of one change in land use in all time periods (2 mm–2016)	
	ha	% of study area	ha	% of study area
Arable land	2177.7	23.6	3013.1	32.7
Permanent grassland	26.1	0.3	37.3	0.4
Vineyard	59.5	0.6	228.7	2.5
Forest	305.9	3.3	353.3	3.8
Water area	1.7	0.0	4.0	0.0
Built-up area	169.8	1.8	215.7	2.3
Total	2747.7	29.8	3852.1	41.7

Aside from the acreage of each land use category, their special distribution has changed too. The result of overlay analyses of land use data layers in six time series is a new map. This map (Figure 5) locates and quantifies areas in the landscape where over the period under study was no change

in land use in the Hustopeče (study area) under scrutiny. This map is classified according to individual land use categories (Figure 5). The resulting map shows area's use in the same manner in all periods under scrutiny.

Figure 5 Areas without any change in landuse (in all time periods) – Hustopeče area



The analysis implies that some 30% of the Hustopeče model area has been used without change of land use (from 2nd military mapping to 2016) (Table 3). Nearly 42% of Hustopeče area was used with maximum of one change in land use in all time periods. The most represented area without any change in land use are arable land - 23.6%. This area is used long-term (historically) mostly for farming. Forests and meadow cultures have already surrendered land to production interests in the past – mostly arable land. Stable areas (core areas) in terms of landscape structure and ecological importance are forests in the Hustopeče area. Historically, the forests were on an area of 1288 hectares. At present, their area is only 853 ha. There are only 306 hectares (3.3% of the Hustopeče area) of forest with no change in land is use (for the period from the first half of the 19th century to the present). A similar situation is with permanent grassland. Their area was historically 2051 ha. At present only 474 ha. The location of permanent grassland without land use change is only 26 ha (0.3% of Hustopeče area). Historic vineyards have survived to the present on a very small area 60 ha (0.6% of Hustopeče area). The landscape character of the settlement is represented by smaller villages. Built-up areas (without change in land use) are located on 170 ha (1.8% of Hustopeče area). These are particularly the original cores of towns and villages that constitute the base of their built-up areas to date.

CONCLUSION

Analysis of landscape structure brings precious information on the development of land and the current needs of society. The social pressure on the landscape has been accelerating in recent centuries. We can perceive a notable change in the landscape structure in Czech conditions particularly as of the second half of the 20th century. This is when large-scale agricultural expanses came into existence due to the collectivisation of agriculture – by ploughing over small landscape elements and thus creating vast arable soil fields. Information on the historical development of the Hustopeče area under scrutiny (South Moravian region) were obtained from historical maps whose quality and valuable information are fundamental for such analyses. The results of the study clearly show there have been many changes to land use in the last few decades. Locations without such changes to landscape structure, occur only rarely. Finding that there was at least one change in about 70% of the area cover under scrutiny points to the fact that changes in landscape happen very often and on a large scale. From the first period of the 19th century to the present day, the area of arable land was the most preserved without changes in the use of the territory. In some 24% of the model area, the culture of arable land has not changed over the time series at all. On the other hand, this arable land is currently concentrated into large blocks of several dozen or even hundreds of hectares. This arable land used to be formed

into small fields of much smaller size in the past. Arable land is the most extensive category in all-time series. The area of permanent grassland and forest has been significantly reduced. Due to the construction of the "Nové Mlýny" water reservoirs, the ecologically stable areas have been largely lost.

ACKNOWLEDGEMENTS

This study was supported by the Internal Grand Agency Faculty of AgriSciences MENDELU No. AF-IGA-IP-04/2017 "Degradation of agricultural land resources in selected areas in the South Moravian region" and research project MZE RO0217.

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GIS ANALYSIS OF POTENTIAL LOCATIONS FOR RAIN GARDENS IN VILLAGE ALEKŠINCE

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Abstract: Climate change leads to the creation of landscape measures for its stabilization. One of the steps of sustainable land-use is the measures aimed at processing rainwater in settlements. Rain gardens represent a way of storm water treatment at settlement level. It is necessary to follow several rules – for proper functioning which include natural conditions and site parameters. The aim of study was the establishment of methodology for determining the suitable locations for rain gardens, in application to settlement – village Alekšince. Selection of potential sites is based on the attributes of soil properties, slope, land use, ownership and ground water level in the given area, that are processed in GIS software QGIS. The methodology used is based on implemented methodologies of last years. The result is raster map which shows the suitability of village location for rain garden based on the spatial analysis with the use of overlaying of the value raster of the mentioned attributes.

Key Words: rain garden, GIS analysis, water management, stormwater management

INTRODUCTION

Water management in landscape requires various approaches in accordance to land use (Halaj et al. 2012, Muchová et al. 2016). Evaporation rates may be affected in various ways in the real conditions (Zarzycki et al. 2015, Żarnowiec et al. 2016). The planting helps to eliminate and treat the storm water (Stiffler 2013) as the water quality may be threatened nowadays (Bedla and Misztal 2014, Policht-Latawiec et al. 2015). Tóth et al. (2014) states water as an important part of green infrastructure.

Rain gardens (RG), as an important storm water management practice, are common tool in landscape creation of public spaces. Their ecological characteristics include collection, processing, infiltration, transpiration and filtration of storm water, which are increased by strong economic aspects because of its relatively low-cost demand and final money savings. These low terrain depressions planted with perennials, shrubs and small trees are notable aesthetic and landscape element used by landscape architects worldwide. Soils in urban areas have significantly altered the natural structure and impaired functionality that greatly extends the time of infiltration of storm water. Increasing input of water to the sewerage system may result in flood conditions.

The study is aimed at determining the methodology for selection of suitable site for RG based on natural conditions and conditions of the current state of locations, in application to settlement – village Alekšince.

MATERIAL AND METHODS

Materials for spatial analysis

Data processing and spatial analysis was performed in software QGIS v. 2.12.0. and used materials below.

- WMS servers: Map of parcels and municipality borders, Map of soil units, Base map of Slovakia (contours),
- WFS servers: map of administrative units (boundaries of cadastres),

- Web GIS pages: Soil maps of Slovakia, Map of dominant soil units, Map of underground water level, Map of village parcels in the village with the orthophotomap layer,
- Parcel ownership identification: Ownership of the village parcels (community/private land),
- Spatial planning documentation,
- Map of underground water levels.

Spatial analysis methodology

Analysis of the spatial and ecological parameters of the land in the studied area was based on work of authors - McCormack (2015), Rokus (2005) and Marney (2012). They focused on various input parameters affecting the suitability of the location for RG (Table 1–3). Each author assigned values (on the scale 1–5 or 1–3) to selected parameters, multiplied them by a weighting coefficient. Studied areas were divided into cells – depending on used methodology were recalculated and resulted in raster map of suitable localities for RG.

Marney's (2012) methodology used values on scale 1–3 for selected parameters of partial and land elements (Table 3) – soil, slope of area, depth of the ground water, proximity to structures (buildings, roads), ownership and land use. For final calculation of suitable RG locality, he used formula: $Y_{DZ} = \sum_{i1=}^{N}(W \times C_i)$, (1), where YDZ is the final suitability of the site for RG, W is the weight of the parameter, Ci is value of the particular parameter (Rokus 2005).

Table 1 The input parameters for analysis to determine a suitable location for RG by McCormack (2015)

Parameter	Coefficient	Parameter value				
		1	2	3	4	5
Hydrological soils group	25%	N/A	D - very high absorption	C - high absorption	B - slow absorption	A - very slow absorption
Minimal soil depth	35%	0–14 inch	-	20 inch	-	20–80 inch
Slope	25%	12–700%	9–12%	6–9%	3–6%	0–3%
Exposure to the cardinal points	10%	0–90°, 270–360°	90–112.5°, 247.5–270°	112.5–135°, 225–247.5°	135–157.5°, 202.5–225°	157.5–225.5° (south)
Use of area	5%	-	-	N/A	-	road, housing, commercial

Table 2 The input parameters for analysis to determine a suitable location for RG by Rokus (2005)

Parameter	Coefficient	Parameter value		
		1	2	3
% of impervious surface	1/3	designed using complex calculations from the number of parameters		
Soil permeability	1/3	designed using complex calculations from the number of parameters		
Slope	1/3	0–18%	18–25%	> 25%

Table 3 The input parameters for analysis to determine a suitable location for RG by Marney (2012)

Parameter	Coefficient	Parameter value		
		1	2	3
Soil	0.25	loam, clay-loam	loamy, loamy-sand	sandy
Slope	0.25	0–1.9%	5–8%	2–4.9%
Depth to ground water	0.10	0–1.9 ft	2–4.9 ft	>5 ft
Proximity of structures	0.15	0–9.9 ft	10–14 ft	>15 ft
Public/private	0.15	private	-	public
Use of area	0.10	housing	industry	commercial

The parameters used in the analysis process

According to available data, we focused on the categories, which are directly involved in the process of proper functionality of RG in the surrounding village Alekšince. Categories were then assigned the values, which were adjusted with the coefficient by relevance of given parameters in the process of RG creation (Table 4).

Table 4 Input parameters for creating GIS analysis to determine a location for RG

Parameter	Coefficient	Parameter value		
		1	2	3
Soil	0.25	light soils	medium heavy and moderate soils	very heavy and heavy soils
Slope	0.25	2–4.9%	5–8%	0–1.9%
Ownership of parcels	0.20	owned by the municipality	co-ownership	private property
Use of area	0.20	public greenery	private greenery	industry
Depth to ground water	0.10	more than 2.00 m	2.00–1.00 m	less than 1.00 m

Process of analysis

Workflow analysis to the development and subsequent assessment in the form of map output included following steps:

• vectorization of village boundaries and parcels with the use of cadastral map and Land Register portal map, assigning the parameter values,

• vectorization of soil types boundaries in cadaster using maps and Guide for Valuated Soil – Ecological Units (Linkeš et al. 1996), assigning the parameter values for categories of soils in accordance to the main unit of soil and soil granularity classifier,

• vectorization of contour lines, assigning the parameter values for slope categories, creation of a digital terrain model (DTM) created from the nodes extracted from the contours of the area,

• vectorization of ground water levels using Map of underground water levels, assigning the parameter values for categories of water depths levels,

• vectorization of buildings and paved surfaces using cadastral maps, orthophoto maps and Land Registry portal maps,

• vectorization of land use zones using a land use plan and a field survey, assigning the parameter values of the land use.

RESULTS AND DISCUSSION

Result maps that show the values of particular parameters within the boundaries of Alekšince were developed by processing the input data according to the methodological model. There were created 9 maps that were combined to create the result map of suitability of sites for RG placement.

Soil type and its characteristics were considered as one of the most important parameters in RG construction process. Using Valuated Soil - Ecological Units maps and Soil map of Slovakia, different soil types in area were plotted (Figure 1). According to the analysis, it can be concluded that the territory is covered with two types of soils – brown modals and black earth soils which predominate. Using Map of soil types (Figure 1) and converting it into three soil categories based on process model – light soils, medium and heavy soils with their associated values, we obtained the Map of soil categories (Figure 2). The map shows there were located just medium heavy and moderate soils. We can state that soils in studied area are suitable for RG.

Digital elevation model was created after vectorization of contours and extraction of their nodes (Figure 3) using TIN interpolation. The model shows that the urban area is sloping downward to the central part (around water streams area). Total elevation difference is approximately 56 m. The highest parts are located in most south village area called Lahne. Digital elevation model was used to create the map of slope, which was afterwards categorized and reclassified to model values (Table 4). Sites, where the slope was exceeding 8%, were considered as unsuitable for RG placement.

Map of ownership within the boundaries of the village Alekšince (Figure 5) was created with the use of ownership parameter. Map of land use (Figure 6) displays the area divided into 3 categories - public greenery - the most suitable areas for RG, private green areas (housing gardens, vineyards, meadows and permanent grassland) and industrial sites. Industrial zones were considered as impropriate for RG because of high proportion of the paved surface and special business. There were also areas totally excluded from analysis process – forest area, water streams zones, area of cemetery and areas in Lahne, which are zones of building process and it is not possible define the final state.

Map of ground water levels (Figure 7) describes division of area into two underground water zones. Both of them are suitable for RG placement. Main area is situated in water depth of 2 m and more. For more detailed elaboration it would be suitable to perform local measurements.

Zones excluded from the possible locations for RG (Figure 8) are paved areas - asphalt roads and buildings, these layers were subsequently merged into a single layer - areas excluded from the analysis (Figure 9). The roof area of the buildings was extended with a buffer distance of 3 m; roads were extended with 1 m buffer. It is generally considered inappropriate to install RG

beyond this limit, as they could lead to waterlogging of subsoil and construction disturbances of structures of buildings.

Raster map of suitability was reclasificated to contain value 0–1 = 1; 1–2 = 2; 2–3 = 3, after that it was vectorizated to create a set of polygons with correspondent category of suitability. Value of area size was calculated for each category in MS Excel environment (Table 5).

The result of this study consists of raster map (Figure 10) which shows division of area to three categories in respect to their suitability to RG placement. There were not areas with perfect suitability, the best areas for RG are represented with value 1.25, red areas with value 2.55 the less suitable.

Table 5 Size of areas suitable for RG placement in village Alekšince

Value of area	Area size [m^2]
1.25	10028
1.575	32364
1.9	204292
2.225	203456
2.55	82096
Sum of area size	532236

Based on the present methodology and implemented process of analysis can be summarized than the application of procedure in conditions of municipalities can help to identify suitable locations for RG placement.

In territory of Alekšince, there were localities suitable for RG placement depending on local conditions. Specific value of the parameter and its suitability must be supported with further research in order to create more detailed categorization suitability. The given fact is related to verification of individual methodologies McCormack (2015) and Marney (2012) in the field. Also, it is appropriate to apply a specific field measurements in selected localities focused mainly on soil conditions. Linking the issue with practice it is based on the verification of results - creation of demonstrative RG.

Figure 1 Soil types in Alekšince cadastral area

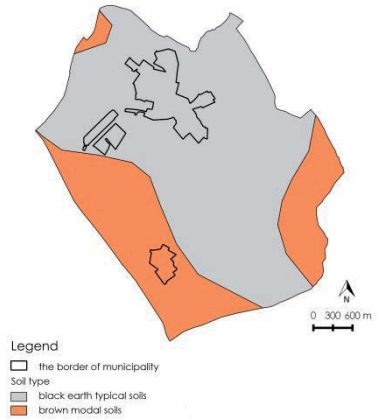


Figure 2 Map of soil categories

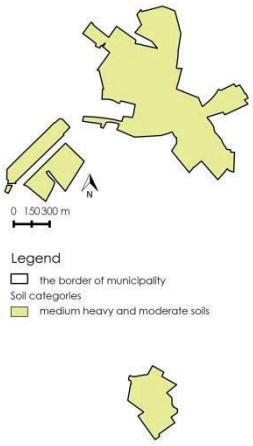


Figure 3 Digital elevation model

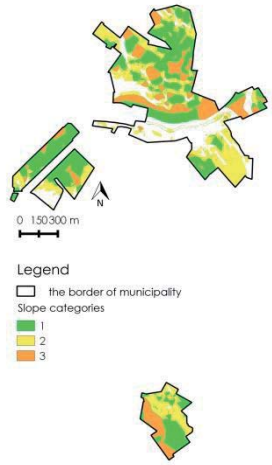


Figure 4 Map of slope categories

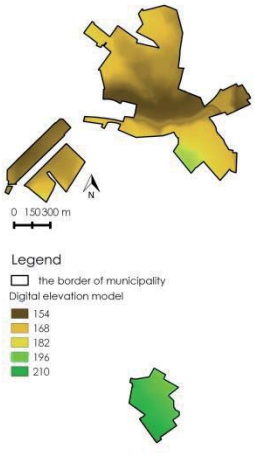


Figure 5 Map of ownership of parcels

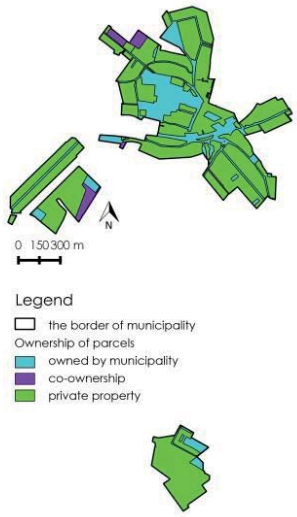


Figure 6 Map of land use

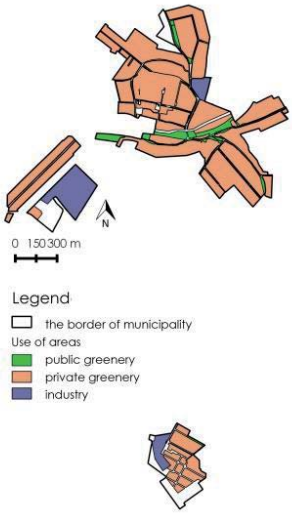


Figure 7 Depth to ground water level

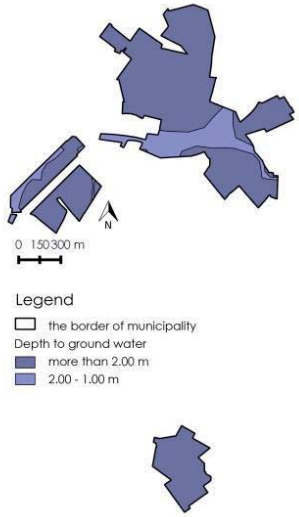


Figure 8 Map of paved area

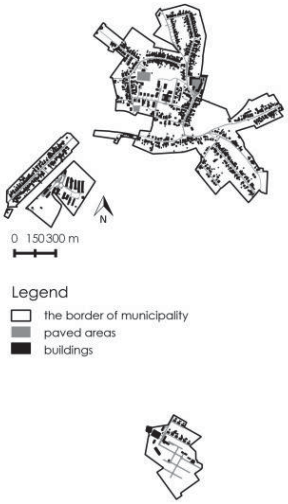


Figure 9 Areas excluded from the analysis

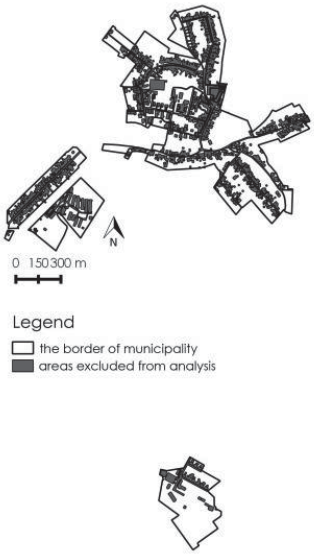
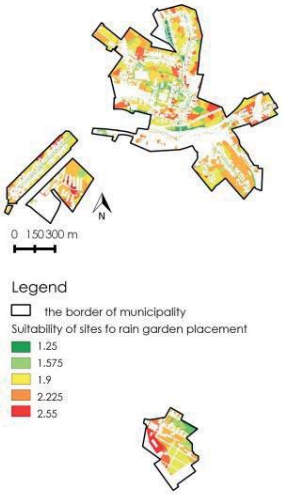


Figure 10 Map of the site suitability for RG design within the boundaries of the village Alekšince



CONCLUSION

This paper focused to propose a solution of storm water management in public spaces of smaller settlement of the Nitra region - the village Alekšince through rain gardens. Solution aims to establish a methodology for identification of the suitable locations for RG creation after analysing the conditions of each locality. The methodology sets the values of necessary parameters for evaluation of suitability of the areas, the weight value, and impact on the overall suitability of the sites.

ACKNOWLEDGEMENT

This study was supported with the grants and projects - APVV-15-0562: Effective irrigation management as a device of changing climate and APVV-16-0278: Use of hydromelioration structures for mitigation of the negative extreme hydrological phenomena effects and their impacts on the quality of water bodies in agricultural landscapes.

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USE OF HEMP (*CANNABIS SATIVA* L.) IN MANAGEMENT OF LANDFILL LEACHATE: PRELIMINARY ANALYSIS AND REACTION ON LEACHATE IRRIGATIONS

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Abstract: Municipal solid waste (MSW) landfills constitute one of the largest sources of anthropogenic pollutions. Landfill sites act as biological reactors, in which waste undergoes physical, chemical and biological transformation. Significant problem associated to landfills is the production of leachates. Leachate is an emergent source of pollutants. Leachate handling typically involves treatment either on site or at a wastewater treatment plants but phytoremediation, using plants is cost-effective and sustainable remediation strategies for removing or detoxifying contaminants. The present study is a part of a larger project on assessing the potential of hemp (*Cannabis sativa* L.) for phytoremediation of pollutants (e.g. heavy metals) from leachate. The principal aim of this paper is to describe preliminary analysis and reaction of hemp on leachate irrigations in a real condition experiment. For the experiment were chosen two varieties of *Cannabis sativa* L., Bialobrzeskie and Monoica. During the measurement, the Bialobrzeskie varieties watered with rainwater were 26% taller on average, and the Monoica varieties were up to 34% taller than plants watered with leachate. The leachate does not stimulate plant growth, which is why the growth was much smaller.

Key words: municipal solid waste, *Cannabis sativa* L., heavy metals, remediation strategies

INTRODUCTION

As people's quality of life has improved, the volume of municipal solid waste generated has increased concurrently. A large amount of MSW is generated everyday around the world (Vaverková et al. 2017a, Voběrková et al. 2017) and landfills are still the most prevalent waste disposal method. MSW landfills constitute one of the largest sources of anthropogenic pollutions (Vaverková and Adamcová 2014, Gworek et al. 2015, Koda et al. 2015, Adamcová et al. 2016a, Koda et al. 2017, Adamcová et al. 2017, Vaverková et al. 2017a, Rong et al. 2017, Wang et al. 2017). MSW (without significant recycling activity) predominantly includes food wastes, market wastes, yard wastes, plastic containers and product packaging materials, and other miscellaneous solid wastes from residential, commercial, institutional, and industrial sources (Rong et al. 2017). Landfill sites act

as biological reactors, in which refuse undergoes physical, chemical and biological transformation (Samadder et al. 2017).

The environmental impacts of the many existing landfills cannot be ignored. Many studies have proved that landfill leachate is a significant source of pollutants as a consequence of the leaching of hazardous substances (Li at al. 2012, Melnyk et al. 2014, Gworek et al. 2015, Adamcová et al. 2016a, Koda et al. 2017, Rong et al. 2017, Samadder et al. 2017). Landfill leachates are complex; heavy metal components are undoubtedly the most harmful because of their persistence and toxicity (Aronsson et al. 2010, Rong et al. 2017, Samadder et al. 2017). Control of heavy metals in leachates has therefore become a focus of landfill management. Moreover, the quantity and quality of leachate is primarily influenced by the amount, waste composition and its solubility, moisture content of the solid waste, as well as by local factors such as hydrogeological conditions, climate, and height and type of landfill (Koda et al. 2015, Samadder et al. 2017). Landfill leachate is generally a dark coloured liquid, with a strong smell, which carries a high organic and inorganic load (Yao 2017). Generally, landfill leachate contains substantial amounts of dissolved organics [(biochemical oxygen demand (BOD5) and chemical oxygen demand (COD)], inorganic salts, ammonia, heavy metals and xenobiotic organic compounds (XOCs) that are originated from personal care products, pharmaceuticals, industrial and household chemicals (Ghosh et al. 2017).

Leachate handling typically involves treatment either on-site or at a wastewater treatment plants (Koda et al. 2013, Vaverková et al. 2017b) but cost-effective and sustainable remediation strategies for removing or detoxifying contaminants in landfill leachate are urgently needed. Phytoremediation, using plants to eliminate leachate contaminants, is a cost-effective, reliable, and promising technology. Phytoremediation covers a wide range of pollutants like inorganic chemicals including heavy metals and metalloids, many organic substances including persistent organic pollutants and radioactive elements (Meagher 2000, Radziemska et al. 2013, Pandey et al. 2016, Yao 2017). In this case, plants grow on polluted medium, extract the toxic substances and accumulate them in the upper parts of the plant (Radziemska et al. 2017). They are then harvested, and consequently the medium is cleaned up (Ligner et al. 2002).

The present study is a part of a larger project on assessing the potential of hemp for phytoremediation of heavy metals from landfill leachate (Vaverková et al. 2017b). The plant we chose for our approach was hemp (*Cannabis sativa* L.). Hemp is one of the world's oldest cultivated annual crops (Salentijn et al. 2015). Hemp was found out to be effective in removing metals under model conditions (Vaverková et al. 2017b) but its evaluation in field conditions is missing. Therefore, the principal aim of this paper is to describe preliminary analysis and reaction of hemp (*Cannabis sativa* L.) on landfill leachate irrigations in a real condition experiment.

MATERIALS AND METHODS

Site description

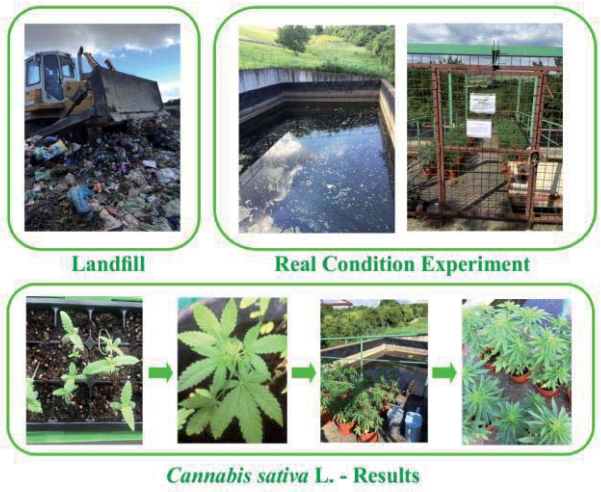
Zdounky-Kuchyňky landfill (49.2490778N, 17.3121181E) has been in operation as a disposal facility, permitted to receive commercial and MSW, since 1996. This site is located in a triangular space delimited by main roads connecting the villages of Zdounky, Nětčice and Troubky-Zdislavice (Figure 1) (Adamcová et al. 2016b, Voběrková et al. 2017). The site is based on cultivated soil, natural clay and semi-waterproof clay layer. Before the operation of the landfill site, the clay layer was pressed tightly, usually 1 ton/m^3 in density, to prevent leachate movement. The dredge tubes were installed for collecting the leaching liquid, which was discharged out into the leachate collection pond. Landfill anatomy include several protective layers, such as: (1) prepared subgrade, (2) compact clay layer, (3) geomembranes of high-density polyethylene (HDPE), (4) leachate collection pipe system, (5) filter geotextile, (6) leachate collection layer. Each of these parts is designed to address specific problems that are encountered in a landfill.

Figure 1 Location of Zdounky-Kuchyňky landfill and surrounding region

Setup of the experiment

Two monoecious varieties of industrial hemp were chosen for the experiment – Bialobrzeskie, a Polish variety registered in 1968 and Monoica, a Hungarian variety registered in 2006 (Bjelková 2011). Seeds provided by Agritec Plant Research Institute Ltd., Šumperk, Czech Republic (CR) were sown into trays with common horticultural substrate from AGRO CS Inc and left to germinate. After two weeks of regular watering in the greenhouse of Mendel University in Brno, newly grown seedlings (10–15 cm) were transported into 10-liter plastic pots with the same substrate. Four plants were put into each pot. There were 18 pots for each of four experimental groups created. Bialobrzeskie irrigated with landfill leachate and irrigated with rainwater, Monoica irrigated with leachate and with rainwater. All pots were labeled to be distinguished for the future measurements (Figure 2).

Figure 2 Experiment set up in a real condition

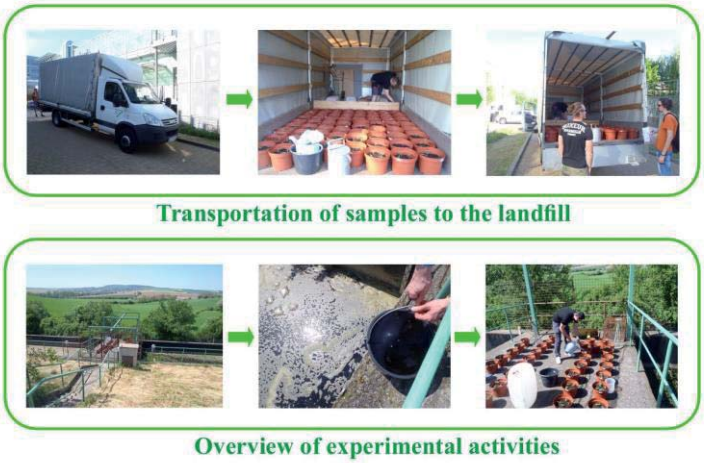


On 18 May 2017, the plant seedlings were transported to the Zdounky-Kuchyňky MSW landfill. All the plants were placed on the paved concrete part of the leachate pond (Figure 3). The landfill leachate pond area is secured against the intrusion of unauthorized persons. This area has been marked with an information sign about the experiment.

The plant pots were evenly distributed over the paved surface so as to avoid confusion when watering. *Cannabis sativa* L., in Monoica and Bialobrzeskie varieties intended for watering with contaminated leachate were placed on the left site, and the same plants intended for watering

with rainwater were placed on the right site. The plants were watered with 2 liters of the required water regularly once a week. Depending on the weather, in order to prevent the substrate from drying and the plants from wilting, the plants were watered with extra rainwater. During the experiment, drainage plates were added to the pots to contain the water and prevent its deficiency. In the case of pots intended for watering with rainwater, the problem was that the substrate dried quickly and the weight of the pots was too low. In unfavorable and windy weather, the plant pots would fall, be blown away and fall into the seepage water basin. For this reason, certain safety measures were implemented. More substrate was added to each pot, a supporting rod was added, and the plants were tied to the rod. The pots were weighed down with stones and the rail around the paved part of the basin was equipped with a protective net.

Figure 3 Overview of the experiment activities

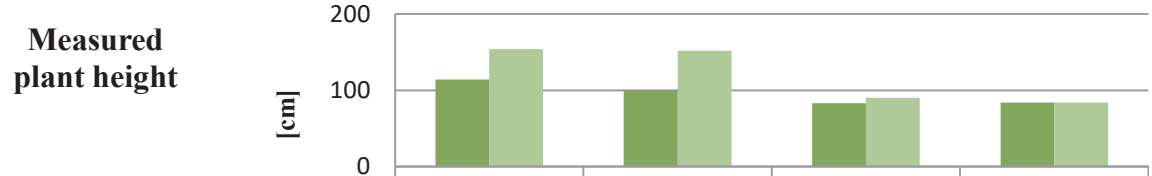


RESULTS AND DISCUSSION

Plant height measurement

Double measurements of the highest plant in each pot were gradually performed, and the data was recorded in the recording sheet. Their relationship is graphically depicted in Figure 4. The pot that irreversibly fell into the basin was not included in the calculation of the average height. On the other hand, dead samples were also included, where the recorded height was zero. The experiment is still ongoing, and the samples will be further modified and prepared for additional procedures and analyses.

Figure 4 Measured plant height



	Bialobrzeskie - RW	Monoica - RW	Bialobrzeskie - L	Monoica - L
1. measurement - average height	114	100	83	84
2. measurement - average height	154	152	90	84

Legend: RW – rain water, L – leachate

Landfill leachate toxicological evaluation is essential to examine the impact of leachate potentially discharged to the environment (Ghosh 2017, Vaverková et al. 2017b). Several studies highlight that leachate can induce both positive and negative responses in the plants (Mor et al. 2013, Suliasih et al. 2010, Vaverková et al. 2017b). Our results indicate that leachate can severely inhibit plant growth. The toxic effect of leachate on plants depends on several factors including the plant species itself (Suliasih et al. 2010, Vaverková et al. 2017b). The tested varieties differed in their response to the leachate treatments. As it can be seen on Figure 4 variety Monoica seems to be more sensitive

to leachate irrigation compering to variety Bialobrzeskie. In regard to the phytotoxicity assessment of leachate a number of studies conducted on *Cannabis sativa* L. revealed that it can accumulate a considerable amount of heavy metals making it a good candidate for remediation (Vaverková et al. 2017b).

CONCLUSION

In order to approximate real conditions, the samples of *Cannabis sativa* L., were placed in pots in the landfill site. According to the methodology, the pots were irrigated weekly with two liters of rain or leachate water in each pot, and sometimes with additional rainwater depending on the weather conditions. Double measurements of the plant heights were conducted over a two-and-a-half-month period, and the results were plotted graphically. The values indicate that the leachate inhibits the growth of the Bialobrzeskie and Monoica varieties of *Cannabis sativa* L., in comparison with the rainwater. In addition to the smaller height of the plants, several samples died. During the second measurement, the Bialobrzeskie varieties watered with rainwater were 26% taller on average, and the Monoica varieties were up to 34% taller. The leachate does not stimulate plant growth, which is why the growth was much smaller. The calculation also included samples that died between the first and second measurement. For this reason, the average growth values were lower. In the Bialobrzeskie variety, the plant height increased by 8%. Due to the death of Monoica samples, the average percentage of the plant height did not change. Leachate contains pollutants, e.g. heavy metals and other contaminants that are an environmental burden. It is for this reason that they inhibit the growth of the plant samples. The samples (obtained biomass) will be subjected to further procedures and analyses in subsequent stages of the experiment, where the content of harmful substances accumulated in the plant will be determined.

ACKNOWLEDGMENTS

The research was financially supported by the IGA FA MENDELU No. TP 5/2017.

We would like to express our great appreciation to the management of the landfill DEPOZ, Ltd. Namely, we are very grateful to Ing. Ivan Mohler and his colleagues for their assistance and their willingness to provide their time so generously.

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FOOD TECHNOLOGY

THE OPTIMIZATION OF METHODS FOR PHENOLIC COMPOUNDS DETERMINATION IN ELDERBERRY (*SAMBUCUS NIGRA* L.)

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Abstract: Phenolic substances belong to the most important secondary metabolites of plants; they have high antioxidant activity useful for the prevention of cardiovascular diseases, bacterial growth or cancer. Phenolic acids and flavonoids obtained by the means of different extraction methods were studied in elderberry flowers, namely chlorogenic acid, caffeic acid, rutin, hyperoside, isoquercetin, quercetin and kaempferol. The plants were extracted in order to obtain three types of phenolic compounds: free, bound and esterified ones. The compounds of interest were analyzed by the means of UHPLC liquid chromatograph with a PDA detector at the Research Institute of Brewing and Malting, PLC. The obtained results indicate that the extraction of free phenolic compounds seems to be the best method for obtaining phenolic substances. Following phenolic substances are used in the food industry: chlorogenic acid, rutin, hyperoside, isoquercetin, quercetin and kaempferol.

Key Words: phenolic compounds, phenolic acids, flavonoids, elderberry flowers

INTRODUCTION

Phenolic acids - also called “benzoic acids”, because they are derived from benzoic acid - make up about one-third of the polyphenols in human diet. Thanks to the presence of aromatic phenolic cycle that stabilize and delocalize unpaired electrons in the aromatic ring, these acids have significant antioxidant properties. They are covalently bound to polymer cell wall (Gallardo et al. 2006, Fardet et al. 2008, Amaki and Saito 2011). Phenolic acids are effective against the oxidation of LDL cholesterol in the organism, they prevent spreading cancer cells by complexation with carcinogens and thus preventing their entry into the DNA and creating a mutation during transcription (Havlík and Marounek 2013, Volf and Andrs 2008). For their general action in a human body, flavonoids are often referred to as “bioflavonoids”. In plants, these compounds are located especially in glycoside-bound form or dissolved in the cytoplasm in vacuoles. They are supposed to be involved in redox processes in organism (Velíšek and Hajšlová 2009, Kovács 2016).

Elderberry is a deciduous bush or a smaller tree with a thin trunk growing up to a height of 9 meters. Flower - *Flos sambuci* and fruit - *Fructus sambuci* are the parts that are collected. The most important use of the flower - *Flos sambuci* - is as a diaphoretic or diuretic. It is used against all respiratory diseases, colds, coughs and angina. It dissolves the mucus, calms the mucous membranes and promotes expectoration. It is a universal remedy for neuralgias, it drains excess of uric acid from the body and is used as a treatment of the digestive tract diseases, as well as against kidney or bladder diseases. Thanks to the rutin content, elderberry has also a beneficial effect on the elasticity and strength of blood vessels. It regulates blood pressure and is also used in dermatology for bangades against skin diseases. Elderberry gargle is used for inflammation of gums and tonsils. (Jirásek and Starý 1986, Kresánek and Krejča 1988, Janča and Zentrich 1994). Elderberry can reduce the risk of diabetes by increasing the antioxidant protection of the body, by reducing the concentration of fibrinogen and lipid oxidation processes. Thanks to polyphenolic compounds such as kaempferol,

caffeic acid or naringenin, elderberry can also increase the metabolism of muscle glucose uptake and insulin secretion (Sidor and Gramza-Michalowska 2015).

Elderberry blossoms contain almost exclusively flavonols - quercetin glycosides (1.5–3%), especially rutin (up to 1.9%), quercetin, isoquercetin, hyperoside, naringenin and astragalin. The essential oil (0.03–0.15%) has high content of free fatty acids. It contains also chlorogenic acid (3%), *p*-coumaric acid, caffeic acid, ferulic acid and their beta-glucose esters, traces of sambunigrin, esterified triterpenes, triterpenic acids, mucus, tannins, sterols, beta-sitosterol, campesterol and stigmasterol, as well as tannins, mucilagens, pectin and sugars (glucose and fructose) (Tomko et al 1999, Bodlák 2000).

MATERIAL AND METHODS

Phenolic substances were monitored in elderberry (*Sambucus nigra* L.) obtained at the Field Experimental Station AF MENDELU in Žabčice. Extraction of free phenolic substances from the flowers was performed, as well as acid and alkaline hydrolysis. The determination of phenolic compounds content was carried out according to a modified method by Krygier et al. (1982), originally designed for the isolation of phenolic acids from oilseeds. The method has been described in detail in the paper by Karabín et al. (2013).

The homogenized drug was quantitatively transferred into a plastic centrifuge tube, 5 ml of hexane was added and the sample was shaken for one minute. The resulting suspension was processed on a centrifuge to separate the excess hexane from the sample; it was removed by the means of Pasteur pipette. The samples were dried overnight and the next day they were prepared for extraction via multi-step hydrolysis. The first step was the extraction of free phenolic substances, which allows to obtain biologically available phenolic compounds contained in the plants. The dried sample was extracted with 70% acetone; the extraction were repeated three times until the majority of the free phenolic substances were washed from the sample. The resulting suspension was separated in a centrifuge and the aqueous phase was transferred to a centrifuge tube after pH adjustment. The extracted solid was dried and used later for alkaline hydrolysis. The aqueous phase was acidified to pH 2 with hydrochloric acid and extracted with diethyl ether/ethyl acetate (1:1 w/w). The extraction was carried out 3 times with this solvent mixture. Collected organic layer was evaporated to dryness in a pear-shaped flask in a rotation vacuum evaporator. The residue was dissolved in 1 ml of 20% methanol and passed through a nylon filter (porosity 0.2 μm) into prepared and labeled vial.

Alkaline hydrolysis

Alkaline hydrolysis was performed as follows. The aqueous and solid fractions obtained in the preparation of first extraction step were quantitatively transferred into a plastic centrifuge tubes and 10 ml of 4 M NaOH containing EDTA and ascorbic acid was added. The mixture was shaken for two hours. After hydrolysis, pH of the samples was adjusted to 2 with hydrochloric acid. The extraction with the mixture with diethyl ether and ethyl acetate (1:1, w/w) was preformed. Extraction was carried out 3 times with this a mixture of solvents. The organic layer was removed, transferred to a pear-shaped flask and evaporated to dryness in a vacuum evaporator. The residue was dissolved in 1 ml of 20% methanol and passed through a nylon filter (porosity 0.2 μm) into prepared and labeled vial. The aqueous phase obtained in this extraction step was subjected to acidic hydrolysis.

Acid hydrolysis

Acid hydrolysis was performed as follows. To the solid and aqueous fractions obtained after the alkaline hydrolysis, 10 ml of 1 M hydrochloric acid was added. The aqueous phase was transferred to a 50 ml plastic centrifuge tube and 10 ml of 1 M hydrochloric acid were added. The opened tube was placed in a 70 °C water bath and kept at constant temperature for 45 minutes. After that the tube was cooled to room temperature and extracted for 3 times with a mixture of diethyl ether and ethyl acetate (1:1, w/w). Separated organic layer was transferred to a pear-shaped flask and evaporated to dryness in a vacuum evaporator. The residue was dissolved in 1 ml of 20% methanol and passed through a nylon filter (porosity 0.2 μm) into prepared and labeled vial. The samples were prepared in four repetitions, stored in vials and then measured by the means of a Waters Acquity Ultra

High Performance Liquid Chromatograph with a PDA detector at 250 or 300 nm wavelengths, according to the analyte type.

RESULTS AND DISCUSSION

The variance analysis of the data obtained for elderberry samples harvested in the years 2015 and 2016 clearly indicates that the vintage statistically very significantly influenced the content of all investigated phenolic compounds. The extraction method also statistically very significantly affected all monitored phenolic compounds. The interaction of both factors statistically very significantly influenced all phenolic substances of interest.

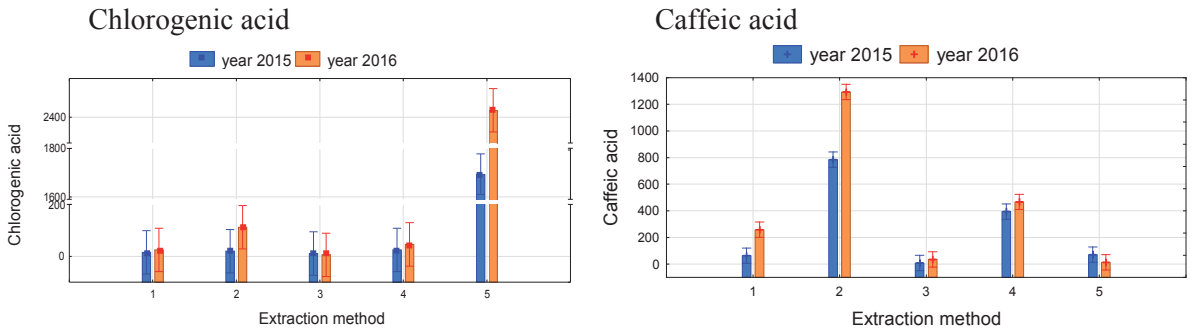
Table 1 The average content [mg/kg] of selected phenolic compounds in elderberry samples obtained by different extraction methods in the investigated years 2015 and 2016

Year	Extraction method		Chlorogenic acid	Caffeic acid	Rutin	Hyperoside
2015	Acid hydrolysis of solid fraction	1	15.11 a	63.34 a	55.52 a	8.56 b
	Alkaline hydrolysis of solid fraction	2	19.92 a	784.84 d	106.27 ab	43.60 d
	Acid hydrolysis of liquid fraction	3	10.63 a	8.49 a	98.86 ab	9.62 b
	Alkaline hydrolysis of liquid fraction	4	24.95 a	395.03 c	170.87 bc	16.99 c
	Free phenolic compouds	5	1693.28 b	70.88 a	548.36 d	9.70 b
2016	Acid hydrolysis of solid fraction	1	24.91 a	258.69 b	51.65 a	1.57 a
	Alkaline hydrolysis of solid fraction	2	112.35 a	1293.05 e	103.40 ab	1.80 a
	Acid hydrolysis of liquid fraction	3	5.93 a	35.37 a	83.66 ab	1.55 a
	Alkaline hydrolysis of liquid fraction	4	45.76 a	467.19 c	249.20 c	0.98 a
	Free phenolic compouds	5	2426.94 c	13.43 a	1282.38 e	8.33 b

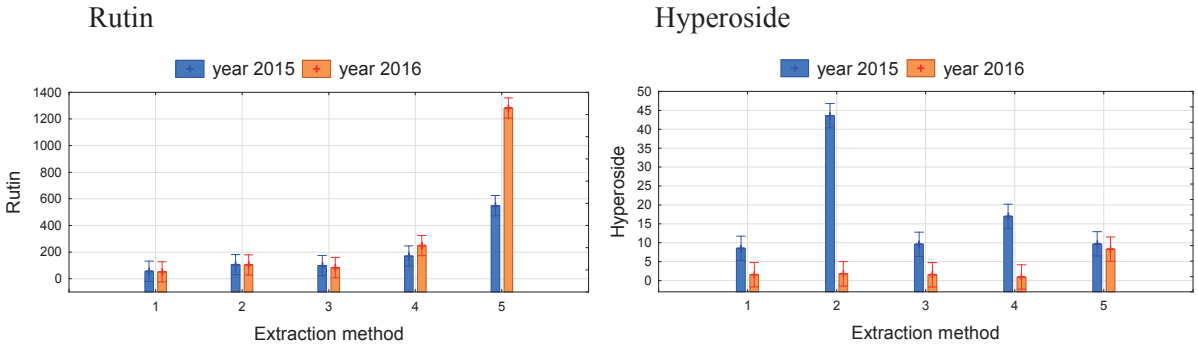
Year	Extraction method		Isoquercetin	Quercetin	Kaempferol
2015	Acid hydrolysis of solid fraction	1	34.55 ab	15.0 f	5.73 d
	Alkaline hydrolysis of solid fraction	2	88.69 c	8.16 cde	5.79 d
	Acid hydrolysis of liquid fraction	3	16.17	8.95e	5.79d
	Alkaline hydrolysis of liquid fraction	4	56.32	6.89bc	5.80d
	Free phenolic compouds	5	138.43	8.41de	5.84d
2016	Acid hydrolysis of solid fraction	1	69.23 c	2.60a	3.05 c
	Alkaline hydrolysis of solid fraction	2	339.08 e	5.89b	2.49 b
	Acid hydrolysis of liquid fraction	3	21.68 a	5.72b	2.21 ab
	Alkaline hydrolysis of liquid fraction	4	88.71 c	1.44a	2.99 c
	Free phenolic compouds	5	336.48 e	7.54cd	1.96 a

The following biologically active substances: chlorogenic acid, caffeic acid, rutin, hyperoside, isoquercetin, quercetin and kaempferol have been monitored in elderberry (*Sambucus nigra* L.). Several of these compounds are used in the food industry: chlorogenic acid, rutin, hyperoside, isoquercetin, quercetin and kaempferol. Caffeic acid is only available in low concentrations by the extraction of free phenolic substances.

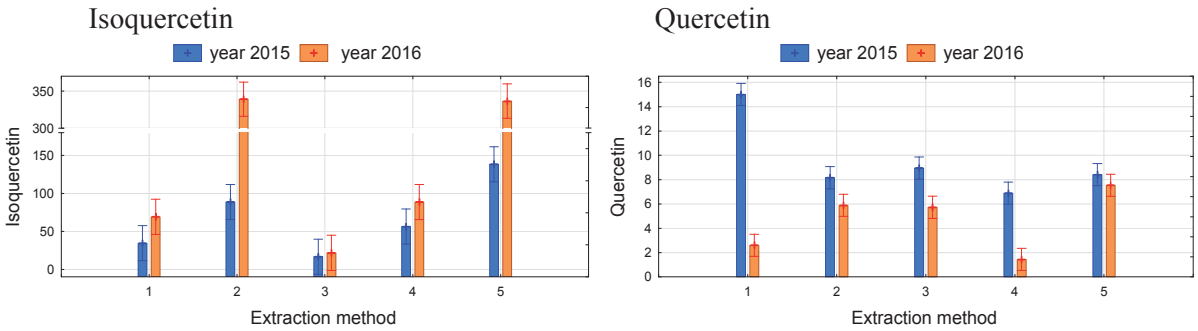
Figure 1 The average content of bioactive compounds in elderberry samples obtained by different extraction methods in the investigated years 2015 and 2016



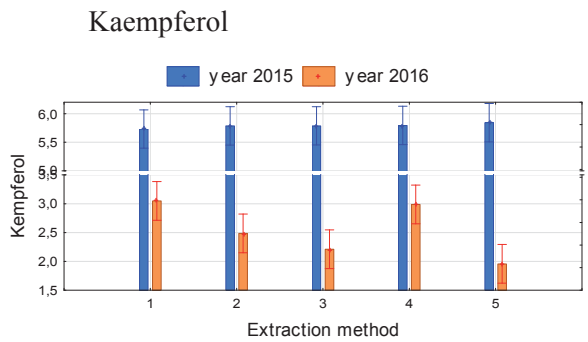
Legend: 1. Acid hydrolysis of solid fraction, 2. Alkaline hydrolysis of solid fraction, 3. Acid hydrolysis of liquid fraction, 4. Alkaline hydrolysis of liquid fraction, 5. Free phenolic compounds



Legend: 1. Acid hydrolysis of solid fraction, 2. Alkaline hydrolysis of solid fraction , 3. Acid hydrolysis of liquid fraction, 4. Alkaline hydrolysis of liquid fraction, 5. Free phenolic compouds



Legend: 1. Acid hydrolysis of solid fraction, 2. Alkaline hydrolysis of solid fraction, 3. Acid hydrolysis of liquid fraction, 4. Alkaline hydrolysis of liquid fraction, 5. Free phenolic compouds



Note: 1. Acid hydrolysis of solid fraction,, 2. Alkaline hydrolysis of solid fraction, 3. Acid hydrolysis of liquid fraction, 4. Alkaline hydrolysis of liquid fraction, 5. Free phenolic compounds

Chlorogenic acid

As the Figure 1 shows, the content of chlorogenic acid obtained by the extraction of free phenolic substances was the statistically most significant. The amounts of 2426.94 mg/kg and 1693.28 mg/kg were found in the years 2016 and 2015, resp.

Caffeic acid

By means of the alkaline hydrolysis of the solid fraction, the statistically significantly highest content of caffeic acid was gained, 1293.05 mg/kg and 784.84 mg/kg in the years 2016 and 2015, resp. In both monitored years the content of caffeic acid obtained by alkaline hydrolysis of the liquid fraction was also statistically significant; the content of caffeic acid did not change fundamentally during those years.

Rutin

The highest content of rutin was obtained using the statistically most significant method – the extraction of free phenolic compounds. The amounts of 1282.38 mg/kg and 548.36 mg/kg were found in the years 2016 and 2015, resp. Other methods were statistically insignificant.

The most prominent method of obtaining another monitored analyte, hyperoside, was alkaline hydrolysis of the solid fraction; 43.60 mg/kg and 1.80 were found in the years 2016 and 2015, resp. Alkaline hydrolysis of the liquid fraction gave the yield of 16.99 mg/kg in the year 2015. Almost identical amounts of hyperoside were obtained by extraction of free phenolic substances in both years.

Isoquercetin

The most statistically significant extraction method for isoquercetin was the extraction of free phenolic substances, with the yield 336.48 mg/kg and 138.43 mg/kg in the years 2016 and 2015, resp. Alkaline hydrolysis of the solid fraction also proved to be a statistically significant method, 339.08 mg/kg of isoquercetin was obtained in the year 2016.

Quercetin

The statistically significantly highest content of quercetin was obtained in 2015 by acidic hydrolysis of the solid fraction, 15.00 mg/kg. By extraction of free phenolic substances, similar amount of quercetin was obtained in both years, 8.41 mg/kg and 7.45 in 1015 and 2016, resp.

Kaempferol

In the harvest year 2015, the type of extraction did not have a statistically significant effect, because almost identical results were obtained by all methods. However, 5.84 mg/kg of kaempferol was obtained by the extraction of free phenolic compounds. On the other hand, statistically significant differences in the content of kaempferol obtained by different extraction methods were found in the year 2016.

CONCLUSION

The paper specifically deals with the optimization of the extraction methods for phenolic compounds in elderberry. Phenolic substances are found in plants in the free, bound and esterified forms, while the only biologically available ones are those in the free form. So far, there is no track record in the available literature to compare the obtained results with. The extraction of free, bound and esterified phenolic compounds occurs in barley and malt. This topic was studied by Běláková et al. (2010) who monitored the change in ferulic acid content in brewing raw materials. From the obtained results it can be assumed that the above-given extraction of free phenolic substances appears to be the most ideal way to extract phenolic compounds. Following biologically active substances were determined in elderberry (*Sambucus nigra* L.): chlorogenic acid, caffeic acid, rutin, hyperoside, isoquercetin, quercetin and kaempferol.

ACKNOWLEDGEMENTS

The research was carried out in the framework of the project TACR TE02000177 „Centre for Innovative Use and Strengthening of Competitiveness of Czech Brewery Raw Materials and Products“.

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EFFECT OF EXTRACTION SOLVENTS ON PHENOLIC COMPOUNDS CONCENTRATION, ANTIOXIDANT ACTIVITITY AND COLOUR PARAMETERS OF SELECTED MEDICAL PLANTS

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Abstract: The impact of extraction solvents on concentration of selected polyphenols, antioxidant activity and colour parameters was evaluated for group of 10 the most popular medical plants conventionally produced in the Czech Republic. High Performance Liquid Chromatography, Electron Paramagnetic Resonance and Ultra Violet Visible Near Infrared Spectroscopy were employed. The entire experimental characteristics were processed by multivariate statistical methods in order to assess the influence of extraction conditions on monitored characteristics and for mutual differentiation of medical plant samples according to the extraction solvent used. Results obtained clearly proved the successful differentiation of medical plant samples by means of canonical discrimination analysis, reaching 96.7% correctness, as well as by k-th nearest neighbour method reaching 100% for k = 1 and 60% for k = 2. The results unambiguously confirmed also the importance of selection of the most proper extraction conditions to obtain the highest possible yields of target compounds/molecules with health-promoting and antioxidant properties.

Key Words: medical plants, solvent extraction, phenolic compounds, antioxidant activity

INTRODUCTION

Medical plants are natural valuable source of phytochemicals, many of which possess also antioxidant activity. The most abundant group of antioxidants in herbs are phenolic compounds, comprising flavonoids, phenolic acids, tannins, chalcones, coumarins or stilbens (Huanq et al. 2010). Polyphenols and flavonoids are of considerable interest to scientists, manufacturers and consumers due to their health promoting properties (Milevskaya et al. 2017, Sytar et al. 2016).

Extraction is the first important step to separate bioactive constituents from plant materials. Further selection of a suitable extraction technique is considerable for the standardization of plant products. However, extraction yield and antioxidant activity not only depend on the extraction method but also on the solvent used for extraction (Dhanani et al. 2017). The most suitable solvents for extraction phenolic compounds are aqueous mixtures containing ethanol, methanol, acetone, ethyl acetate, dimethyl sulfoxide (DMSO), methanol-DMSO mixtures, and dimethylformamide (Magwaza et al. 2016, Ngo et al. 2017). Ethanol has been known as a good solvent for polyphenol extraction and is safe for human consumption and water is certainly the safest and the most environmentally friendly and accessible solvent. It is also significantly less expensive and effective in extraction of phenolic compounds than organic solvents (Addai et al. 2013, Tan et al. 2014).

The main aim of this contribution was to determine the effect of extraction solvent on the concentration of phenolic compounds, their antioxidant properties and colour parameters by using modern analytical techniques (Ultra Violet Visible Near Infrared

Spectroscopy - UV-VIS-NIR, Electron Paramagnetic Resonance - EPR and High Performance Liquid Chromatography - HPLC) with combination of multivariate statistical analysis.

MATERIAL AND METHODS

Medical plant material and its pre-treatment

Ten selected medical plants, the most commonly used in the Czech Republic were analyzed (Table 1). The samples originated from the Medical Herbs Centre in Brno and were harvested in their full ripeness by experienced botanists preferably in the sunny morning time (8:00–10:00) during the summer and autumn 2016. The weather conditions at localities were more-less constant (temperature: 20–28 °C; rainfall: 5–15 mm; humidity: 40–80%). After harvest each herbal sample was air-dried on trays at 30 °C and stored separately in paper bags and dark room until analysis.

Table 1 List of medical plants used in the study

Abbreviation*	Botanical name	Family	Part used
LA	*Lavandula angustifolia*	Lamiaceae	flower
CO	*Calendula officinalis*	Asteraceae	flower
HP	*Hypericum perforatum*	Hypericaceae	flower
SS	*Salvia sclarea*	Lamiaceae	flower
MO	*Melissa officinalis*	Lamiaceae	leaf
GO	*Galega officinalis*	Fabaceae	flower
HO	*Hyssopus officinalis*	Lamiaceae	flower
MP	*Mentha piperita*	Lamiaceae	leaf
SO	*Salvia officinalis*	Lamiaceae	leaf
SM	*Silybum marianum*	Asteraceae	seed

*Legend: * For practical reasons, the abbreviations of medical plants based on their botanical name are further used throughout the text.*

Medical plant extraction

0.5 g of homogenized herbal sample was placed into centrifuge tubes for solvent extraction. 20 ml of extraction solvent (three different solvents were tested: water; 50% (v/v) ethanol water solution; dimethylsulfoxide) was poured over the respective herbal sample. Mixture was shaken on laboratory shaker for 1 hour at 150 rpm at laboratory temperature. After that, the mixture was centrifuged using the laboratory ultracentrifuge at 5 000 rpm and 20 °C for 10 minutes, filtered through a fluted filter into a dark vial. The repeated filtration of sample through 0.22 μm nylon filter was applied prior the HPLC experiments. Four extracts from each herb were prepared.

Determination of total phenolic content, total flavonoid content and colour by UV-VIS-NIR

The entire UV-VIS-NIR experiments were performed using UV-VIS-NIR spectrophotometer Shimadzu 3600 with accessories.

Total phenolic compounds content (TPC) was determined applying the Folin-Ciocalteu method (Tobolková et al. 2014). Standard solutions of gallic acid were used for calibration curve construction and the results were expressed as gallic acid equivalents (GAE, g/kg).

Total flavonoids content (TFC) was determined following the modified method using 2-aminoethyl-diphenylborate reagent (Tobolková et al. 2014). Standard solutions of rutin were used for calibration curve construction and the results were expressed as rutin equivalents (RE, g/kg).

Colour characteristics were determined directly from the measured spectra by means of Colour Lite Panorama Shimadzu software under the standardized conditions: D65 day light illuminant and 10° standard observer. Every spectrum was recorded at wavelength range 200–1000 nm, with 0.5 nm interval and slot width 1 nm. For these purposes, 1 mm quartz cuvette was used for measurements. Colour parameters *L*a*b** were used to assess the objectively the colour of medical plant extracts. Additionally, the chroma (C*) and hue angle (h°) were calculated according to the procedure suggested by Kortei et al. (2015).

Determination of antioxidant activity by EPR

The entire experiments were performed using a portable X-band EPR spectrometer e-scan with accessories. The experimental EPR spectra processing and evaluation was carried out using WINEPR.

Antioxidant activity of extracts was tested by 2,2´-azino-bis(3-ethyl-benzothiazoline-6-sulphonic acid cation radical ($ABTS^{\bullet+}$) assay. Radical scavenging activity of respective sample was expressed as TEAC (Trolox equivalent antioxidant capacity) value in the same manners as previously described by Polovka and Suhaj (2010).

The capability of extracts components to terminate the hydroxyl radicals ($^{\bullet}OH$) generated by chemical reaction directly in the experimental system via the thermal decomposition of potassium persulfate radical initiator in the presence of DMPO as spin trap was investigated according the method described by Butorová et al. (2017). The antioxidant/prooxidative activity of the samples tested by the spin trap technique in the presence of DMPO/$K_2S_2O_8$ was expressed as % RS (% of radicals scavenged). The % RS was calculated for samples in deionized water and DMSO according to the equation (1):

$$\%RS = \left(1 - \frac{I_{sp(15)OH\cdot} - I_{sp(1)OH\cdot}}{I_{ref\,(15)OH\cdot} - I_{ref\,(1)OH\cdot}}\right) \cdot 100 \quad (1)$$

Where: $I_{sp/ref\ (15)OH\cdot}$ represents the intensity of the 15th EPR spectra of the $^{\bullet}OH$ of the sample respectively reference

$I_{sp/ref\,(1)OH\cdot}$ represents the intensity of the 1st EPR spectra of $^{\bullet}OH$ of the sample respectively reference

For samples in 50% ethanol, the% RS was calculated according to the equation (2) due to the presence of both $^{\bullet}OH$ and carbon-centered radicals:

$$\%RS = \left(\frac{I_{sp(15)C\cdot}}{I_{sp(15)OH\cdot}} - \frac{I_{ref\,(15)C\cdot}}{I_{ref\,(15)OH\cdot}}\right) \cdot 100 \quad (2)$$

Where: $I_{sp/ref\,(15)C\cdot}$ represents the intensity of the 15th EPR spectra of the $C^{\bullet}$ of the sample respectively reference

$I_{sp/ref\,(15)OH\cdot}$ represents the intensity of the 15th EPR spectra of $^{\bullet}OH$ of the sample respectively reference

Determination of individual phenolic compounds by HPLC

HPLC Agilent 1260 Infinity apparatus equipped with diode array detector (DAD) with 10 mm absorption cell, autosampler, quaternary pump, column thermostat and degasser was used.

Individual phenolic compounds (gallic acid, chlorogenic acid, caffeic acid, ferrulic acid, sinapic acid, catechin, rutin, hesperidin, myricetin, quercetin, luteolin) were separated on Poroshell 120 Agilent C18 column (150 mm × 4.6 mm, particle size 2.7 nm) at 45 °C. The mobile phase consisted of acetonitrile (solvent A) and 2.5% solution of formic acid (v/v) (solvent B) was used. The following gradient was used for the determination of phenolic compounds: 92% B, 0–25 min; 85% B, 25–30 min; 88% B, 30–45 min; 85% B, 45–53 min; 80% B, 53–56 min; 92% B, 56–65 min. Additional parameters: flow rate, 0.75 ml/min; injection volume, 5 µl; wavelengths of detection: 280, 290, 330, 350 nm; scan range: from 200 to 450 nm. The data were collected by the Agilent 1260 Infinity chromatography data system. Identification of the individual phenolic compounds was based on the comparison of the retention times and the UV spectra obtained by DAD of unknown peaks to those of reference authentic standards. Quantification of individual phenolic compounds was performed via calibration curve of respective standard.

Statistical analysis

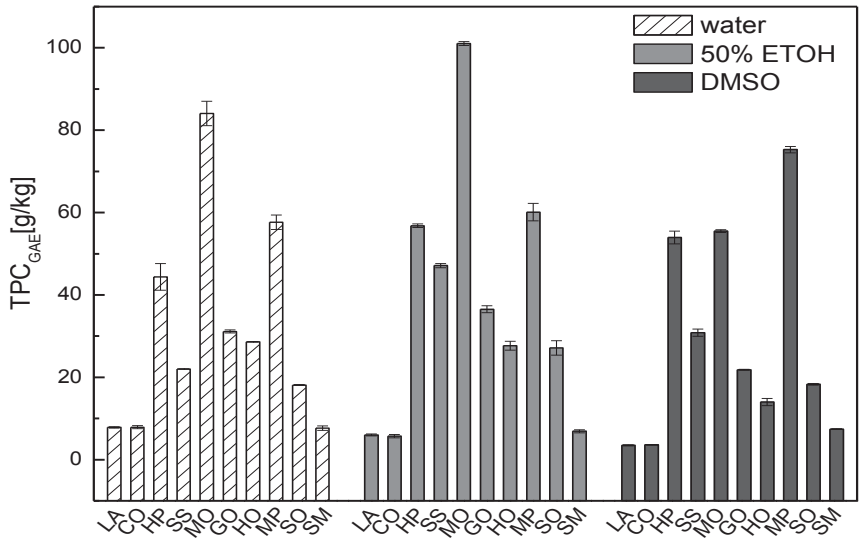
The results were evaluated and visualized using OriginPro v. 7.0 and are expressed as mean ± standard deviation ($n = 4$). The statistical analysis was performed using the statistical package Unistat v. 6.0. The analysis of variance using Tukey's HSD test was used for determination of differences among samples. A probability value of $P \leq 0.05$ was accepted for statistically significant results. Due to high number of experimental characteristics, the entire experimental dataset was

processed by multivariate statistical analysis canonical discrimination analysis (CDA) and k-th nearest neighbour discrimination.

RESULTS AND DISCUSSION

Obtained results confirmed expected dependence of all the monitored characteristics on solvent used, as previously also proved for isolation of phenolic compounds from basil leaves (Złotek et al. 2016). The total content of polyphenols and flavonoids, antioxidant properties, colour parameters as well as individual content of phenolic compounds of herbs under study are inversely proportional to the values of relative permittivity of the solvents, taken as a measure of polarity (Butorová et al. 2015). The observed tendency is demonstrated on Figure 1 proving the decrease in total phenolic compounds in the following order 50% ethanol > distilled water > DMSO. This trend was similarly observed for parameters TFC, TEAC, %RS as well as for individual phenolic compounds. Based on the results we can conclude that 50% ethanol was the most appropriate solvent for the extraction of phenolic compounds selected herbs. It allows obtain the maximum content of phenolic compounds and antioxidants from herbs and therefore it is suitable for routine analytical work and due to its harmlessness as well as for implementation into food industry (Tan et al. 2014).

Figure 1 Dependence of total phenolic content of medical herbs extracts on extraction solvent



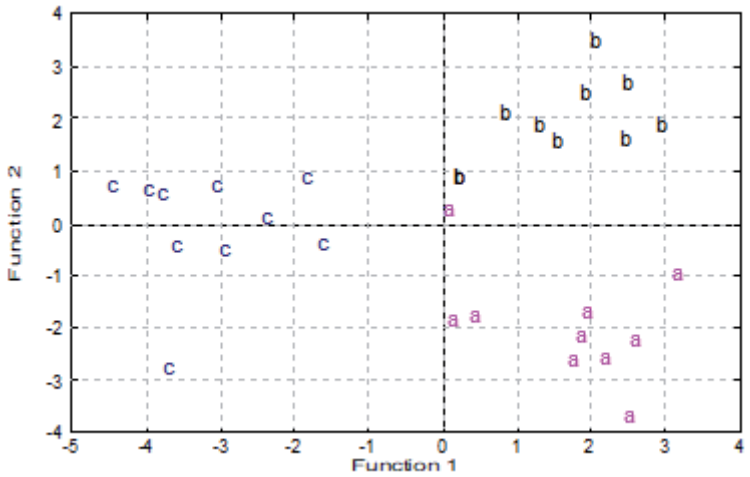
Legend: TPC_{GAE} – Total phenolic compounds content express as gallic acid equivalents, ETOH – ethanol, DMSO – dimethylsulfoxide, LA – Lavandula angustifolia, CO – Calendula officinalis, HP – Hypericum perforatum, SS – Salvia sclarea, MO – Melissa officinalis, GO – Galega officinalis, HO – Hyssopus officinalis, MP – Mentha piperita, SO – Salvia officinalis, SM – Silybum marianum

However, it can also be seen from Figure 1 that the differences in total phenolic content among the individual solvents at the first sight are not so high. Even the analysis of variance in the case of multiple comparison found statistically significant differences only in parameters a* and %RS from all studied parameters when the extraction solvent factor was used. In the case of $^{\bullet}OH$ scavenging assay express as %RS statistically higher values were found in 50% ethanol extracts. Also two types of spin aducts (dominant DMPO-$OH^{\bullet}$ adduct and minor by product DMPO-CH_2-CH_2-$OH^{\bullet}$/ DMPO-$CX^{\bullet}$) were detected in 50% ethanol extracts. On the other hand, only DMPO-$OH^{\bullet}$ aduct was identified in water and DMSO herbal extracts. Further the negative values of %RS in DMSO and water extracts of individual herbs were found indicate pro-oxidative properties of the extracts, which can be caused by higher concentration of transition metals such as iron, copper and manganese in these extracts (Nasri and Rafieian-Kopaei 2014).

For the visualization of the differences among extraction systems canonical discrimination analysis was used. CDA results according solvent type constructed from all 20 experimental parameters (containing parameters TPC, TFC, TEAC, %RS, colour parameters L*, a*, b*, h°, C* and 11 individual phenolic compounds) show the differentiation of samples into three discrete zones.

From Figure 2 it is obvious that DMSO extracts (aprotic solvent) differ the most from water and ethanol extracts (protic solvents), although all three types of solvents are different from each other (Figure 2). As regards the influence of individual characteristics on discrimination function construction parameters C*, b*, TEAC and TFC are the most discriminating markers for distinguishing medical plants according extraction solvents. CDA classified samples according to extraction system with 96.7% correctness, also k-th neighbour analysis classified samples according to solvents with 100% correctness for k = 1, for k = 2 classification scores decrease to 60%.

Figure 2 Canonical discrimination analysis of medical plants according to extraction solvents. All 20 experimental parameters were used for discrimination function construction



Legend: a – distilled water, b – 50% ethanol, c – dimethylsulfoxide

CONCLUSION

The obtained results demonstrate high potential of combination of analytical techniques with multivariate analysis for differentiation and classification of herbal samples according to extraction systems and evaluation data in complex way. The differences in experimental characteristics were sufficient for successful differentiation and classification of medical plants according to extraction solvents. Based on the results, also 50% ethanol was chosen as the most suitable extraction system for the extraction of substances with antioxidant potential, which will be used in conjunction with industrial practice for further application into food products.

ACKNOWLEDGEMENTS

This publication is the result of the project implementation "Improvement of nutritional and sensorial parameters of fruity and vegetable drinks via an inert gases application – ITMS 26220220175" supported by the Research and Development Program funded by the ERDF. The Medical Herbs Centre in Brno is gratefully acknowledged for samples provision and cooperation.

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THE EVALUATION OF WALNUT OIL EXTRACTION PARAMETERS

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Abstract: Walnut is known because of its high polyunsaturated fatty acids content, they reduce blood pressure, total and LDL cholesterol. In the walnut oil production, it is very important to find appropriate parameters to pressing the oil from seeds. In this study, walnut oil was obtained by pressing by screw press UNO FM 3F by Farmet Company, Czech Republic. The effects of pressing frequency of 30, 50, 70, and 90 rpm and nozzle size of 6, 8, 10 mm in pressing experiments were monitored. The walnuts oil density was 876.3 kg/m^3 and seed oil content was determined by extraction ranges 70.17% in dry basis. The results have confirmed that when the screw rotation speed is changed from 30 to 90 rpm, the press capacity increases on average from 0.71 to 2.05 kg/h but simultaneously the oil yield reduces from 41 to 12%. Finally, the optimal pressing parameters were determined to 30 rpm and nozzle size of 6 mm.

Key Words: walnuts seeds, walnuts seeds oil, screw press, rotation speed, efficiency

INTRODUCTION

Walnut kernels have a high oil content which varies from 52 to 72% (Poggetti et al. 2017, Labuckas et al. 2014, Martínez et al. 2006, Zwarts et al. 1999). Walnut is well known because of its high polyunsaturated fatty acids (PUFA) content (Mehmet 2009, Crews et al. 2005). Regular consuming of walnuts reduces the risk of diabetes (Kendall et al. 2011), has a positive effect on brain function (Hou et al. 2014) and reduces the amount of cholesterol in the blood (Kodad et al. 2016, Uzunova et al. 2015, Avanzato 2010, Park et al. 2008).

Oil from walnut kernel can be obtained in three ways, mechanical extraction (pressed oil), chemical or solvent extraction and supercritical CO_2 extraction (Singh and Bargale 2000). The extraction by mechanical screw presses is typical for lower proportion of collected oil. Screw pressing has been studied for a large variety of oilseeds (linseed, canola, crambe, chi seeds and others) (Ezeh et al. 2016, Ling et al. 2016, Wang et al. 2016, Mridula et al. 2015, Savoire et al. 2013).

But benefits of screw pressing are to produce high-quality oil containing bioactive compounds, without using organic solvent. Screw pressed method have low investment costs for equipment compared with supercritical fluid extraction method. Benefits of screw pressing is providing a simple and reliable method of processing small batches of seed. The amount and quality of pressed walnut oil are critical for efficiency of commercial production (Jokic et al. 2016, Labuckas et al. 2014, Teh and Birch 2013, Martínez et al. 2008, Turkmen et al. 2006, Koski et al. 2002).

Considering the increasing demand of new sources of high quality vegetable proteins, this study evaluates the effect of different oil extraction parameters conducted by screw pressing.

MATERIAL AND METHODS

Walnut kernels

Walnut kernels were vacuum-packed and purchased in the supermarket chain originated in Czech Republic. Seeds were selected a mixed together. They were ground in a stainless-steel mill

with pro-homogenization sample to the fraction of size from 0 mm to 6 mm. Then, the material was pressed.

Screw press parameters

The screw press type UNO FM 3F made by the Farmet Company in Czech Republic was used for experimental measurements. This model is suitable for pressing all kinds of oilseeds. The drive is configured for three-phase voltage with variable speed of the main drive using a frequency converter, which enables better optimization of pressing parameters. The press components are: an electric motor (1.5 kW power), transmission, pressing device, motor starter and frequency converter (this allows precise adjustment of rpm). The screw rotation speed was adjusted on 30, 50, 70 and 90 rpm. The pressing device components are: a matrix, 220 mm screw, head, heating mantle, nozzle holder and nozzles of different in diameter (from 6 mm up to 10 mm).

Determination of water content of walnut kernels and density of walnuts oil

Water content of walnut kernels was determined by dehydration at 103 °C (in a drying oven type FN 120, Nuve, made in Ankara, Turkey) according to the CSN EN ISO 665 (461025) Oilseeds - Determination of moisture and volatile matter content. Analysis was made on 5 g of grinded sample, weighted with an accuracy of 0.1 mg. Results are expressed as the ratio of water loss per gram of dried sample. Density of oil was determined pycnometrically according to the CSN EN ISO 6883. This international standard specifies a method for the determination of the conventional mass per volume ("litre weight in air") of vegetable fats and oils. Determination of water content and density was performed in triplicate.

Determination of the total fat content in the seeds and cakes through extraction

To determine the total fat content, we used the Soxhlet extractor with hexane as a solvent. Crushing the walnut kernels always took place immediately prior to the oil extraction and from the pressed cakes directly after the pressing. For this purpose, the IKA MF 10 basic on the sieve with the average of 3 mm was used. Emphasis was always placed on precise cleaning of the grinder in order to avoid distorting the results. The temperature of the extraction mixture was kept by the heating mantle closely around the boiling point of hexane (70 °C). Extraction was always carried out for 8 hours. Subsequently, the hexane was evaporated on the vacuum evaporator, type IKA RV 10 control at the pressure of 200 kilopascals until the hexane was evaporated. After that, the pressure was lowered down to 60 kilopascals for another 2 hours at the constant temperature of 40 °C. The weight of total fat was then measured on the scales type KERN EG 2200-2NM.

Statistical analysis

Analytical determinations were done in triplicate and data were reported as means ± standard deviation. Analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) tests were conducted to determine the differences among which means that the statistical significance was declared at $p \leq 0.05$. These statistical evaluation methods were applied using the computer software package "Statistica 12.0" (StatSoft Inc., USA).

RESULTS AND DISCUSSION

The input raw material had the following values of the main parameters. The water content was at 2.95 ± 0.21%, which corresponds to the values conventionally recommended and recommended for storage (Wco 2017, Poggetti et al. 2017, Christopoulos and Tsantili 2015). The total fat content was set at 70.17 ± 0.455%, which is rather higher in comparison with other authors (Poggetti et al. 2017, Labuckas et al. 2014, Martínez et al. 2006, Zwarts et al. 1999).

The walnut oil density was 876.3 ± 5.3 kg/m^3. Özcan, (2009) indicates value 972.1 kg/m^3 and Gharibzahedi et al. (2014), in different cultivars, from 921.2 to 921.8 kg/m^3. Demirbas (2008) indicates significantly different value, which is 912.0 kg/m^3.

The pressing of walnut kernels was carried out at speed 30, 50, 70, and 90 rpm and nozzles 6, 8 and 10 mm were used. These two parameters significantly affected the press capacity. This means that one kilogram of kernels may produce 0.41 to 0.12 kg (41–12%) of crude oil (see Table 1). The results

match with the results of Gharibzahedi et al. 2013 who indicates the highest yield crude pressed oil 34.9% and Labuckas et al. (2014) who states oil extraction in between 41.0–44.4%.

The results (Table 1 and Figure 1) indicate that at higher speeds of the pressing and higher mean of nozzle, the oil yield drops. Specifically, speed change affects extraction up to 57% and nozzle change up to 53%. This effect could be attributed to the conveying capacity of the press that increases with screw rotation speed and increased mean of nozzle (Vadke and Sosulski 1988, Poustkova et al. 2010, Savoire et al. 2013).

Table 1 Values of pressing time (h), yield of oil (kg) and press capacity (kg/h), the amount of oil in pressed cake and the amount of sediment depending on the speed and diameter of the nozzle

Mean of nozzle (mm)	Speed (rpm)	Time of pressing 1 kg kernels (h)	Yield of oil from 1 kg kernels (kg)	Press capacity (kg/h)	The weight of pressed cakes (kg)	The amount of oil in pressed cakes (kg)	The weight of sediment (kg)
10	30	0.37 ± 0.0043^{h}	0.28 ± 0.0042^{b}	0.76	0.46	0.18	0.24
10	50	0.27 ± 0.0017^{d}	0.24 ± 0.0018^{f}	0.89	0.56	0.31	0.20
10	70	0.18 ± 0.0010^{b}	0.16 ± 0.0016^{a}	0.89	0.70	0.46	0.14
10	90	0.17 ± 0.0025^{a}	0.12 ± 0.0014^{d}	0.71	0.78	0.52	0.10
8	30	0.33 ± 0.0104^{g}	0.27 ± 0.0053^{b}	0.82	0.50	0.26	0.23
8	50	0.27 ± 0.0042^{d}	0.31 ± 0.0052^{g}	1.15	0.42	0.19	0.26
8	70	0.20 ± 0.0017^{c}	0.20 ± 0.0037^{e}	1.00	0.62	0.37	0.18
8	90	0.17 ± 0.0025^{a}	0.15 ± 0.0016^{a}	0.88	0.71	0.42	0.13
6	30	0.20 ± 0.0029^{c}	0.41 ± 0.0040^{j}	2.05	0.29	0.03	0.35
6	50	0.19 ± 0.0025^{b}	0.38 ± 0.0085^{i}	2.00	0.30	0.04	0.33
6	70	0.22 ± 0.0025^{f}	0.33 ± 0.0060^{h}	1.50	0.36	0.11	0.29
6	90	0.00 ± 0.0000^{e}	0.00 ± 0.0000^{c}	0.00	0.00	0.00	0.00

Legend: Values are means ± standard deviations of triplicate determinations. Means followed by different lowercase letters in the same column indicates significant differences ($P < 0.05$) between treatments involving pressing

Table 1 shows clearly, that the amount of oil in pressed cakes depends on the extraction parameters from 0.03 up to 0.52 kg (3–52%). The higher values are caused by inappropriate combination of the rotational speed of press and nozzle, which would not be seen in the commercial use. Labuckas et al. (2014) states, that in the pressed cakes there is leftover in between 27.7–31.1% of oil and Jokić et al. (2014) states, that the value of residual oil in press cakes was in the range from 7.95 to 17.57% depending on applied pressing parameters. This oil can be obtained from the following extraction. Moreover, the amount of sediment was found in the range between 0.1–0.35 kg (10–35%).

The sediment is made by the imperfect separation of the oil from the pressed cakes. It usually appears in small quantities and can be separated by settling the oil and filtering it. The higher amount of this sediment is again caused by an inappropriate combination of press parameters and should be avoided in regular operation.

Figure 1 Effect of the number of revolutions and mean of nozzle of the press on the yield of oil (kg)

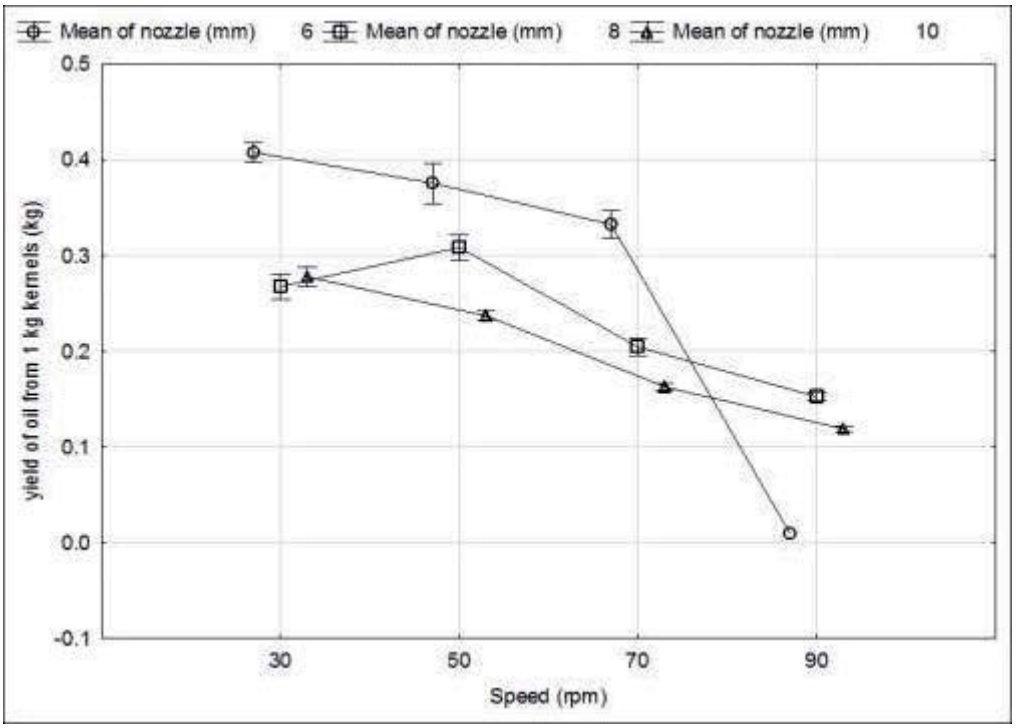
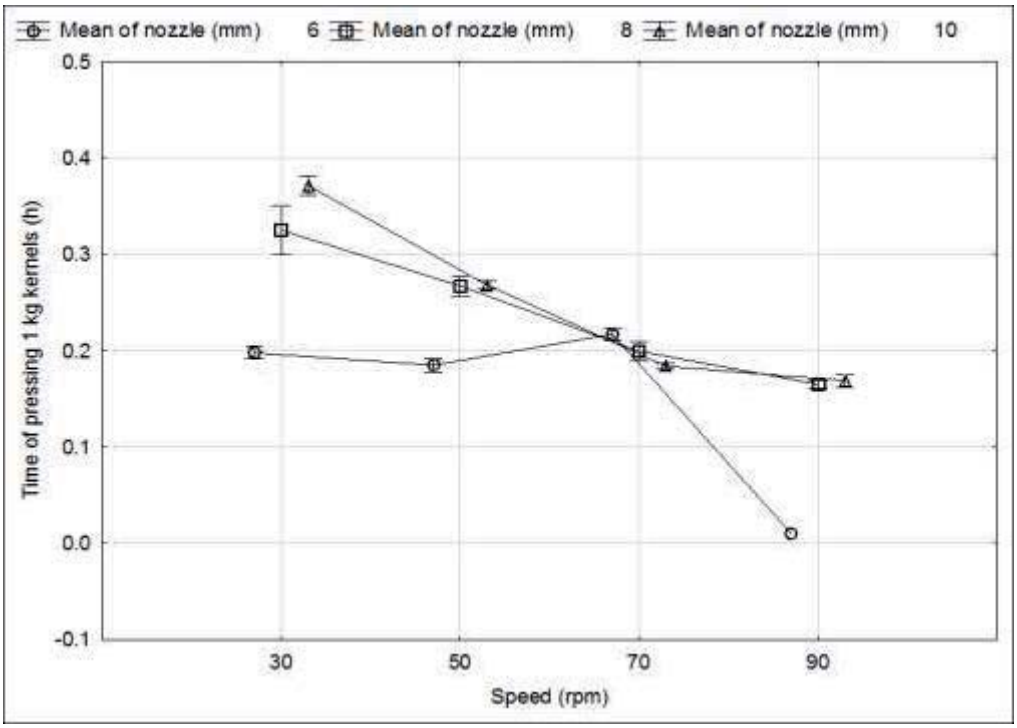


Figure 2 Effect of the number of revolutions and mean of nozzle of the time of pressing (h)



The oil yield depends on the pressing speed, attained pressure, the length of pressure action, conditions of outflow of oil at a maximum pressure, viscosity, and oil temperature (Black and Bewley 2000). During this study, it was found out that the combination of 6 mm nozzle and the speed of 90 rpm made the extracting not possible as the press was becoming blocked.

CONCLUSION

The benefit of screw pressing is to provide a simple and reliable method of processing small batches of seed. From the results of this study, it is followed that the optimal combination of press parameters uses 6 mm nozzle and 30 rpm. It is thus possible to achieve the highest moulding of more than 400 g of oil per kilogram of walnuts kernels, which means that a press capacity is 2.05 kg/h. In this setting, only about 3% of the oil remains in the pressed cakes. Part of this oil can still be obtained during the post-process. The measured results can be used in commercial practice for optimizing the pressing process for pressing of oil from walnuts kernels.

ACKNOWLEDGEMENTS

The research was financially supported by the project IGA - ZF/2017 - AP004 - Possibilities of pressing oil from seeds of fruit crops.

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VARIABILITY OF THE CONTENT AND COMPOSITION OF LAVENDER MEDICAL (*LAVANDULA ANGUSTIFOLIA* P. MILL.) ESSENTIAL OILS OF DIFFERENT ORIGIN

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Abstract: The aim of this work was the determination of variability of the essential oil content in lavender obtained from different sources. The results indicate that significant differences were found both in the essential oil content and in its composition. Only the samples of the *Lavandula angustifolia* P. MILL (*L. officinalis* CHAIX) were used to obtain the essential oils. According to the Czech Pharmacopoeia 2014, the drug (lavender flowers) should contain at least 1.3 ml of essential oil in 100 g of anhydrous drug and lavender essential oil should contain following ingredients: limonene < 1.0%, cineol < 2.5% (eucalyptol), 3-octanone < 2.5%, camphor < 1.2%, linalol 20.0% to 45.0%, linalylacetate 25.0% to 46.0%, terpinene-4-ol 1.2% to 6.0%, lavandulylacetate > 1.0%, lavandulol > 0.1%, α-terpineol < 2.0%. The average content of the essential oil found in samples from three cultivation sites in Hvar was 9.58 ml in 100 g sample. However, statistically significant differences were found between samples obtained from Hvar. The content of limonene in the analyzed samples was highest in the sample from France and the sample from Brno Královo Pole. The content of cineol also complied with the Pharmacopoeia, except for the sample Žabčice II. It is evident that the samples from Hvar and France had several times higher cineol content than required by the Pharmacopoeia.

Key Words: lavender, essential oil, variability, essential oil content, essential oil composition

INTRODUCTION

Lavender (*Lavandula angustifolia* Mill.) is a plant with direct, partially woody stalks that grows as a subshrub; it belongs to the *Lamiaceae* family (Schönfelder I. and Schönfelder P. 2004). Several literature sources present a wide range of lavender species - Slavík (2000) lists 20 species, Kuťková (2009) 35–40 species, Upsan and Andreros (2004) shows 39 species. According to the International Plant Index, 94 species of the lavender genus were registered on April 23rd, 2015 (www.ipni.org). Lavender is a 0.4–0.8 m high herb with branched stems. The leaves are linearly lanceolate, 20–40 mm long, with entire margins; young plants have hairy white hairs. The flowers are composed of 6–10 blossoms that create the top verticillastrum. Lavender is blooming at the beginning of July. Glandular calyx has numerous trichomes, it is gray-violet and trumpet-shaped; corolla bilabiata is of violet-blue color. The fruits are black shining nuculae (Felková and Kocourková 2003). South Europe is the original home of lavender, but it has spread throughout the world as an ornamental, aromatic and medicinal plant (Schönfelder and Schönfelder 2004). It grows on dry, rocky soils with high calcium content, on sunny slopes with southern exposure. In the Czech Republic, we can even find it in the wild, but only in warm regions of South Moravia - Křtiny, Buchlovice, etc. (Neugebauerová 2006). Lavender is one of the polycarpic plants that have fruits annually for several years. After creation of fruits, the plants have ability to survive even at frosts of -2 to -20 °C (Kocourková et al. 2015). It is a heliophilic plant with the metabolism, anatomical and morphological structure adapted to sunny habitats. As for the water requirements, lavender belongs to xerophyte plants, i.e. plants growing in dry sites where it is actively able to withstand a longer dry period. It can

adapt even to a more permanent lack of soil and air humidity. The plant is adapted to the drought thanks to the leaves that can bind the vents inwards.

The most important lavender ingredient is the essential oil. It is assumed that the lavender essential oil serves to reduce the transpiration by evaporating near the transpiring plant. Lavender essential oil is traditionally used and approved by the European Medicines Agency (EMA) as a herbal remedy to relieve stress and anxiety. The essential oil contains lynalyl acetate, lonalone, borneol, isoborneol, cineol, geraniol, camphor and other terpenes. In addition, essential oil contains also tannins, anthocyanins and bittering agents (Janča a Zentrich 1995). According to the Czech Pharmacopoeia 2014, lavender essential oil contains following ingredients: limonene < 1.0%, cineol < 2.5% (eucalyptol), 3-octanone < 2.5%, camphor < 1.2%, linalol 20.0% to 45.0%, linalyl acetate 25.0% to 46.0%, terpinene-4-ol 1.2% to 6.0%, lavandulyl acetate >1.0%, lavandulol > 0.1%, α-terpineol < 2.0%.

Ancient Greeks and Romans used lavender as a bath ingredient. The very name of lavender (*Lavandula*) comes from the Latin word *lavare*, "to wash oneself". Lavender keeps long-lasting scent and has the ability to repel insects. In the past it was used for the production of gloves used to touch the upper part of the skin. Lavender was also used as a protection against bubonic plague. People used the essential oil for centuries and it is still accepted both in the form traditional products and in the modern health care system. Lavender and its active substances are classified into a phytotherapeutic group as a nervinum, sedativum and antiseptic agent. It is used in the cosmetics industry to produce soaps, perfumes, lotions, as an ornamental plant, as well as odor corrigens. For use in cosmetics, a hybrid variety (*Lavandula angustifolia* MILL x *Lavandula latifolia* VILL) is cultivated, often referred to as lavandil. This crossbreed has stems up to 1.0 m long and can be multiplied only vegetatively; it is grown mainly in France (Neugebauerová 2006).

Lavender oil is considered to be one of the best-selling medicines for anxiety, stress and depression (Lopéz et al. 2017). It is useful for problems with the gastrointestinal tract, rheumatism and neurological disorders (Hassiotis et al. 2010). Hassiotis et al. (2010) also state that lavender essential oil and its components are rapidly absorbed into the skin; linalool and linalyl were found in plasma already 19 minutes after the application.

The standard, composition and quality of the essential oils are affected by climatic conditions, the soil composition, the time of harvest and technological method of processing. In addition, the variability in the content of essential oils depends on the genetic basis of the plant (Felková and Kocourková 2003).

MATERIAL AND METHODS

Only the samples of the *Lavandula angustifolia* P. MILL (*L. officinalis* CHAIX) were used to obtain the essential oils. According to the Czech Pharmacopoeia 2014, the lavender flowers drug should contain at least 1.3 ml of essential oil in 100 g of anhydrous drug. The essential oil is obtained by steam distillation; the principle is to convert the liquid into steam by heating, let the steam pass through the sample and then condense. When water vapor under high pressure passes through the plant material, aromatic components are extracted into the steam. Volatile compounds are carried by steam into the upper part of the column and the cooler, where they condense together with the water. The essential oils are then separated from the water phase due to different densities (Trepková and Vonášek 1997).

To determine the amount of essential oil in the plant drug, 20.0 g of dried drug was weighted and 500 ml of water was added. The drug was distilled for 2 hours at the flow-rate of 2 mL/min to 3 mL/min in a 1000 mL round bottom flask. For each lavender sample, two distillations were performed.

The samples for the determination of the essential oil content (according to the Czech Pharmacopoeia 2014) in lavender (*Lavandula angustifolia* P. MILL) were selected from six different places of origin, all from the harvest year 2017. The first three samples were obtained from different parts of Croatian island Hvar (HVAR I[a], HVAR II[b] and HVAR III[c]). The sample obtained from the company "levandulova.cz" originated from France. The study was focused mostly on lavender samples cultivated at the Field Experimental Station AF MENDELU in Žabčice. These

samples were taken from lavender grown for 1, 2, 3 and 4 years (Žabčice I, Žabčice II, Žabčice III and Žabčice IV, resp.). The last sample was hobby-grown in a private garden (Brno, Královo Pole)[d]. Two samples (n = 2) were obtained from each place of origin. The essential oil content was determined by steam distillation under the standardized conditions. Samples were always taken from the plants in the dried state and after thorough grinding. The results are expressed in ml and related to 100 g of the dried drug.

$$x\,\% = \frac{100 \,.\, a}{n}$$

a – essential oil volume [ml]

n – sample weight [g]

x – essential oil volume in 100 g of dried drug

Essential oils were analyzed using the technique of gas chromatography coupled with a mass detector (GC/MS). It is a commonly used separation method based on the separation of the individual components of the sample in the column. The gas chromatograph Trace GC Ultra Finnigan coupled with a mass detector Trace DSQ Thermo Finnigan was used for the determination of the content of the studied analytes in the essential oil samples. Separation was performed on a capillary column SLB5MS (60m x 0,25 mm x 0,25 mm; Supelco, USA), a carrier gas was helium. For the measurement was used this temperature program: T_1 = 50 °C, t_1 = 0,1 min, 3 °C/min to T_2 = 150 °C, t_2 = 10 min, 10 °C/min to T_3 = 200 °C, t_3 = 5 min. Injection temperature was 250 °C with split 1 min. Calibration curves and analyses performed by the method of external standard were processed and evaluated by the means of the Xcalibur software. Following components of the lavender essential oil were determined in the samples: β-pinene, myrcene, limonene, cineol, linalool, camphor, borneol, α-terpineol, terpinene-4-ol, linalyl acetate, lavandulyl, farnesene.

Characteristics of the places of origin for all investigated samples:

- HVAR I: Gdinj, Split-Dalmatia county, Croatia; 43.1245383N, 17.1085633E
- HVAR II: Split-Dalmatia county, Croatia; 43.1581617N, 16.7633631E
- HVAR III: Brusje, Split-Dalmatia county, Croatia; 43.1862011N, 16.5021694E
- „levandulova.cz“: the farm „Eveline Popee“; 44.1053889N, 5.4012778E;84390 Route du Ventoux, Sault, France. levandule@techonice.cz
- Žabčice I - one-year lavender growth in the Field Experiment Station AF MENDELU in Žabčice
- Žabčice II - two-year lavender growth in the Field Experiment Station AF MENDELU in Žabčice
- Žabčice III - three-year lavender growth in the Field Experiment Station AF MENDELU in Žabčice
- Žabčice IV - four-year lavender growth in the Field Experiment Station AF MENDELU in Žabčice
- BRNO-Královo Pole, 49.2294639N, 16.6037308E

The evaluation of results was performed using the statistical program STATISTICA (data analysis software system), StatSoft, Inc. (2013), version 12.

RESULTS AND DISCUSSION

Table 1 Average essential oil content in ml per 100 g of sample in lavender samples of various origin

Provenance	HVAR I	HVAR II	HVAR III	FRANCE	Žabčice I	Žabčice II	Žabčice III	Žabčice IV	Brno
Essential oil in 100 g of sample	12.5 d	8.75 c	7.5 bc	2.25 a	3.88 a	3.88 a	3.88 a	5.38 abc	4.75 ab

Legend: The average values marked with different letters in the columns differ statistically significantly at P = 0.05

The results shown in Table 1 indicate that the highest content of essential oils was found in lavender grown on the island Hvar in Croatia. The average content of three cultivation sites in Hvar

was 9.58 ml in 100 g sample. However, statistically significant differences were observed between the samples obtained from Hvar, the highest content was in HVAR I, which differed from that of HVAR II and HVAR III samples by 3.75 ml and 5.0 ml, resp. In the lavender samples grown in Žabčice the average amount of essential oil was 3.87 ml in 100 g sample. Lavender samples from Žabčice grown for one year, two years and three years did not differ in the content of essential oils. However, the essential oil content in four-year lavender was statistically significantly different, it was higher by 1.5 ml. This result is comparable to that one found in the sample from Brno Kr. Pole. The lowest content of essential oil was found in the sample obtained from the company "levandulova.cz"[e]. All evaluated samples meet the requirements of the Czech Pharmacopoeia.

Table 2 Average essential oil composition in lavender samples of various origin

Provenance	Essential oil composition											
	β-pinene	myrcene	limonene	cineol	linalool	camphor	borneol	terpinen-4-ol	α-terpineol	linalyl acetate 1	lavandulyl	farnesene
HVAR I	**0.64c**	1.35b	0.59c	12.15e	56.61b	1.01bc	5.04c	5.1d	1.97b	9.00d	0.72ab	0.71b
HVAR II	0.04a	0.34a	0.11a	**18.75f**	59.71bc	2.2c	**8.46e**	3.95c	0.37a	4.84b	0.36a	0.07a
HVAR III	**0.65c**	1.06ab	0.89d	15.30ef	56.62b	1.96c	**6.763d**	**6.62e**	1.84b	6.19c	1.05b	**1.05c**
FRANCE	0.31b	1.91b	**1.27e**	**19.49f**	25.38a	**24.92e**	5.65cd	5.37d	3.13c	14.20e	2.00bc	0.01a
ŽABČICE I	0.02a	**2.61c**	0.61c	6.19c	50.84bc	0.16a	1.57b	**6.40ef**	**4.13cd**	**17.91f**	**8.96fg**	0.70b
ŽABČICE II	0.219b	0.10a	0a	1.20a	**73.51d**	0.26ab	0.056a	3.03ab	0.43a	**17.58f**	3.62d	0a
ŽABČICE III	0a	**2.48c**	0.83d	7.35cd	55.56b	0.3a	1.59b	**7.13f**	**4.58d**	13.78de	**6.396f**	0a
ŽABČICE IV	0a	1.19ab	0.35b	2.30b	**78.04d**	0a	0a	2.62ab	0a	10.58d	4.91e	0a
Brno Kr. Pole	0a	0.60ab	**1.26e**	7.57d	60.90c	**14.15d**	3.02bc	1.78a	0.53a	0.75a	5.47ef	0.37ab

Legend: The average values marked with different letters in the columns differ statistically significantly at P = 0.05

The components of lavender essential oil used in a wide range of industries are given in Table 2. The content of limonene in analyzed samples was highest in the sample from France and the sample from Brno Kr. Pole. The content of the cineol also met the requirements of the Czech Pharmacopoeia, except for the sample Žabčice II. It is evident that the samples from Hvar and France had several times higher cineol content than required by the Pharmacopoeia. Linalool is a naturally occurring terpene alcohol contained in many types of medicinal plants. It has a wide commercial use, especially for its pleasant scent (floral, with a little spice aroma). Due to the properties of lavender essential oil, linalool is the most often used compound obtained from lavender. The samples from Hvar had the average content of 57.35%; the samples from Žabčice had the average content 64.49%, which is > 5% more compared to the Hvar sample. The samples from Žabčice had also high contents of other lavender essential oil components, such as linalyl acetate and lavandulyl. Differences between the samples were statistically significant.

CONCLUSION

The results of the determination of essential oil content and composition in different lavender samples (from Hvar, France, Žabčice and Brno) showed statistically significant differences in the content of the essential oil and its composition. The content of essential oil in Hvar samples differed statistically significantly from the samples from cultivation in Žabčice and Brno (Hvar – 9.58 ml, Žabčice – 4.23 ml, Brno – 4.75 ml per 100 g). Linalool is the most represented compound in the essential oil with an average content of 57.46%, with statistically significant differences between the provenances. The highest values were found in the samples from Žabčice (73.51% and 78.04%). Other essential oil components also showed statistically significant differences according to provenance. The results from growing in Žabčice are obviously interesting

from the viewpoint of further use of the essential oil (high content of following ingredients was found: myrcene, linalool, terpinene, α-terpineol, linyl acetate and lavandulyl); however, the results are available only from one harvest year yet.

ACKNOWLEDGEMENTS

The research was carried out in the framework of the project TACR TE02000177 „Centre for Innovative Use and Strengthening of Competitiveness of Czech Brewery Raw Materials and Products“.

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THE USE OF SATURATED MIDDLE-CHAIN FATTY ACIDS IN THE TECHNOLOGY OF WINE PRODUCTION

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Abstract: Reducing the amount of sulphur dioxide in wine has been one of the main subject of interest to winemakers for several years. Recent research has shown the efficacy of saturated middle chain fatty acids (MCFAs) as inhibitors of alcoholic fermentation; even in the production of wine with higher residual sugar. In this study, the effectiveness of the octanoic (C_8), decanoic (C_{10}) and dodecanoic (C_{12}) mixture of MCFAs as a complement to sulphur dioxide was monitored with a view to reducing the dosage of sulphur dioxide required to ensure effective inhibition of *Saccharomyces cerevisiae* yeasts, which in turn halts alcoholic fermentation. The progression of alcoholic fermentation was observed across twenty-four variants and combinations of middle chain fatty acid mixtures and sulphur dioxide. The development and conclusion of alcoholic fermentation was controlled for ten days, after application of the MCFAs mixtures. The process was monitored through daily measurement of residual sugar content. The results were statistically evaluated. A mixture of the C_8, C_{10} and C_{12} MCFAs in the ratio 2:7:1, in concentration 10 mg/l, can reduce the required dosage of sulphur dioxide by several tens of mg/l. The results prove that the application of 10 mg/l of MCFAs mixture with 30–40 mg/l of sulphur dioxide has the same efficacy as the dosage of 60 mg/l of sulphur dioxide alone.

Key Words: alcoholic fermentation, octanoic acid, decanoic acid, dodecanoic acid, sulphur dioxide

INTRODUCTION

One of the main subject of interest in the wine world in recent years has been the reduction of sulphur dioxide in wine. This is due to the possible health risks associated with the consumption of SO_2. Whilst the total exclusion of sulphur dioxide without any substitution is, in most cases, impossible, the dosage of SO_2 could be significantly reduced. Sulphur dioxide has many important functions in the winemaking process – it acts as an antioxidant, deactivates enzymes and has antimicrobial effects. Furthermore, SO_2 affects the organoleptic characteristics of wine, both positively and negatively (Baroň 2013, Guerrero and Cantos-Villar 2015, Henderson 2009).

Whilst the health risks associated with the consumption of SO_2 are a serious cause for concern, the issues associated with using no SO_2 in the winemkaing process forced us to consider alternatives. Much attention had been focused on identifying alternatives or substitutes which were effective in protecting wine and minimising or eliminating the use of sulphur dioxide, without any negative influence on human health. However, to the extent that such chemical or physical alternatives had been identified, those alternatives were prohibitively expensive and/or technically demanding and therefore unavailable to most winemakers (Baroň 2013).

A breakthrough in this area has been made through the exploration of saturated MCFAs, which are perfect alternatives to sulphur dioxide. MCFAs can replace the SO_2 in respect of antimicrobial function. Fatty acids with longer chains (C_{16} and C_{18}) work like activators of alcoholic fermentation (Rodriguez-Nogales et al. 2013), whereas MCFAs (C_8, C_{10} and C_{12}) have the opposite effect, halting alcoholic fermentation. MCFAs are also good at inhibiting undesirable malolactic fermentation (Guilloux-Benatier et al. 1998, Viegas and Sá-Correia 1997). The functioning of MCFAs C_8, C_{10} and C_{12} is very straightforward. They penetrate the body of the yeast, changing the structure of the yeast resulting in the yeast cell becoming permeable to other substances. Thus, the yeast becomes non-functional and alcoholic fermentation is halted. These MCFAs are able to prevent refermentation which could otherwise occur in the wine bottle as a result of the presence of residual sugar. Accordingly,

MCFAs could assist with the storage of wine. The question is, are there residues of the MCFAs in wine as a result of this process? This can be answered very easily. A significant proportion of the MCFAs added to the wine are absorbed or assimilated by the yeasts. The balance of MCFAs are esterified and remain in the wine as residue; however, this is a negligible quantity in the order of tenths (maximally units). MCFAs are fixed on the dead-yeast bodies and are removed gradually during the winemaking process. No significant difference has yet been found between residues of MCFAs and their esters in wine treated with or without MCFAs. Simultaneously, no increased occurrence of volatile compounds was registered. The aim of this study was to explore these new issues, which appeared in the wine sector. The research followed the idea of using MCFAs in winemaking technology and the storage of wine (Baroň 2013).

The main goal of this experiment was to confirm or disprove the efficacy of MCFAs – octanoic, decanoic and dodecanoic – as inhibitors and stoppers of alcoholic fermentation.

MATERIAL AND METHODS

Design of experiment

The progression of alcoholic fermentation was compared across twenty-four different variants and combinations of dosages of MCFAs mixtures and sulphur dioxide. The evolution of alcoholic fermentation and its conclusion was controlled for ten days, after application of MCFAs mixtures and SO_2. The term of stopping alcoholic fermentation was determined at the time, while the content of fermentable sugar in the fermenting must was 25.6 mg/l (in this case it is called "the day 0"). The process was monitored daily through the measurement of residual sugar content. The results were statistically evaluated.

Table 1 Analytical parameters of must at the moment of addition of MCFAs mixture and SO_2

PARAMETER	Titratable acids	pH	Alcohol content	Content of residual sugars
VALUE	9.43 g/l	3.12	11.15% vol.	25.6 g/l

Material

The must from the *'Laurot'* (rosé) variety was chosen for this experiment.

Sulphur dioxide was used in solid form – as $K_2S_2O_5$ (also called pyrosulphate), which was quantitatively converted into a pure form (from a practical point of view).

The mixture of MCFAs: for this experiment a MCFAs mixture with the ratio $C_8 : C_{10} : C_{12} = 20 : 70 : 10$ was chosen for every combination with or without sulphur dioxide (except the control variant, which was without any treatment). 100 mg of MCFAs (in the appropriate ratio) was dissolved in an aqueous solution of potassium hydroxide (volume 1L).

The variants and combinations of dosages of SO_2 and MCFAs are shown in Table 2 below.

Table 2 Variants and combinations of dosages of MCFAs and SO_2

MCFAs/SO_2 [mg/l]	0	20	30	40	50	60
0	0/0	0/20	0/30	0/40	0/50	0/60
5	5/0	5/20	5/30	5/40	5/50	5/60
10	10/0	10/20	10/30	10/40	10/50	10/60
20	20/0	20/20	20/30	20/40	20/50	20/60

Methods

Determination of analytical parameters of must was completed after the application of the MCFAs mixture and SO_2, and was measured by the ALPHA analyser, which is working on principle infrared spectroscopy with Fourier's transformations (FTIR) using the sampling technique Attenuated Total Reflection (ATR). Basic analytical parameters were established on this analyser; these parameters were: titratable acids, pH, alcohol content and residual (fermentable) sugar content (Table 1).

The residual sugar content after treatment (application of MCFAs and SO_2) was measured with Rebelein's method. The principle of this method consists of iodometric determination based on the difference in consumption of sodium thiosulphate on titration of a defined concentration of the copper cation and its balance after reaction with reducing sugars of wine without removal of interfering substances (Balík 1998).

RESULTS

This study examines the identification of a substance to complement sulphur dioxide as a wine additive, which could mean a significant reduction of sulphur dioxide dosages. One of the very problematic aspects of making wine with a higher content of residual sugar is stopping alcoholic fermentation. This step usually needs a higher dose of SO_2. The reduction of this dose could be supported by the application of a MCFAs mixture (C_8, C_{10} and C_{12}). This MCFAs mixture is able to stop alcoholic fermentation, so the dose (and thus the content) of sulphur dioxide could be greatly decreased.

The most important parameter monitored in this experiment was the change in residual sugars content. By observing this change, the effectiveness of the MCFAs mixture in stopping alcoholic fermentation could been determined. The efficacy of the MCFAs mixture (octanoic, decanoic and dodecanoic acid) is summarized and commented upon in the figures below.

Table 3 Key to the charts

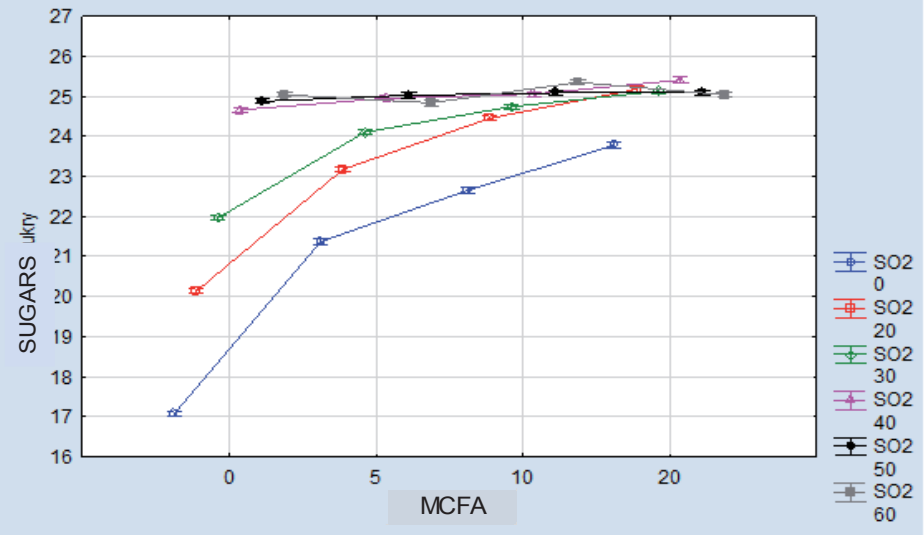
ABBREVIATION	EXPLANATORY NOTE	ABBREVIATION	EXPLANATORY NOTE
MCFA 0	Without addition of MCFAs	SO_2 0	Without addition of SO_2
MCFA 5	Addition of 5 mg/l MCFAs	SO_2 20	Addition of 20 mg/l SO_2
MCFA 10	Addition of 10 mg/l MCFAs	SO_2 30	Addition of 30 mg/l SO_2
MCFA 20	Addition of 20 mg/l MCFAs	SO_2 40	Addition of 40 mg/l SO_2
Sugars	Content of residual sugars [g/l]	SO_2 50	Addition of 50 mg/l SO_2
Days	Measurement 20. – 29. 11. 2015	SO_2 60	Addition of 60 mg/l SO_2

The efficacy of sulphur dioxide depending on the different doses of MCFAs mixture

MCFAs were investigated for the purpose of reducing the dosage (and the content) of sulphur dioxide in wine; not necessarily the total elimination of SO_2. Through analysis of the content of residual sugar after the addition of the MCFAs mixture and SO_2, it was possible to compare the efficiency of MCFAs and SO_2 during the halting of alcoholic fermentation. Through testing different variants and combinations of dosages of MCFAs mixture and SO_2, it was possible to find the appropriate solution for reducing the amount of sulphur dioxide required to be added. The following chart (Figure 1) shows the efficacy of sulphur dioxide in combination with MCFAs and the mutual influence of these substances. It can be said that the higher the content of fermentable sugars, the higher the effectiveness of the inhibitors. The SO_2 dose could be up to several dozen of mg/l lower in a suitable combination with the MCFAs, which confirms the idea that MCFAs enhance the efficiency of SO_2. The experiment was designed to consider the parameters influencing the development of fermentable sugar content - time, sulphur dioxide dose and medium chain fatty acid mixture.

The obtained results are shown at Figure 1 and from that follows the conclusion that the efficacy of sulphur dioxide in dosages more than 40 mg/l SO_2 (colours pink, black and grey) was almost identical in these variants. These dosages of SO_2 were able to stop alcoholic fermentation without the addition of the MCFAs mixture. What is more interesting is that the dosages of SO_2 in concentration 20 and 30 mg/l in combination with MCFAs mixture in concentrations 10 and 20 mg/l seem very promising, insofar as the inhibition of alcoholic fermentation is more than noticeable. The combination of a convenient dose of MCFAs mixture and a reduced dose of SO_2 (less than 40 mg/l) is more effective than a dose of the SO_2 by itself, even in the case of a higher concentration of SO_2. This supports the conclusion that a reduction in the dosage of sulphur dioxide is possible using the MCFAs mixture.

Figure 1 The degree of influence of SO_2 on the inhibition of the yeasts according to doses of MCFAs mixture is shown by the development of content of residual sugars (g/l)

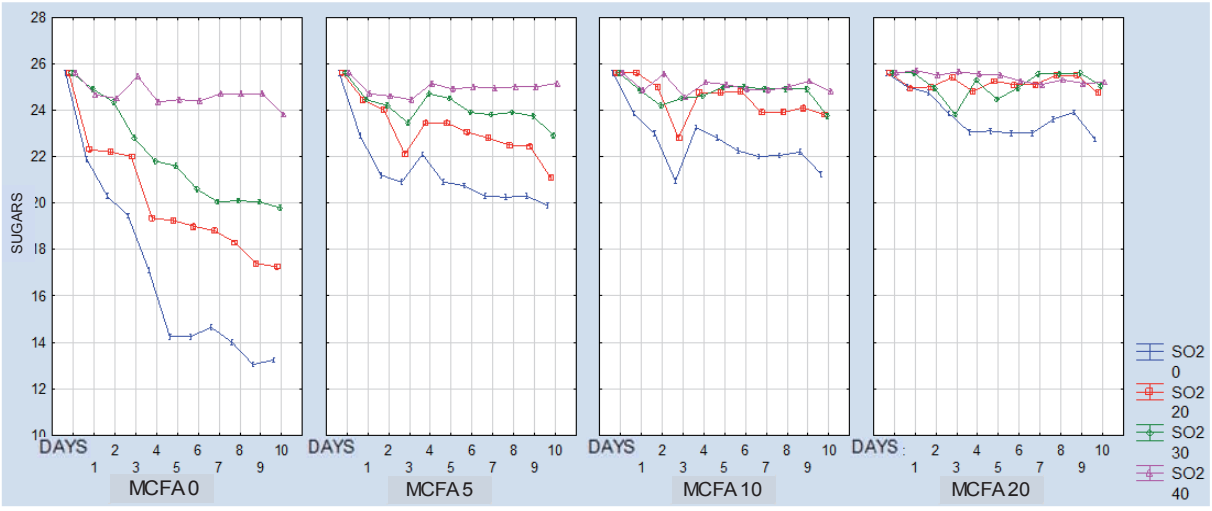


The development of the content of residual sugar depending on all monitored parameters

Figure 2 (below) provides the most comprehensive view of the entire experiment. The development of the content of residual sugars for all samples with the addition of sulphur dioxide 0–40 mg/l (included) is shown. The samples with doses of 50 and 60 mg/l were not considered in this evaluation, because those doses of SO_2 can stop alcoholic fermentation without the addition of the MCFAs mixture (Figure 1).

The experiment was designed to consider the parameters influencing the development of fermentable sugar content: time, sulphur dioxide dose and dose of MCFAs mixture. Applying those parameters, it was possible to find the most effective combination of the inhibitors (i.e. the MCFAs and SO_2) and select the best variant for stopping alcoholic fermentation, with the aim of making wine with a higher residual sugar content.

Figure 2 The development of residual sugar content depending on the added amount of MCFAs mixture, SO_2 and on time



The most significant results for the intended purpose are contained in the box labelled "MCFA 10" (Figure 2); this is the dose 10 mg/l of MCFAs mixture in combination with 20–40 mg/l of SO_2 (colours red, green and pink). This concentration is optimal for wineries, even if the goal of production is a wine with a higher residual sugar content. The change of residual sugars was, in this case, minimal. A little change can be attributed to the phasing of alcoholic fermentation when the measure was made, or potential measurement errors. Slowing of alcoholic fermentation can be observed from the dose 5 mg/l of MCFAs mixture even with zero addition of sulphur dioxide. Obviously, the variants

with the highest addition of MCFAs mixture and SO_2 were the most effective (the box "MCFA 20"). There are only minimal differences in effectiveness between the different SO_2 dosages applied in this variant (units percent). The one outlier is where the MCFAs mixture was applied by itself, without SO_2. Also in this case we can see obvious slowing of alcoholic fermentation.

Table 4 Statistical evaluation – Fisher's test LSD

CONTENT OF SUGAR	AVERAGE MCFA	A	B
20	0.000000	****	
23	2.500000	****	****
24	7.500000	****	****
25	8.928571		****

The evaluation in Table 4 shows that the MCFAs averages (with the dose 30–40 mg/l SO_2) at similarity level $\alpha = 0.05$ were divided into three homogeneous groups. The MCFAs dose 0 mg/l is group A, the doses of MCFAs 2.5 and 7.5 mg/l are the group AB and the dose of MCFAs 8.93 mg/l (rounded) is the group B. The difference between the doses 0 and 8.93 mg/l of MCFA is confirmed by this test.

DISCUSSION

The MCFAs C_8, C_{10} a C_{12} were investigated many years ago (Garbay et al. 1995, Guilloux-Benatier et al. 1998, Viegas and Sá-Correia 1997, Cabral et al. 2001) and their ability to inhibit alcoholic fermentation and possibly halt undesirable malolactic fermentation was confirmed in several studies (Baroň 2013, Budínová 2016).

The structure of the experimental part of this study was relatively simple, but the results are conclusive. The efficacy of MCFAs was confirmed. A handicap of this experiment was a small spectrum of monitored factors that could affect the effectiveness of MCFAs.

The results confirm previous findings that a dose of MCFAs mixture for wine production of 10 mg/l is adequate to enable a reduction in the dose of sulphur dioxide by several tens of milligrams per litre (Baroň 2013). However, several questions remain. How high a dose would be required at a later stage (for example, during storage) to ensure microbial stability without refermentation. This problem relates to the possible development of resistance of the yeasts to MCFAs, because yeast cells can build their own defence mechanism. According to Cabral (2001), yeasts that have been exposed to the sub-lethal effects of octanoic acid at their inception are more resistant to the inhibitory effect of medium chain fatty acids. Further, he presents a very interesting finding about so-called "cross-resistance"; if a cell is infected by MCFAs and the permeability is increased, ethanol can flow into the body of the yeast and, whilst it could kill the yeast, the moderate stress induced by ethanol can also force the yeast to activate its own defence mechanism. Further, the yeast can use this increased permeability for faster transport of octanoic acid out of the cell. The issues of toxicity and time of effectiveness of the MCFAs require further clarification.

Budínová (2016) states in her thesis that the dose 20 mg/l MCFAs mixture without adding SO_2 was not sufficiently effective and, consequently, disadvantageous for the winemakers. This proposition is consistent with the results of our study. However, where the results differ is in the variant with 5 mg/l MCFAs and 60 mg/l SO_2. While this dose (5 mg/l) of MCFAs mixture (and also SO_2 in concentration 60 mg/l itself) was quite sufficient in our study, Budínová's research suggested that these doses were less effective than our study suggested. This difference could be explained by the content of residual sugar contained in the tested wines differing at the moment when the MCFAs and SO_2 were applied. In our experiment, the content of residual sugar was lower by 10–15 g/l than in Budínová's experiment.

It is very difficult to discuss results where there are only a few comparable studies, even where those studies resulted in similar conclusions.

CONCLUSION

This research was focused on identifying alternative substances to sulphur dioxide. Going back to the basics – SO_2 can stop alcoholic fermentation and ensure microbial stability of the wine. However,

the risk of the yeast and the other microorganisms developing resistance is considerable, in addition to the possible health risks. It is no wonder that the reduction of SO_2 is one of the most discussed topics in the contemporary wine world. Our study proves that the amount of sulphur dioxide can be reduced by tens of milligrams thanks to the MCFAs mixture even if the aim is to produce a wine with a higher content of residual sugar. The ideal concentration of the dose of the MCFAs mixture is 10 mg/l. This dose and the method of application is acceptable, easy and inexpensive. The MCFAs mixture is a new method for the production of wine with a higher content of residual sugar. This method is not technologically difficult and therefore it is accessible to smaller winemakers. This method has minimum risks, because MCFAs are naturally occurring and fit for human consumption. The amount of MCFAs residue after the application of the MCFAs mixture as a treatment with the aim to inhibit yeasts differs only fractionally from wine which has not had the MCFAs mixture added. MCFAs cannot be detected in the organoleptic properties, if they are used sparingly. The middle chain fatty acids mixture seems like an ideal aid in the wine production process.

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PHTHALATE ESTERS IN SOUSAGES PACKAGED INDIVIDUALLY

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Abstract: This study deals with the occurrence of phthalic acid esters in sausages and their packaging depending on the time. The identified phthalic acid esters were dibutyl phthalate (DBP) and diethylhexyl phthalate (DEHP). DBP and DEHP are the most used plasticizers. It exist a risk of migration phthalic acid esters to the food. In sausages was found concentration of DBP from 7.01 to 18.79 μg/g of original raw material, concentration of DEHP from 4.81 to 23.45 μg/g of original raw material. But the statistically proven difference between concentrations in sausages established immediately after purchase and on expiration date was not found. The concentration in packaging was detected DBP from no detected level to 83.92 μg/dm^2, DEHP from 0.62 to 247.91 μg/dm^2. Statistically prove different between concentrations established immediately after purchase and on expiration date was found only for the concentration of DBP per gram of plastic, and per dm^2 in plastic casing, then for wrap with label for concentration of DEHP per gram of plastic, and per dm^2.

Key Words: contaminant, wrap, pollutant, DBP, DEHP

INTRODUCTION

Plastic contains its polymer and other components: antioxidants, antistatic agents, plasticizers, stabilizers. Some components, mostly plasticizers and residual monomers of plastic, migrate from food packaging into food. Main plasticizers are used esters of phthalic acid (Rupp 2003). Plasticizers impart better processability and flexibility to plastic material (Han and Gennadios 2005). Plasticity, elasticity and shrinkage are the important properties of plastics. Plastic polyvinyl chloride (PVC), widely used in non-softened plastic foils, contains up to 5% plasticizer, the most commonly used plasticizers are dibutyl phthalate, di-(2-ethylhexyl) phthalate, dioctyl adipate (Kačeňák 2001). The migration limits for food plastic contact materials are regulated by Commission Regulation (EU) No 10/2011, for DEHP 1.5 μg/g of food, for DBP 0.3 μg/g of food, an overall migration limit is set of 10 mg per 1 dm^2 (EU 2011). Phthalates are lipophilic, therefore foods with higher fat content have higher phthalate content to tens of μg phthalates/g, other foods contain up to units μg/g (Velíšek and Hejšlová 2009). The highest concentration of DBP was found in cheese and sauces of 62 μg/g and the highest concentration of DEHP in fish 32 μg/g (Clark et al. 2003). The amount of phthalates in food depends on contamination and the process of processing, not just on the amount of fat (Sharman et al. 1994). Esters of phthalic acid are carcinogenic and teratogenic in acute toxicity cause dizziness, nausea, somnolence, hallucinations (Velíšek and Hajšlová 2009). According to the Decree of the Ministry of Agriculture of the Czech Republic No. 69/2016 Coll. sausages belong to heat-treated meat products, heated at a minimum of 70 °C in all parts for 10 minutes (Česká Republika 2016). Storage must be at temperature max. 5 °C (Pipek 2012). Covers for meat products can be used natural or artificial casing. The natural casings are better shaped, and they are used for the highest quality products. The natural casings are for example: pork casing, mutton casing, bovine intestine.

Artificial casings, which are non-consumable, are particularly suitable for cooked products, the coating is resistant to heating (Fletcher and Bretherton 2013).

MATERIAL AND METHODS

Chicken sausages for analysis were purchased in the Czech Republic. Chicken sausages and their packaging were analyzed. Sausages were type of sausage wrapped separately in an artificial coat and the sausages in the artificial coats were packaged in the next package (outer package). Frankfurters were analyzed in the six repeat immediately after purchase and in the six repeat on the day of expiration. Packages of these sausages were analyzed in three reps immediately after the purchase of sausages and on the day of sausages expiration. The covers were divided into plastic casing, outer wrap, outer wrap with label. The plastic packaging was analyzed by HPLC according to the method of Gajdůšková et al. (1996), sausage samples according to the method of Jarošová et al. (1999). Software Agilent Chemstation for LC and LC/MS systems was used for evaluation of concentrations. The results were prepared by Microsoft Excel and STATISTICA. Shapiro-Wilk's test for normality was used ($\alpha = 0.05$), followed by t-test for dependent samples; or nonparametric test to compare two dependent samples. The DBP and DEHP concentrations after purchase with concentrations at the time of expiration were compared for the same types of samples.

RESULTS AND DISCUSSION

The concentration (Table 1 and Table 2) of DBP in sausages was ranged from 7.01 to 18.79 μg/g of original raw material, the concentration of DEHP from 4.81 to 23.45 μg/g of original raw material. The values of DBP in sausages measured immediately after purchase was determined at 12.80 ± 3.68 μg/g of original raw material, DEHP at 10.13 ± 3.78 μg/g of original raw material. The concentration of DBP in sausages on expiration date was detected to 12.43 ± 3.44 μg/g of original raw material, DEHP to 11.74 ± 5.62 μg/g of original raw material. Statistically was found no difference between the concentration of DBP and DEHP in sausages determined immediately after purchase and on expiration date ($p > 0.05$).

Table 1 Concentration of DBP and DEHP in sausages immediately after purchase

Sample	DBP μg per gram of fat	DEHP μg per gram of fat	DBP μg per gram of dry matter	DEHP μg per gram of dry matter	DBP μg per gram of original raw material	DEHP μg per gram of original raw material
1	135.34	87.52	52.54	33.97	18.79	12.15
2	65.17	51.21	25.16	19.77	9.07	7.12
3	100.90	114.73	38.12	46.63	13.48	16.49
4	115.11	62.52	45.21	24.55	16.08	8.73
5	68.20	74.99	29.22	32.13	10.43	11.47
6	61.85	33.26	25.36	13.64	8.95	4.81

Table 2 Concentration of DBP and DEHP in sausages on expiration date

Sample	DBP μg per gram of fat	DEHP μg per gram of fat	DBP μg per gram of dry matter	DEHP μg per gram of dry matter	DBP μg per gram of original raw material	DEHP μg per gram of original raw material
7	57.93	70.63	23.96	29.21	8.30	10.12
8	104.89	95.72	41.47	37.84	14.36	13.11
9	120.49	54.22	45.55	20.49	15.90	7.15
10	107.43	70.25	43.41	28.39	14.78	9.66
11	99.90	164.61	41.70	68.71	14.23	23.45
12	50.04	49.78	20.42	20.31	7.01	6.97

The value (Table 3 and Table 4) DBP concentration of plastic casing immediately after purchase was detected by 18.52 ± 2.427 μg/g of plastic, and on expiration date by 4.11 ± 0.567 μg/g of plastic. The value DEHP concentration of plastic casing immediately after purchase was detected to 8.66 ± 4.895 μg/g of plastic, and on expiration date to 3.33 ± 1.425 μg/g of plastic. DBP concentration of outer wrap was determined at 40.08 ± 4.164 μg/g of plastic after purchase, on expiration date at 26.65 ± 21.496 μg/g of plastic. DEHP concentration of outer wrap was determined to 5.97 ± 2.376 μg/g of plastic, on expiration date to 3.94 ± 1.676 μg/g of plastic. And the concentration of DBP in wrap with label was found after the purchase to be 18.65 ± 13.189 μg/g of plastic, on the date of expiration to be 28.15 ± 14.108 μg/g of plastic. The concentration of DEHP in wrap with label was determined at 34.46 ± 32.955 μg/g of plastic after the purchase, on the date of expiration at 98.43 ± 27.663 μg/g of plastic. The concentration DBP per gram of plastic, and per dm^2 in plastic casing after the purchase and on the date of expiration was found statistically significant difference, by DEHP was not found statistically significant difference. In outer wrap for DBP and DEHP concentration was not found significant difference. But for wrap with label was found statistically significant difference for concentration DEHP per gram of plastic, and per dm^2 after the purchase and on the date of expiration. The concentration of DBP for wrap with label was not significantly different.

Table 3 Concentration of DBP and DEHP in the packagings of sausages immediately after purchase

Samples	DBP μg per gram of packaging	DEHP μg per gram of packaging	DBP μg per dm^2	DEHP μg per dm^2
1) plastic casing	20.12	1.76	7.13	0.62
2) plastic casing	20.34	11.62	7.80	4.46
3) plastic casing	15.09	12.60	6.13	5.12
1) outer wrap	34.29	9.33	24.72	6.72
2) outer wrap	43.91	4.14	31.52	2.97
3) outer wrap	42.05	4.45	31.76	3.36
1) wrap with label	ND	81.00	ND	144.03
2) wrap with label	27.80	13.33	48.88	23.44
3) wrap with label	28.15	9.05	50.50	16.23

Legend: ND no detected

Table 4 Concentration of DBP and DEHP in the packagings of sausages on expiration date

Sample	DBP μg per gram of packaging	DEHP μg per gram of packaging	DBP μg per dm^2	DEHP μg per dm^2
4) plastic casing	3.33	3.15	1.32	1.25
5) plastic casing	4.34	5.16	1.79	2.13
6) plastic casing	4.66	1.68	1.85	0.67
4) outer wrap	52.64	2.95	39.86	2.24
5) outer wrap	ND	2.57	ND	1.97
6) outer wrap	27.31	6.30	21.17	4.89
4) wrap with label	25.67	135.90	46.82	247.91
5) wrap with label	12.25	69.96	22.16	126.57
6) wrap with label	46.54	89.42	83.92	161.26

Legend: ND no detected

Puškárová et al. (2013) reported in the study that the amount of DEHP and DBP in the packaging of meat product ranged from 5.56 to 32.83 μg/dm^2. In our case, the sum of DBP and DEHP was 1.97–294.74 μg/dm^2. DBP was ranged from no detected to 83.92 μg/dm^2, DEHP from 0.62 to 247.91 μg/dm^2.

CONCLUSION

It can be a positive finding for the health of food consumer, that the concentration of phthalates in sausages did not change rather, that it did not increase during storage. The statistically significant difference was determined for the concentration of DBP in the plastic casing and for the DEHP concentration in the wrap with label. On the date of expiry the DBP concentration in the plastic casing was lower than in the day of purchase and the DEHP concentration in the wrap with label was lower in the day of purchase than on expiration date.

ACKNOWLEGEMENTS

The research was financially supported by the grant IGA FA MENDELU No. IP 11/2017 ″Phthalates in packaged foods and used packaging″

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THE SENSORY EVALUATION OF YOGHURTS WITH CHIA FLOUR, QUINOA FLOUR, NOPAL POWDER, APPLE FIBER AND BAMBOO FIBER

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Abstract: This work deals with sensory evaluation of yoghurt with the addition of fibers and raw materials with natural high fiber content, which brings many positive effects on human health. Chia flour, quinoa flour, nopal powder, apple fiber and bamboo fiber were added to the yoghurt in the amounts of 1%, 3% and 5%. The work shows the evaluation in the first week after production of the yoghurt. Freed whey, acceptability of color, sour aroma, acceptability of aroma, viscosity, texture, stickiness, sandiness, intensity of sourness, acceptability of taste and total impression were used as the descriptors. Very badly assessed were the yoghurts with 5% and 3% quinoa flour and with 5% and 3% nopal powder and very well evaluated in overall impression were the yoghurts with 1% bamboo fiber and nature yoghurt. Therefore, according to this study, the addition of 1% bamboo fiber to nature yoghurt as fiber fortifier are recommendable, but the highest concentrations of quinoa flour and nopal powder to yoghurt cannot be suggested.

Key Words: dietary fiber, health, dairy product, sensory evaluation, yoghurt

INTRODUCTION

Milk and milk products have positive impact on human health, especially as it contains calcium, which decreases the risk of hypertension, colon cancer and osteoporosis. It also contains conjugated linoleic acid (CLA), which protects against obesity, cardiovascular disease, cancer and improves immunity (Miller et al. 1999). Yoghurt counts as a functional food, because it contains probiotics. Probiotics are living microorganisms, which bring health benefits to the consumer are: bifidobacteria, lactic acid bacteria, some yeasts, etc. (Plocková 2012). A functional food must have at least 10^6 viable microorganisms per gram of food to have a positive effect. Positive effects of probiotics include the restoring of positive microflora in the colon, strengthening the immune system, reducing lactose intolerance, increasing calcium absorption, synthesizing some vitamins, bacteriocins, lowering total cholesterol and LDL cholesterol (Kalač 2003).

Into prebiotics, an indigestible food ingredient (fiber) that promotes growth of the colon microflora, belong indigestible oligosaccharides, e.g. inulin. Inulin is split into oligofructosans with 3 to 8 units of fructose, which were used into non-alcoholic beverages, yoghurts, other dairy products, pastries and marmalades (Kalač 2003). Food fiber that belongs to carbohydrates are not digested and absorbed in the intestine, but are decomposed in the colon by symbiotic microorganisms (Komprda 2009). It is recommended to receive 0.3 g of prebiotics per kilogram bodyweight for men and 0.4 g per kilogram bodyweight for women. This fiber intake contributes to the growth of desirable microflora in the intestines, reducing consumer energy consumption, eliminating constipation, strengthening the immune system, preventing rectal and colon cancer, improving calcium utilization, and lowering cholesterol levels (Kalač 2003). The dietary intake of fiber decreases the occurrence of obesity and disease of cardiovascular system (Slavin and Lloyd 2012). Synbiotics are the products containing both prebiotics and probiotics (Plocková 2012).

Chia seeds are a good source of dietary fiber, omega-3 fatty acids and antioxidants, which protects against cancer, ageing, and liver and heart diseases. Chia does not contain gluten. Chia seed also has a positive effect on hypertension, dyslipidemia, diabetes, depression, immunity and vision and other (Ullah et al. 2016).

Dehulled quinoa flour, a cultivar grown in Colorado, USA contains about 58.1% starch, 2.7% sugar, 15.6% protein, 4.6% fat, 8.9% total dietary fiber (insoluble 7.7%, soluble 1.2%) and 2.3% ash. Minerals included are particularly potassium, calcium, magnesium, phosphorus, iron, from the vitamins B-group vitamins (Ranhotra et al. 1993). Quinoa does not contain gluten and flour of the quinoa is used for example in bakery products (Bavec and Bavec 2007).

The dietary intake of fiber decreases occurrence of obesity and disease of cardiovascular system (Slavin and Lloyd 2012).

Nopal cactus, thanks to contents of vitamins, polyphenols, amino acids and polyunsaturated fatty acids, shows antioxidant, antimicrobial, neuroprotective, hypoglycemic and anti-inflammatory effect (El-Mostafa et al. 2014). In the study (Uebelhack et al. 2014) was found that cactus fiber was binding on the dietary fat and caused fat to be removed from the body, which can reduce the weight of consumer.

In the study (Jensen et al. 2014) was found that fibers from apple peel have an anti-inflammatory and an antioxidation effect. Also apples and apple products protects against asthma, cancer and Alzheimer's disease (Hyson 2011). Healthy properties were also contributed by the phytochemicals included in apples (chlorogenic acid, catechin, quercetin, phloridzin), which had shown strong antioxidant activity (Boyer and Liu 2004).

In the study (Li et al. 2016) was discovered that bamboo fiber from shoot reduced more effectively weight of mice than the intake of cellulose fiber. Bamboo fiber was absorbed very good in water (Yueping et al. 2009).

MATERIAL AND METHODS

The raw materials were purchased in the Czech Republic. The yoghurts were made at the Department of Food Technology at Mendel University. The milk for preparation of yoghurt originated from the south Moravian region, from Holstein dairy cows. The percentage values of composition was determined lactose to 4.50%, protein to 3.42%, fat to 3.50% and titratable acidity was established to 6.7 SH by the stirred coagulated method. The milk was pasteurized by 85 °C for 5 minutes, then the milk was cooled down to 36 °C, after which it was inoculated with a (0.5% by weight) starter culture of original Bulgarian yogurt (bulgaricus.cz, GENESIS LABORATORIES, Bulgary) and fermented at 36 °C for 18 hours. Thereafter was yoghurt homogenized for 5 min. Yoghurt was divided into 16 groups, one group stayed plain yoghurt, into the others were added chia flour, quinoa flour, nopal powder, apple fiber and bamboo fiber in amount of 1%, 3% and 5% by weight. The yoghurts were stored at 4 °C.

The sensory evaluation was conducted at the Department of Food Technology at Mendel University, in premises designed for sensory assessment according to ČSN ISO 8589 (560036). The evaluation was done by eleven trained sensory assessors. This descriptors were evaluated: freed whey, acceptability of color (0 = unacceptable,100 = acceptable), sour aroma (0 = unintensive, 100 = very intensive), acceptability of aroma (0 = unacceptable,100 = acceptable), viscosity (0 = sparse, 100 = dense), texture (0 = grainy, 100 = smooth), stickiness (0 = without stickiness, 100 = extreme), sandiness (0 = without sandiness, 100 = very sandiness), taste of sour intensity (0 = without sour, 100 = very sour), acceptability of taste (0 = unacceptable, 100 = excellent), total impression. The scales for sensory evaluation used was a graphical (0–100) and a categorizing scale (for freed whey and total impression). The sensory evaluation was performed weekly for 3 weeks. However, this study included a sensory assessment at the first week, because yoghurts with chia seeds could not assessable at the second week and at the third week yoghurt with 5% quinoa flour could not be assessed either. The statistic comparison of mean values with evaluations in first, second and third week for the description the taste acceptability was detected congruity of mean values ($\alpha = 0.05$).

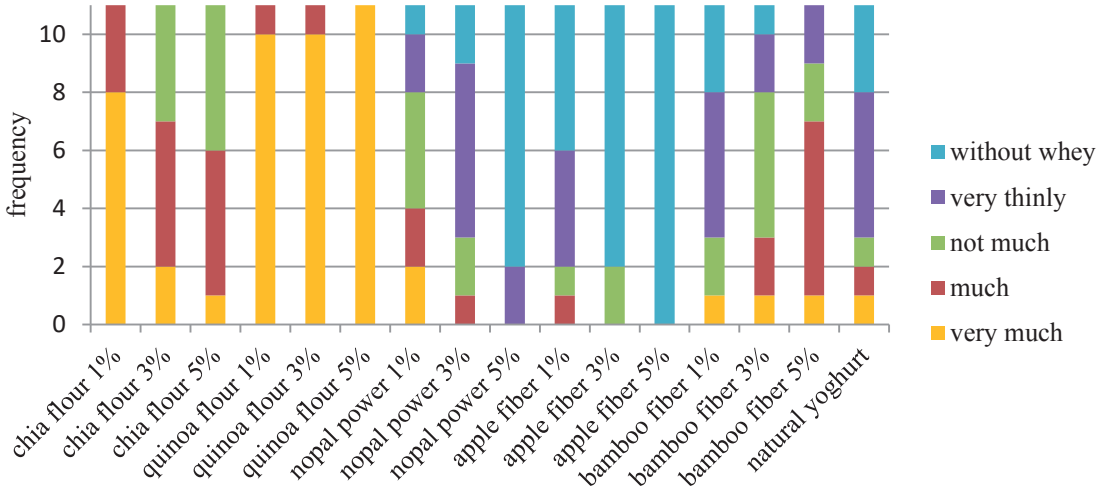
The results were evaluated using Microsoft Excel and STATISTICA.

This research was carried out partially in Biotechnology Pavilion M, financed by the OP VaVpI CZ.1.05/4.1.00/04.0135 project at the Department of Food Technology at Mendel University in Brno.

RESULTS AND DISCUSSION

The whey in yoghurt with 5% quinoa flour was evaluated with all 11 assessors as very much loosed, and in yoghurt with 5% apple fiber as without whey. The yoghurts with quinoa flour all three variants was assessed whey as very much loosed. All frequency for description freed whey are shown in Figure 1.

Figure 1 Frequency of description freed whey for yoghurts with chia flour, quinoa flour, nopal powder, apple fiber, bamboo fiber and for natural yoghurt, evaluated 1 week after production

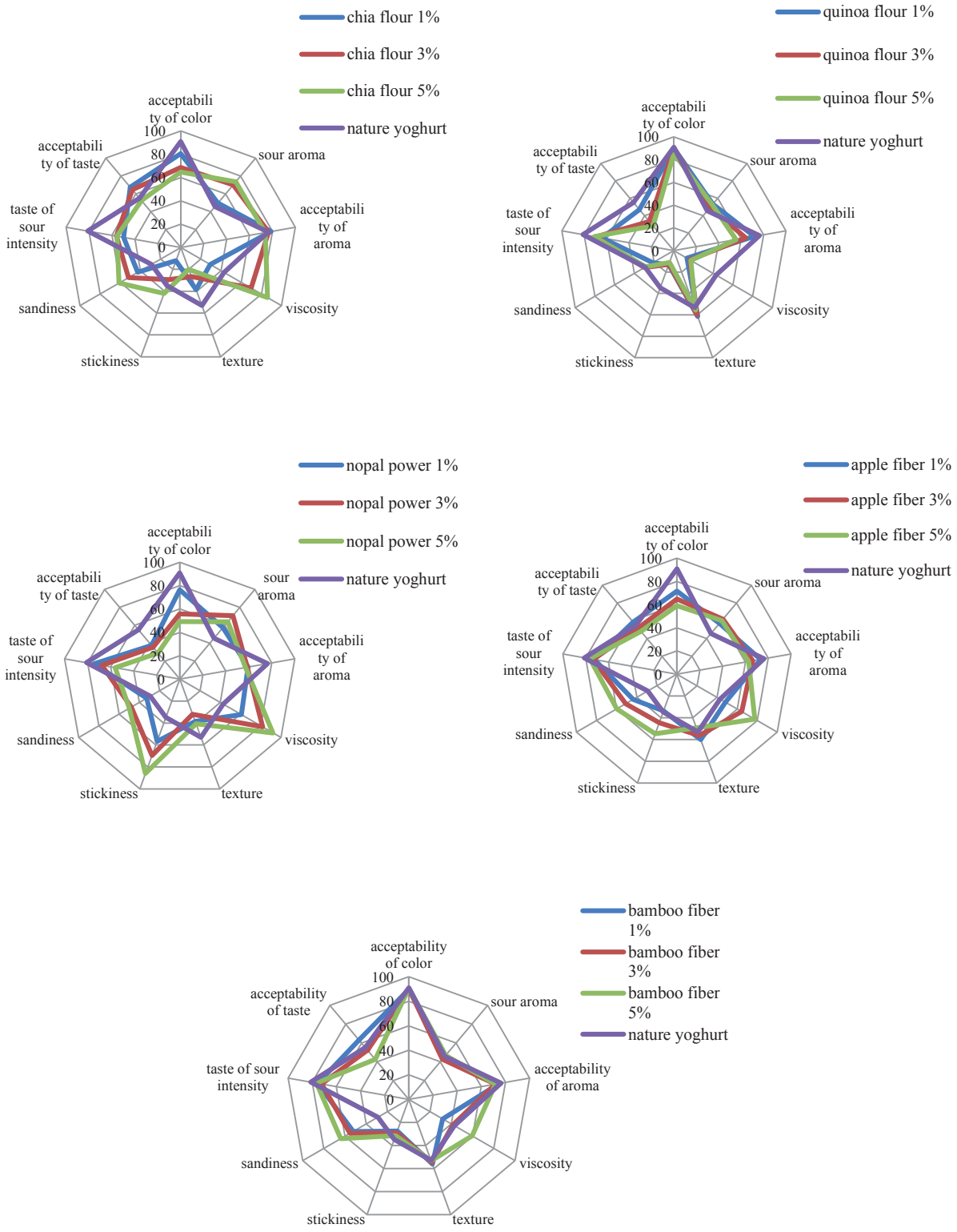


The yoghurts with nopal powder (Table 1 and Figure 2) were badly evaluated for color, which was detected as green, and the yoghurts with white color were determined higher values: nature yoghurt and with bamboo fiber. Aroma was evaluated good by yoghurts with chia flour and bamboo fiber. The densest were yoghurts with nopal powder and the sparsest all yoghurts with quinoa flour. The grainy was discover by yoghurts with chia flour. Sandiness was found typically for yoghurts with bamboo fiber. In yoghurts with chia flour was ascertained low intensity of sour taste. Great taste was evaluated by yoghurts with chia flour and unfavourable by quinoa flour.

Table 1 The yoghurts with the highest and the lowest value for followed descriptors

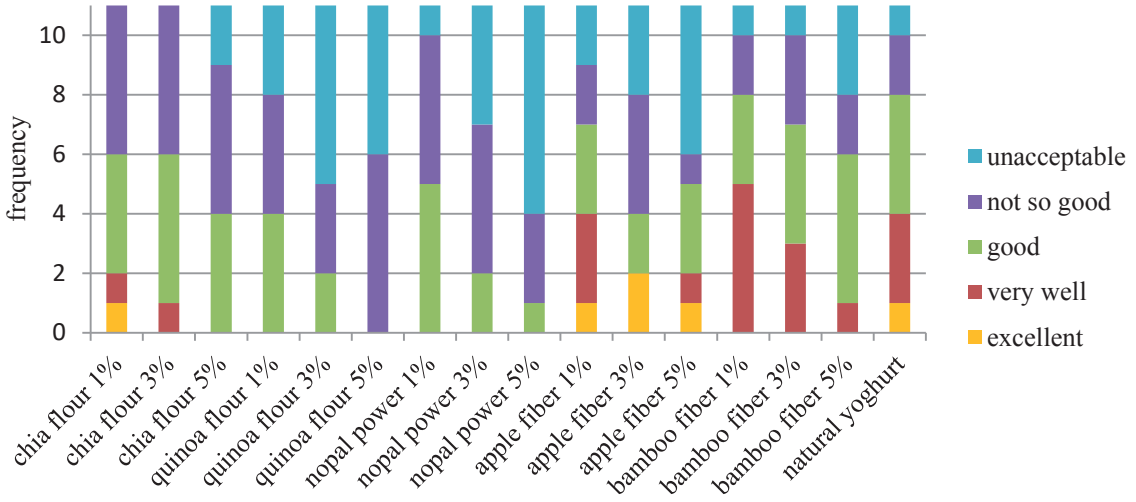
Descriptor	The highest value	The lowest value
Acceptability of color	natural yoghurt	nopal powder 5%
Sour aroma	chia flour 5%	bamboo fiber 3%
Acceptability of aroma	chia flour 1%	quinoa flour 5%
Viscosity	nopal powder 5%	quinoa flour 1%
Texture	quinoa flour 1%	chia flour 5%
Stickiness	nopal powder 5%	quinoa flour 5%
Sandiness	bamboo fiber 5%	quinoa flour 1%
Taste of sour intensity	natural yoghurt	chia flour 1%
Acceptability of taste	chia flour 1%	quinoa flour 5%

Figure 2 Sensory evaluation for yoghurts, divided into groups according to the added raw material, in descriptors: acceptability of color, sour aroma, acceptability of aroma, viscosity, texture, stickiness, sandiness, taste of sour intensity, acceptability of taste



In description total impression (Figure 3) was evaluated very badly the yoghurt with 5% quinoa flour and with 5% nopal powder. Very positive was assessed the yoghurt with 1% bamboo fiber and nature yoghurt.

Figure 3 Frequency of descriptor total impression for yoghurts with chia flour, quinoa flour, nopal powder, apple fiber, bamboo fiber and for natural yoghurt, evaluated 1 week after production



In the study (Staffolo et al. 2004), which explored the rheological and sensory properties of yoghurts with wheat, apple, bamboo fiber and inulin was concluded, that yoghurts with bamboo and wheat fiber had a firm texture (too high values of compression force) and that the firm texture caused better evaluation of texture descriptor. However, in our study the densest yoghurts (yoghurts with nopal powder) were evaluated in the total impression as the worst, which was probably caused by the green color or because of the stickiness of nopal yoghurts.

CONCLUSION

The yoghurt, which looked like nature yoghurt (white, similar density) received the best results in total impression - the yoghurt with 1% bamboo fiber. However, the bamboo fiber in higher concentrations caused sandiness. The yoghurt with 5% quinoa flour was evaluated badly, not only for total impression, but also for acceptability of the taste. Besides, the yoghurts with quinoa flours were detected very sparse. The yoghurt with 5% nopal powder, which was evaluated with low total impression, was unacceptable in color, density and had the highest value for stickiness. Low addition of bamboo fiber to yoghurts can be recommended, on the other hand, high concentration of quinoa flour and nopal powder is not very suitable.

ACKNOWLEGEMENTS

The research was financed by the following grant: IGA FA MENDELU No. TP 2/2017 "Effect of additives on the rheological behaviour of food stuffs and raw materials for their production".

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INFLUENCE OF PRE-CULINARY TREATMENT ON MICROBIOME OF EDIBLE INSECT *TENEBRIO MOLITOR*

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Abstract: Entomophagia is currently only a marginal issue in the Czech Republic. It has been gaining more supporters in recent years. Thus, it is necessary to address even its health safety. For this reason, we paid attention to the microbiological quality of the flour beetle and an influence of a pre-culinary treatment on number of microorganisms. Large numbers of microorganisms were found in live larvae. Their highest values were reached by the total plate count (TPC) - 3.3 x 10^8 CFU/g, *Enterobacteriaceae* family 4.8 x 10^7 CFU/g. After sacrificing and drying, the number of microorganisms was reduced to 1.6 x 10^3 CFU/g in TPC and *Enterobacteriaceae* family was completely eliminated.

Key Words: entomophagia, *Enterobacteriaceae,* microbiota, total plate count

INTRODUCTION

In western countries, the popularity of edible insects is growing as an alternative source of food. It is a potential source of high quality animal protein. Not all species of insects can be used for human nutrition. Insect, which is used for human nutrition, is called edible insects. The edible insects can be defined as all kinds of insects whose consumption does not cause a negative influence on the health state of a consumer (Borkovcová et al. 2009). However, there is only a limited amount of expert information on the hazards associated with insect consumption, especially the microbiological safety and durability of live insects sold for human consumption. Microbiome of insect intended for food and feed use can be generally divided into microorganisms naturally associated with insects, and microorganisms contaminating insects during breeding and processing.

Microorganisms present in insect guts are essential for its metabolism and survival. Insects are processed with their intestinal contents for both, food and feed purposes. Even when the intestinal content is emptied, residues remain in the substrate and can contaminate the insects. Microorganisms also occur on the surface of insects, and some of them may be pathogenic to insects. Papers on insect microbial microbes are very limited in the professional literature. Therefore, the question remains whether microbiome of insect intended for food and feed also contain microorganisms pathogenic to humans and livestock (EFSA 2015).

Currently, there are no microbiological parameters for edible insects in the Czech Republic. Grabowski and Klein (2016) report only Belgium and the Netherlands have incorporated microbiological parameters of edible insect products into their legislation.

The aim of the work was to find out the presence of significant groups of microorganisms in *Tenebrio molitor* larvae and influence of the pre-culinary treatment.

MATERIALS AND METHODS

Flour beetle (*Tenebrio molitor*) was purchased for the experiment from a farm engaged in insect farming for feed purposes (Farm PAPEK s.r.o., Hostim Czech Republic). The purchased insects were

held in plastic containers (57 x 39 x 28 cm) with feed substrate consisting of a mixture of wheat bran and wheat flour. Under these conditions, the insects were kept for 5 days to reduce the impact of the previous feed substrate. Afterward, the insect was removed from the substrate and placed for 3 days into an empty plastic container for perfect emptying. Then, live insect sample of 5 g was taken for microbiological analysis. The remaining insects were sacrificed by immersion into boiling water for 30 seconds. 5 g sample was taken from the dead insect. The remaining material was dried in a hot-air oven for 1 hour at 120 °C. After drying, the insects were milled to flour to take 5 g sample. Samples of live and dead insects were homogenized in a friction bowl. All samples were placed in sterile centrifuge tubes supplemented with 45 ml of sterile physiological solution and centrifuged for 1 minute.

In the samples obtained, the following groups of microorganisms were determined by standard procedures: total plate count on PCA (Biokar Diagnostics, France) at 30 °C for 72 hours, *Enterobacteriaceae* family on VRBG (Biokar Diagnostics, France) at 37 °C for 24 hours, *E. coli* and other coliforms on Rapid *E. coli* Agar (Bio Rad, Finland) at 37 °C for 24 hours, micromycetes on Chloramphenicol Glucose Yeast Extract Agar (Biokar Diagnostics, France) at 25 °C for 120 hours and *Enterococcus* bacteria on Slanetz-Bartley agar (Merck, Germany) at 37 °C for 72 hours. After the cultivation, colonies were counted and the results were expressed as CFU/g (colony forming units per one gram). The experiment was repeated three times.

RESULTS AND DISCUSSION

Monitored groups of microorganisms present basic criteria to assess microbiological quality of food. The highest counts were found in samples of live flour beetles. Specifically, it was total plate counts informing on total microbial contamination. Its value was 3.3×10^8 CFU/g. Another group was *Enterobacteriaceae* family, which may indicate faecal pollution, 4.8×10^7 CFU/g. Average counts of monitored microorganisms are stated in Table 1.

Table shows sacrificing of insects by immersing into boiling water has greatly reduced the number of microorganisms, and drying completely suppressed the occurrence of coliform bacteria, the *Enterobacteriaceae* family and the yeast.

Stoops et al. (2015) investigated microbiology of fresh flour beetle larvae. They determined the total plate count, the *Enterobacteriaceae* family, yeasts and moulds. The number of microorganisms per a group was reported in log CFU/g. After conversion to CFU/g, the following values were obtained: total plate count 2.0×10^8 CFU/g, *Enterobacteriaceae* 4.0×10^7 CFU/g and yeast and moulds 5.0×10^5 CFU/g. After comparing with our results, it was found the amount of all microorganisms, except yeasts and moulds, was higher in our samples. It is due to the fact that our samples were purchased from a farm producing insect for animal feed purposes.

Garofalo et al. (2016) investigated the microbiology of the dried flour beetle. They determined the total plate count, the *Enterobacteriaceae* family, yeasts and moulds. All monitored groups, except moulds, were found in counts less than 100 CFU/g. Moulds were detected in counts of 2.0×10^2 CFU/g.

Klunder et al. (2012) dealt with the microbiology of fresh larvae of flour beetle and larvae boiled for 10 minutes. They monitored only the total plate count and *Enterobacteriaceae.* Resulting values in fresh larvae were: 5.0×10^7 CFU/g for TPC and 6.3×10^6 for *Enterobacteriaceae*; and in boiled: 3.2×10^2 CFU/g for TPC and less than 10 CFU/g for *Enterobacteriaceae*. Compared to our values, these values are 10 times lower, which due to the conditions of breeding and longer cooking is in our opinion.

Table 1 Average counts of microorganisms in larvae Tenebrio molitor with different treatment (CFU/g

Category	TPC	*E.coli*/other coliform bacteria	*Enterobacteriaceae*	Yeast	Moulds	Enterococci
Live	$3.3 \pm 5.2 \times 10^{8}$	$0/\ 1.4 \pm 2.3 \times 10^{7}$	$4.8 \pm 7.5 \times 10^{7}$	$2.1 \pm 3.5 \times 10^{4}$	$1.67 \pm 2.5 \times 10^{2}$	$3.0 \pm 4.7 \times 10^{7}$
Scalded (100 °C 30s)	$5.6 \pm 8.4 \times 10^{3}$	$0/\ 3.1 \pm 5.4 \times 10^{2}$	$4.7 \pm 6.2 \times 10^{2}$	0	1.5 ± 2.6	$1.7 \pm 2.5 \times 10^{3}$
dried – flour (120 °C 1h)	$1.6 \pm 2.1 \times 10^{3}$	0	0	0	$2.0 \pm 2.3 \times 10^{1}$	1.52 ± 2.6

CONCLUSION

In our experiment, high microbial contamination of fresh flour larvae has been detected. It can be suppressed by sufficient culinary treatment. In our opinion, it is necessary to continue to address this issue and to set precise microbiological criteria for edible insects and their products. Last but not least, it is necessary to focus on the conditions of insects breeding intended for human consumption and to reduce the microbial contamination already in raw material.

ACKNOWLEDGEMENTS

The research was financially supported by the IGA grant, no. IP 24/2017.

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THE SENSORY QUALITY CHANGES IN BEEF LONGISSIMUS THORACIS ET LUMBORUM AND SEMIMEMBRANOSUS MUSCLES DURING AGING

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Abstract: This study was conducted to determine the effects of long time aging on beef *longissimus thoracis et lumborum* (LL) and *m. semimembranosus* (SM) sensory meat quality traits. Beef LL (n = 18) and SM cuts (n = 18) from slaughtered Czech Fleckvieh animals (n = 6) were assigned to storage at 2 °C for 7, 28, 42, 49 or 56 days. Increased aging time changed colour of LL and SM vacuumed beef steaks to 56 days of storage, SM became darker (L* = 41.16 to 38.02). In our case, total colour difference in SM storage exceeded the value earlier (42 days) than in LL (49 days), when the observer would find the difference between the two samples (ΔE*ab = 2), although there was a bigger change at LL, although at LL it reached higher final values (4.13 versus 3.81). Meat quality traits were affected by long ageing differently. Quality characteristics of Czech Fleckvieh meat from the two different muscles were analysed and surprisingly had an acceptable sensory evaluation.

Key Words: colour, pH, heifers, carcass, Czech Fleckvieh

INTRODUCTION

Post-mortem aging of beef carcasses and cuts is a natural process that usually improves tenderness under refrigerated conditions. Despite efforts by the industry to control the eating quality of beef, there remains a high level of variability in palatability, which is one reason for consumer dissatisfaction (Hocquette et al. 2014).

Flavour is very important component of the eating quality of meat and there has been much research aimed at understanding the chemistry of meat flavour, and at determining those factors during the production and processing of meat which influence flavour quality (Mottram 1998). The color of meat is one of the most important criteria for consumer acceptability, mainly because it is associated with quality (Boakye and Mittal 1996, Mancini et al. 2005). Generally, beef carcasses are usually held for 7−14 days at 3−5°C following slaughter than at 0–1°C (Kameník 2017). Beef aged in vacuum packaging is darker in colour when removed from the package due to the lack of oxygen (Lanari et al. 1987). Beef *m. semimembranosus* has faster stabilization of bright red color during blooming and is more tender. This is valuable information for the producers, since the best quality traits could be achieved after sufficient blooming and aging time (Wyrwisz et al. 2016). Meat aged in vacuum packaged bag have higher weight losses, total bacteria and yeast counts than in dry-aging (Li et al. 2013). Wet ageing (vacuum) is widely used in the meat industry due to its high production yield and convenience in storage and transport (Sitz et al. 2006). Longer aging could potentially be used to replace blade tenderization for *m. longissimus* steaks, but not in other steaks, for example *gluteus medius*. In this case aging at 3.3 °C increase proteolysis (King et al. 2009). Ageing influenced colour components (except b* value), specific activity of cytochrome c oxidase and amount of oxygen consumed (Gašperlin et al. 2001). Czech Flecvieh is a traditional Czech breed of versatile utility with gaining quality meat and milk. The aim of this study was the determination of pH, color and sensory evaluation as a meat quality parameter of long aging *longissimus* and *m. semimembranosus*.

MATERIAL AND METHODS

Sampling

The study was conducted on total of 6 Czech Fleckvieh heifers (age 19 months, Ø weight 400 kg life and Ø weight 215 kg in carcass) and slaughtered in commercial slaughterhouse. The animals came from south region of Moravia. Meat quality measurements were carried out on *m. longissimus thoracis and lumborum* (LL) and *m. semimembranosus* (SM). Total of 72 meat samples (6 animals x 2 half carcass x 2 meat cuts x 3 samples in repetition) were picked from carcass after seven days of dry storage at 4.0 °C and vacuumed in special meat laboratory facility (CZ 22067, Faculty of AgriSciences, MENDELU) and stored at 2.0 °C overall other 28, 42, 49 or 56 days.

Methods

Selected qualitative nutritional indicators were determined in beef meat: dry matter content, ash content, protein content by the Kjeldahl method, and intramuscular fat content according to Soxhlet (Czech National Standard 57 0185, 1963 – arbitration methods). The ultimate pH was measured with a pH meter PORTAVO 907 MULTI pH (KNICK, Germany). The PQM apparatus Fleischtester L191/F (WTW, Germany) was employed to determine specific electrical conductivity (mS/cm). The colour of meat cut surface was measured after the meat had been removed from bag using CM-3500d (KONICA MINOLTA, Japan), values were given in the colour space CIE (CIE 1976) with L* (lightness) and coordinates a* (redness), b* (yellowness).

Group of panellists (n = 8) recruited from Department of Food Technology (Faculty of AgriSciences, MENDELU) were asked to score the samples for colour intensity, fiberness, flavour intensity, tenderness, juiciness, taste intensity, overall flavour, overall taste. Trained sensory panellists evaluated samples for descriptors on 100-mm continuous line scales anchored at the end points (0 = extremely low or dislike, 100 = extremely high or positively). The steaks were cooked in the convention oven (model SCC 61, RATIONAL AG) to achieve a final internal temperature of 77 °C (well done).

Statistical analyses

The measurements were subjected to ANOVA using STATISTICA 12 to determine the effect of storage time (aging) on each cut. The Tukey's honest significant difference (HSD) post hoc test was carried out to identify groups differing significantly ($P<0.05$).

RESULTS AND DISCUSSION

Muscles differed significantly in indicators of nutritional value ($P<0.05$), when LL had higher content of dry matter (28.54 ± 1.3%) and intramuscular fat (4.74 ± 1.25 %) than in SM (26.83 %, resp. 2.94%), which is slightly higher values than she writes in his study Dračková et al. (2014). In the present study, the ultimate pH-value of the LL was significantly ($P<0.05$) higher (from 5.56 ± 0.02 in 7 days to 5.79 ± 0.02 in 56 days) than in SM during the whole period (5.44 ± 0.03, resp. 5.70 ± 0.03). Similarly, but at the end of the ageing period, electrical conductivity of LL has stagnated, while at 56 days in SM the electrical conductivity was exceeded (14.38 mS/cm).

The difference in the quality indicators monitored is confirmed by Florek et al. (2007). The changes in L*, a* and b* values, pH and electrical conductivity during the 56 days of storage are shown in Table 1. Rising pH values may indicate changes caused by natural microflora of meat retarded by vacuum conditions (Borch et al. 1996). In this study lightness and a* and b* colour parameters varied during storage in a separate way (Table 1). Lightness increased in LL (to L* 40.03 ± 1.85), while in SM decreased (L* 38.02 ± 2.94). For these cases, it is more appropriate to use expressions by ΔE*ab (CIE 1976). Total colour difference affected by storage was higher in LL (4.13) against SM (3.81). Oliete et al. (2006) found that lengthening of the vacuum storage time of *m. longissimus thoracis* (1st, 7th and 14th day) resulted in a rise of the a* and b* parameters, i.e. meat became more red and yellow, e the colour saturation was more intensive (higher C*). Gender factor is also important. Florek et al. (2007) published that the meat of heifers aged for 14 days under vacuum was characterised by lower pH, higher specific electrical conductivity, greater natural drip as well redder colour (higher a*) and yellow (higher b*) as compared to the meat of young bulls.

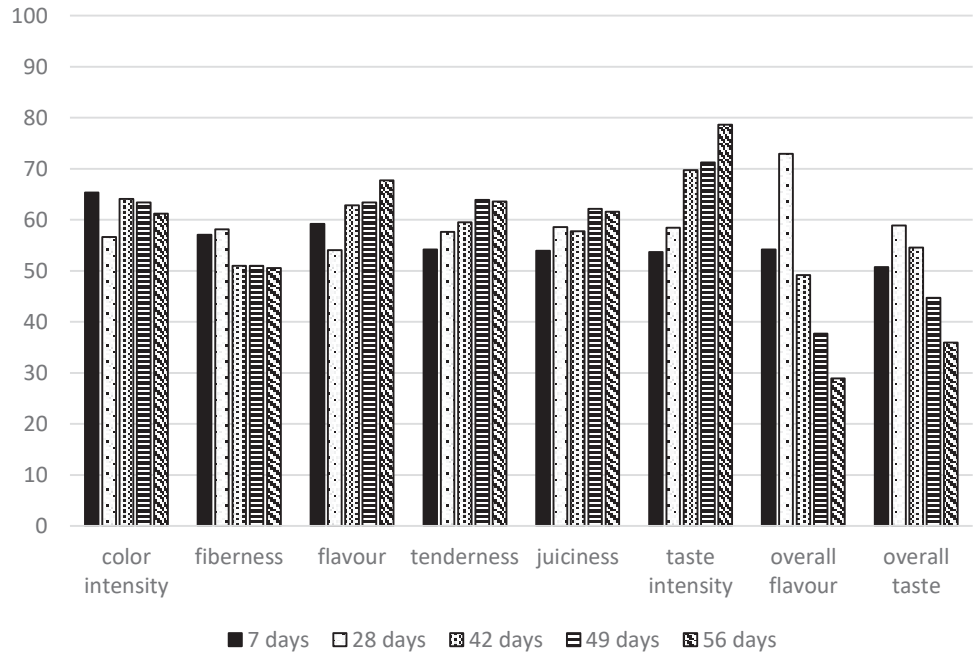
Table 1 Mean (± SEM.) colour parameters (L, a*, b*, ΔE^*_{ab}), pH and electrical conductivity of steaks stored up to 56 days*

Attribute	Muscle	Days of aging				
		7	28	42	49	56
pH	LL	$5.56 \pm 0.02^{a*}$	$5.67 \pm 0.04^{b*}$	$5.72 \pm 0.02^{c*}$	$5.74 \pm 0.02^{c*}$	$5.79 \pm 0.02^{d*}$
	SM	$5.44 \pm 0.03^{a*}$	$5.60 \pm 0.02^{b*}$	$5.66 \pm 0.03^{c*}$	$5.65 \pm 0.02^{c*}$	$5.70 \pm 0.03^{d*}$
Electrical conductivity (mS/cm)	LL	5.28 ± 0.39^{a}	$7.65 \pm 0.40^{b*}$	$12.08 \pm 0.45^{c*}$	$12.77 \pm 0.15^{d*}$	$12.81 \pm 0.44^{d*}$
	SM	4.90 ± 0.14^{a}	$6.29 \pm 0.13^{b*}$	$9.40 \pm 0.23^{c*}$	$11.20 \pm 0.43^{d*}$	$14.38 \pm 0.58^{e*}$
L*	LL	$36.74 \pm 0.84^{a*}$	$37.30 \pm 0.97^{ab*}$	38.85 ± 2.07^{ab}	39.44 ± 1.33^{ab}	40.03 ± 1.85^{b}
	SM	$41.16 \pm 1.78^{*}$	$40.31 \pm 2.83^{*}$	38.71 ± 2.19	39.06 ± 1.04	38.02 ± 2.94
a*	LL	11.15 ± 0.52^{a}	11.12 ± 0.45^{a}	10.03 ± 1.37^{ab}	9.24 ± 0.86^{ab}	$8.68 \pm 1.14^{b*}$
	SM	9.21 ± 1.06	9.28 ± 0.91	10.47 ± 1.15	9.42 ± 1.37	$11.34 \pm 2.85^{*}$
b*	LL	9.29 ± 0.89	9.49 ± 0.94	9.40 ± 0.94	9.48 ± 1.09	9.64 ± 0.91
	SM	8.99 ± 0.93	9.25 ± 0.93	8.54 ± 0.94	8.32 ± 0.90	8.40 ± 0.87
ΔE^*_{ab}	LL	0	0.59	1.30	3.31	4.13
	SM	0	0.89	2.62	2.21	3.81

*Legend: * Statistical significance between LL and SM (P<0.05); ^{a,b,c} – within a days of aging without same superscript for a trait differ (P<0.05); ΔE^*_{ab}*

In our case, total colour difference in SM storage exceeded the value earlier (42 days) than in LL (49 days), when the observer would find the difference between the two samples ($\Delta E^*_{ab} = 2$), although there was a bigger change at LL, although at LL it reached higher final values (4.13 versus 3.81).

Figure 1 Sensory evaluation (100 mm scale) of LL steaks stored up to 56 days

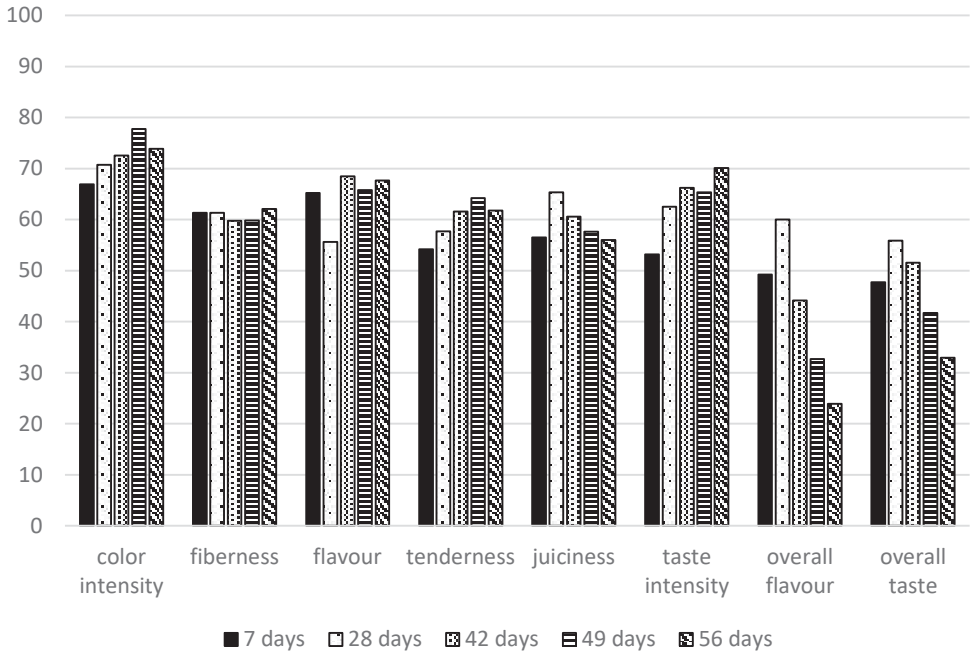


Boakye and Mittal (1996) reports the highest ΔE^*_{ab} in vacuum packed LL. Colorimetry of meat confirms different changes in lightness (L*) in LL and SM muscles. Gašperlin et al. (2001) states in their study high correlation coefficients between sensory properties and instrumental values of beef colour (L* and presence of black R= -0.68, a* and presence of red R= 0.63). Figure 1 show a decrease

in colour intensity in LL and an increase in SM (Figure 2) during storage. These results correspond to the already mentioned values of lightness L* (negative correlation with subjective sensory evaluation), which increase in LL and decrease in SM.

Sensory evaluation of LL and SM shows that hedonic overall flavour and overall taste is affected ($P<0.05$) thanks to their storage time (ageing). Taste intensity was significantly higher in LL in the group of 56 days aged meat ($P<0.05$). Tenderness and juiciness have shown a positive correlation with time of ageing. Overall, vacuumed meat compared with modified packaging atmosphere has longer durability, positively influenced shear force, thawing loss, α-tocopherol content and colour stability, as well as the sensory attributes tenderness, juiciness and to some extent meat flavour (Lagerstedt et al. 2011). The sensory quality of the hedonic scale declined in relation to the length of maturation in SM and LL.

Figure 2 Sensory evaluation (100 mm scale) of SM steaks stored up to 56 days



CONCLUSION

Naturally, meat quality traits were substantially affected by long ageing. Quality characteristics of Czech Fleckvieh meat from the two different muscles were analysed and surprisingly had an acceptable sensory evaluation. Many authors describe the ripening of beef on various meat breeds. Meat quality traits of *longissimus thoracis et lumborum* and *m. semimembranosus* from Czech Fleckvieh showed and confirmed the relationship between meat quality parameters like pH and electrical conductivity with emphasis on those sensory ones as lightness and colour intensity. Steaks aged for 28 days in vacuum had lower colour stability than steaks aged for shorter times.

ACKNOWLEDGEMENTS

The research was financially supported by the OP VaVpI CZ.1.05/4.1.00/04.0135 Teaching and research capacities for biotechnology and infrastructure development.

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EFFECT OF ADDITIVES ON COLOUR STABILITY OF YOGURT

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Abstract: This study monitored the effect of the addition of chia flour, quinoa flour, nopal powder, apple fiber and Bamboo fiber BAF 40 on colour stability of yogurt. It was made yogurt with 1, 3 and 5% of addition. Bovine milk was heated up to 85 °C for 5 min and cooled down to 36 °C and fermented until pH of yogurt was 4.50. The yogurt was divided into 16 groups and into each group was adding 1, 3 and 5% of chia flour, quinoa flour, nopal powder, apple fiber and Bamboo fiber BAF 40 and one group was without additives as natural. Titratable acidity was determined during the incubation period of the samples and storage time up to 15 days. Colour stability was measured during the storage time. The results showed that storage had significant effect to titratable acidity fortified and natural yogurt. There were found some significant different between colour parameters on 1, 8 and 15 days of storage. But these changes were not so bigger and not influence the colour stability of yogurt with additives.

Key Words: fiber, chia, quinoa, nopal, titratable acidity

INTRODUCTION

Milk is a food of almost complete nutrition. Many changes occur to the components of milk during fermentation, although there is no significant difference between the gross composition of unfermented and fermented milk. Considerable progress has been made in demonstrating certain beneficial effects of fermented milk in animals, probably due to the changes occurring in milk during fermentation (Walstra et al. 2006).

It was made a many studies how is influence the yogurt quality by dietary fiber. The effects of commercial fibers from apple, wheat, bamboo, or inulin were studied on sensory and rheological properties of yogurt. The addition of fiber into yogurt changed the rheological characteristic. This addition was acceptable for consumers. Yogurt fortified with apple fiber had a different colour compared with unfortified yogurt (Staffolo et al. 2004). The oat fiber adding in concentration 1.32% improved the body and texture of unsweetened yogurt and decreased the overall flavour quality (Fernández-García et al. 1998).

The colour of food is one of immediately indicators quality of food (Kress-Rogers and Brimelow 2001) such as flavour, naturality, sanity or maturity. The consumers may decide according colour which product is most acceptable for them (Dufossé et al. 2005).

Cerezal Mezquita et al. (2014) used astaxanthin oleoresin to simulate the apricot colour in traditional and diet yogurt. The colour parameters were very stable during four week of storage. It was found high stability of astaxanthin pigment within the protein-lipid matrix of the yogurt. Krammerer et al. (2006) found, that stability of colour from natural sources tend to lost tinctorial strength or disappear with time in storage.

The aim of this was work was monitoring the colour stability of yogurt and monitoring of titratable acidity during storage. It was observed the effect of addition chia flour, quinoa flour, nopal powder, apple fibre and bamboo fibre BAF 40 for colour stability and titratable acidity into yogurt.

MATERIAL AND METHODS

Yogurt for research was prepared from the bovine milk at Mendel University in Brno at Department of Food Technology. This research was carried out in Biotechnology Pavilion M, financed by the OP VaVpI CZ.1.05/4.1.00/04.0135 project at the Department of Food Technology at Mendel University.

The bovine milk used for yogurt was analysed for milk composition. Before analysis was milk heated up prior to 40 °C and then cooled down to 20 °C for better dispersion of fat globules. It was determined titratable acidity and lactose by polarimetry according to Czech state standard No. 57 0530. The protein content was determined by Kjeldahl´s method (EN ISO 8968-1:2002), the fat content was determined by Gerber´s method (ISO 2446:2008).

The yogurt was prepared from milk of Holstein dairy cows from South Moravian region. The milk content: 3.42% of protein, 3.50% of fat, 4.50% of lactose and titratable acidity 6.7 SH by method with stirred coagulated. The milk was heated up to 85 °C for 5 min and then cooled down to 36 °C. After was added into pasteurized milk 0.5% of starter for making original Bulgarian yogurt (bulgaricus.cz, GENESIS LABORATORIES, Bulgary). The milk was fermented 18 hour for 36 °C. After fermentation was coagulum stirred for 5 min. In the next step was yogurt divided into 16 groups. Into yogurt were added chia flour, quinoa flour, nopal powder, apple fiber and bamboo fiber. Each addition was made from three different concentrations. It was made yogurt with 1, 3 and 5% of addition. One group of yogurt was made as natural. The samples of yogurt were storage at the 4 °C.

The titratable acidity of yogurt was determined according Czech state standard No. 57 0530, where yogurt samples (50 g) were diluted with 50 mL distilled water and titrated with 0.25 M NaOH in the presence of phenolphthalein.

The colour determinations were performed using Konica Minolta CM-3500d (Japan). For measure was used mode reflectance. The parameters of measured: illuminant D 65, observer 10 °, SCE and size of gap 30 mm. The colour parameters L* (lightness), a* (red-green axis) and b* (yellow-blue axis) of the yogurt samples were measured at 4 °C. Measurement was carried out from a 200 ml sample which was homogenized and then applied in a layer 1 cm on a cuvette CM-A-128 (17 x 45 mm). Colour was determined in three cups of yogurt per replication 1st, 8th and 15th days of storage.

The results were statistically processed by program STATISTICA 12. For difference between days of storage was used the Scheffe test ($P < 0.05$).

RESULT AND DISCUSSION

The titratable acidity of natural yogurt was the lowest first day of storage, this value was statistically significant (46.9 SH). The next results of titratable acidity are not statistically difference (Table 1). During the storage is not any difference in the L* parameters (mean value of 90.92 to 91.53). Ozcan el al. (2014) stated that value L* during storage increased when in 1st day of storage was L* value 94.45 ± 0.01, while on the 8th days of storage up to 98.52 ± 0.01. The parameter a* was changed at 15th days of storage, this change was statistically significant (-3.10), but this value is not much bigger than other value (-3.00). The parameter b* was changed during storage, this changed was statistically significant from 11.38 till 11.80.

Table 1 Results of titratable acidity and colour parameters natural yogurt (mean±standard deviation)

Storage days	SH	L*(D65)	a*(D65)	b*(D65)
1	46.9±0.4^{b}	91.18±0.04	-3.01±0.02^{a}	11.62±0.08^{ab}
8	51.3±0.4^{a}	91.53±0.35	-3.00±0.02^{a}	11.38±0.13^{a}
15	50.4±0.5^{a}	90.92±0.06	-3.10±0.02^{b}	11.80±0.05^{b}

Legend: $^{a, b}$ *– different superscripts in a column indicate a statistically significant difference at $P < 0.05$*

The titratable acidity of yogurt with chia flour was the lowest first day of storage, titratable acidity was increased during storage. This difference was statistically significant ($P < 0.05$). The highest titratable acidity was in the group with the 5% of chia flour (44.1 SH), but difference

between 8 and 15 storage days in not statistically significant (54.4 to 55.4 SH). During the storage is not any statistical significant difference in the L* parameters (Table 2) for any groups with different concentration chia flour. The parameter a* for yogurt with 1% chia flour was changed from -0.63 at the 1st day to -0.35 at 15th days of storage, this change was statistically significant, equally for 3 and 5% addition. These differences are not so bigger and they were not perceptible for consumers. The parameter b* was changed during storage only for 5% addition of chia flour, for other concentration the difference are not statistically significant. Garcia-Perez et al. (2005) reported that when adding 1 % orange fiber into yogurt, it showed a decrease of value L*, but an increase of value a* and b * was observed. The increase of a* and b* values is attributed to the release of carotenoids from orange fiber during storage at low temperatures.

Table 2 Results of titratable acidity and colour parameters yogurt with chia flour (mean±standard deviation)

Adding	Storage days	SH	L*(D65)	a*(D65)	b*(D65)
1%	1	37.1±0.1^{a}	82.70±0.24	-0.63±0.05^{a}	6.68±0.25
	8	46.8±0,1^{b}	83.55±0.20	-0.56±0.11^{ab}	6.81±0.13
	15	50.4±0.3^{c}	82.46±0.51	-0.35±0.08^{b}	7.14±0.14
3%	1	39.9±0.2^{a}	77.22±0.37	0.46±0.04^{a}	6.77±0.11
	8	48.5±0.3^{b}	76.38±0.29	0.70±0.04^{b}	6.82±0.06
	15	52.4±0.3^{c}	76.27±0.31	0.59±0.06^{ab}	6.78±0.12
5%	1	44.1±0.3^{b}	71.63±0.20	1.22±0.05^{a}	7.33±0.09^{a}
	8	54.4±0.4^{a}	71.73±0.28	1.44±0.03^{b}	7.70±0.08^{b}
	15	55.4±0.3^{a}	71.58±0.08	1.29±0.05^{a}	7.37±0.04^{a}

Legend: $^{a, b, c}$ *– different superscripts in a column indicate a statistically significant difference at P < 0.05*

The titratable acidity of yogurt with 1% quinoa flour was the lowest first day of storage, titratable acidity was increased during storage. The average accrual titratable acidity was 5 °SH for every term of analysis. This difference was statistically significant ($P < 0.05$). The same is valid for 3 and 5% of addition quinoa flour. During the storage is statistical significant difference in the L* parameters (Table 3) for 1% concentration quinoa flour from the 89.75 at the 1st day to 90.19 at 8th day. Difference between 8th and 15th days is not statistically significant. During storage yogurt with 3% quinoa flour, the samples get darker from 87.63 at the 1st day to 86.65 at 15th day of storage. This difference was statistical significant. The lightness is not changed for yogurt with 5% quinoa flour. The parameter a* for yogurt with 1% quinoa flour was changed from -2.30 at the 1st day to -2.49 at 15th days of storage, this change was statistically significant. Bakirci et al. (2017) had similar results in their study when the values a* of the control sample increased from -2.10 in the 1st day of storage to -2.33 at the 14th days of storage. Bakirci et al. (2017) also reported that when adding 1.5 % pumpkin fiber, however, the values a* decreased from 4.22 ± 0.02 in the first day of storage to 3.68 ± 0.01 in the 14th days of storage. The parameter a* for yogurt with 3% quinoa flour was changed from -1.53 at the 1st day to -0.63 at 15th days of storage, this change was statistically significant. The same significant difference was valid for yogurt with 5% of quinoa flour. These differences are not so bigger and they are not perceptible for consumers. The parameter b* was changed during storage only for 3% addition of quinoa flour, for other concentration the difference are not statistically significant.

The titratable acidity was increased during storage for all concentration of nopal powder. This difference was statistically significant ($P < 0.05$). The highest increase titratable acidity was monitored at the yogurt with 5% nopal powder. During the storage is statistical significant difference in the L* parameters (Table 4) for 1% concentration nopal powder. During storage yogurt with 3% and 5% nopal powder was not statistical significant different. The parameter a* for yogurt with 1% nopal powder was changed only very slightly, but this change was statistically significant. The parameter a* for yogurt with 3% and 5% were changed. These changes were

statistically significant too. The parameter b* was changed during storage for all concentration of nopal powder, the difference was statistically significant. These differences are not so bigger and they are not perceptible for consumers.

Table 3 Results of titratable acidity and colour parameters yogurt with quinoa flour (mean±standard deviation)

Adding	Storage days	SH	L*(D65)	a*(D65)	b*(D65)
1%	1	42.8±0.3^{a}	89.75±0.01^{b}	-2.30±0.01^{b}	11.83±0.04
	8	47.4±0.4^{b}	90.19±0.07^{a}	-2.43±0.02^{ab}	11.95±0.02
	15	52.7±0.5^{c}	90.08±0.10^{a}	-2.49±0.08^{a}	11.92±0.19
3%	1	45.2±0.4^{a}	87.63±0.08^{c}	-1.53±0.06^{a}	12.65±0.05^{a}
	8	50.0±0.2^{b}	87.24±0.12^{b}	-0.95±0.01^{b}	12.54±0.09^{a}
	15	57.2±0.5^{c}	86.65±0.15^{a}	-0.63±0.01^{c}	12.88±0.04^{b}
5%	1	46.3±0.4^{a}	86.84±0.05	-1.12±0.01^{a}	12.89±0.05
	8	51.7±0.3^{b}	86.26±0.35	-0.70±0.01^{b}	12.85±0.15
	15	57.8±0.5^{c}	86.45±0.22	-0.55±0,02^{c}	13.12±0.09

Legend: $^{a, b, c}$ – different superscripts in a column indicate a statistically significant difference at P < 0.05

Table 4 Results of titratable acidity and colour parameters yogurt with nopal powder (mean±standard deviation)

Adding	Storage days	SH	L*(D65)	a*(D65)	b*(D65)
1%	1	46.5±0.4^{a}	85.07±0.02^{a}	-1.74±0.02^{ab}	13.80±0.02^{ab}
	8	48.8±0.5^{b}	85.41±0.10^{b}	-1.71±0.01^{b}	13.63±0.02^{a}
	15	51.5±0.4^{c}	84.89±0.07^{a}	-1.76±0.01^{a}	13.98±0.13^{b}
3%	1	50.0±0.5^{a}	78.85±0.05	-1.03±0.00^{a}	17.01±0.05^{a}
	8	52.4±0.4^{b}	78.85±0.05	-0.97±0.03^{a}	16.46±0.26^{b}
	15	56.0±0.4^{c}	78.56±0.09	-0.89±0.00^{b}	17.14±0.03^{a}
5%	1	51.7±0.3^{a}	75.01±0.06	-0.56±0.04^{a}	18.63±0.03^{a}
	8	55.2±0.3^{b}	75.17±0.28	-0.61±0.02^{a}	18.22±0.04^{b}
	15	59.0±0.5^{c}	74.28±0.05	-0.42±0,02^{b}	18.73±0.12^{a}

Legend: $^{a, b, c}$ – different superscripts in a column indicate a statistically significant difference at P < 0.05

The titratable acidity was increased during storage for all concentration of apple fiber (Table 5). This difference was statistically significant ($P < 0.05$). These results were similar as the titratable aciditidy yogurt with the nopal powder. During the storage is statistical significant difference in the L* parameters (Table 5) for 5% concentration apple fiber. During storage yogurt with 1% and 3% apple fiber was not statistical significant different. The parameter a* for yogurt with all concentration apple fiber was not changed during storage, between results was not statistically significant different. The parameter b* was increased with the increase concentration of apple fiber. The statistically significant different was only for yogurt with 3% apple fiber. Staffolo et al. (2004) indicates than color parameters in yogurt with addition apple fiber did not show a significant difference ($P > 0.05$) over time. However, the apple fiber had a pronounced brown color at a lower lightness (L* value).

Table 5 Results of titratable acidity and colour parameters yogurt with apple fiber (mean±standard deviation)

Adding	Storage days	SH	L*(D65)	a*(D65)	b*(D65)
1%	1	42.8±0.6^{a}	79.47±0.08	2.27±0.02	18.63±0.03
	8	46.3±0.4^{b}	80.22±0.57	2.21±0.09	18.40±0.31
	15	50.3±0.3^{c}	79.40±0.14	2.31±0.05	18.90±0.09
3%	1	46.9±0.3^{a}	70.43±0.08	5.46±0.06	24.49±0.17^{ab}
	8	50.0±0.4^{b}	70.66±0.14	5.40±0.05	24.32±0.18^{a}
	15	56.5±0.4^{c}	70.40±0.13	5.56±0.06	24.78±0.02^{b}
5%	1	48.4±0.4^{a}	65.30±0.06^{ab}	7.08±0.01	26.83±0.08
	8	51.4±0.3^{b}	65.89±0.38^{b}	6.97±0.16	26.55±0.37
	15	57.3±0.3^{c}	64.95±0.04^{a}	7.26±0,06	27.31±0.16

Legend: $^{a, b, c}$ *– different superscripts in a column indicate a statistically significant difference at P < 0.05*

The titratable acidity was increased during storage for all concentration of bamboo fiber BAF 40 (Table 6). This difference was statistically significant (P < 0.05). These results were similar as the titratable acidity yogurt with the nopal powder and/or apple fibre. During the storage is statistical significant difference in the L* parameters at 1st day of storage and 8th day of storage. The parameter L* was slightly increased at 8th day of storage and 15th day of storage was L* slightly decreased. The parameter a* was changed during storage, this change was statistically significant for 1% and 5% bamboo fiber BAF 40. For 3% bamboo fiber BAF 40 was not change parameter a*. The parameter b* was not change during all storage but Seçkin et al. (2012) reported values b* was there is a decrease when values b* was in 1st day of storage 20.0 ± 2.8 and in 21st days of storage was values 18.2 ± 3.7.

Table 6 Results of titratable acidity and colour parameters yogurt with bamboo fiber BAF 40 (mean±standard deviation)

Adding	Storage days	SH	L*(D65)	a*(D65)	b*(D65)
1%	1	46.7±0.3^{a}	90.94±0.03^{a}	-2.88±0.01^{a}	11.57±0.09
	8	48.7±0.3^{b}	91.44±0.21^{b}	-2.95±0.04^{a}	11.27±0.28
	15	53.8±0.5^{c}	90.64±0.09^{a}	-3.09±0.02^{b}	11.40±0.13
3%	1	47.1±0.3^{a}	90.55±0.02^{a}	-2.84±0.02	11.32±0.03
	8	49.2±0.5^{b}	91.23±0.33^{b}	-2.75±0.03	11.19±0.21
	15	54.7±0.5^{c}	90.44±0.04^{a}	-2.85±0.05	11.57±0.11
5%	1	47.3±0.4^{a}	90.02±0.06^{a}	-2.68±0.02^{ab}	11.12±0.06
	8	49.7±0.4^{b}	90.57±0.23^{b}	-2.65±0.01^{ab}	11.00±0.21
	15	52.4±0.3^{c}	89.99±0.10^{a}	-2.72±0,03^{a}	11.41±0.03

Legend: $^{a, b, c}$ *– different superscripts in a column indicate a statistically significant difference at P < 0.05*

CONCLUSION

This study monitored effect of different type of additives for colour stability and titratable acidity of yogurt during storage. Colour stability and titratable acidity was monitored 1st, 8th and 15th days of storage. The changes of colour stability were statistically significant in many cases, but these changes were not so big. These changes were not perceptible for consumers. The addition of chia flour, quinoa flour, nopal powder, apple fiber and Bamboo fiber BAF 40 was not influence for colour

stability of yogurt during storage. These additives were not influence for fermented process during storage, the titratable acidity was increased during storage. With the higher concentration of additives was the titratable acidity higher.

ACKNOWLEDGEMENT
This research was supported and financed by project TP 2/2017 "Effect of additives on the rheological behaviour of foodstuffs and raw materials for their production" of Internal Grant Agency FA MENDELU.

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INFLUENCE OF RECIPES OF QUALITY CHOCOLATE PRODUCTS DURING THEIR STORAGE

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Abstract: The aim of the work was to investigate the effect of chocolate composition on its shelf life and to monitor the effect of different storage regimes on the changes in the sensory quality and texture properties of chocolate products with different contents of cocoa butter. Samples of tempered and untempered milk and bitter chocolate were made. The chocolate samples were stored for three months at various temperature regimes: -18 °C, 6 °C, 12 °C and 20 °C. Sensory analysis and texture measurements were performed before and after the samples were stored. In conclusion, the effect of the composition on the shelf life was evaluated, and a measure was proposed to improve the stability and shelf life of chocolate and chocolate products.

Key Words: chocolate, storage, sensory evaluation, quality, texture

INTRODUCTION

The chocolate production involves a complex system of technological operations. The basic raw material for production is cocoa beans obtained from the cocoa tree *(Theobroma cacao).* This species originated from crossing the species *Theobroma pentagonum* and *Theobroma leiocarpum.* The most widely used varieties for making chocolate and chocolate products are Criollo, Forastero, and their hybrid Trinitario. After harvesting, cocoa beans are processed by fermentation, drying, cleaning, pre-roasting, roasting and final grinding when the cell structure breaks down and cocoa butter is released. In the mixing process cocoa beans and other added ingredients become cocoa mass, which is further processed by pressing and rolling, which affects its structure and consistency. Consequently, the chocolate mass passes through the process of conching and tempering, which are very important processes for obtaining quality chocolate with good glossiness, colour, and structure. The actual processing of chocolate and chocolate products includes treatments such as shaping, cooling, packing, storage, and shipping (Hřivna 2014).

The quality of chocolate is influenced by many factors, which can significantly affect the sensory and textural qualities of chocolate (Afoakwa 2010). In order to obtain high-quality chocolate products, chocolate mass composition plays an important role, especially the form of crystallization of cocoa butter. Also important is the technological process of their production, especially tempering and temperature regime during storage (Saltini et al. 2013).

MATERIAL AND METHODS

Two types of chocolate, namely bitter chocolate with 54 per cent of cocoa component and milk chocolate with 35 per cent of cocoa component, were made for the experiment. Table 1 shows the outline of the experiment. A part of the products of each type was subjected immediately after the production to the so-called retempering, which consists in storing the product in a temperature regime of 23 °C for 24 hours. The rest of the production was packed so that always one half of each type of chocolate was packed in aluminium foil and the other half was vacuum-packed into PE foil. Retempered products were packed in the same way. Immediately after packing, all products were stored in different temperature regimes. Control samples were frozen at -18 °C, while the others were stored at 6 °C, 12 °C, and 20 °C. After three months, texture properties were evaluated

on the TIRATEST 27025 testing machine by TIRA GmbH, Germany. A penetration test with a knife-shaped probe was used to test chocolate products. The selected criteria for penetration test of chocolate products by the pressure test were as follows: blade edge length 10 mm, test speed v_1 = 40 mm/min. Sensory analysis was performed before and after storage of the samples. Statistical evaluation of the gained data was done in Microsoft Excel and Statistica 12 programmes. For the calculation, we have used the multifactor ANOVA method.

Table 1 Outline of the Experiment

Variant	Type of chocolate	Temperature regime	Retempering	Packed
1	Bitter	Control	Yes	Foil
2	Bitter	Control		Vacuum
3	Bitter	Control	No	Foil
4	Bitter	Control		Vacuum
5	Bitter	6 °C	Yes	Foil
6	Bitter	6 °C		Vacuum
7	Bitter	6 °C	No	Foil
8	Bitter	6 °C		Vacuum
9	Bitter	12 ºC	Yes	Foil
10	Bitter	12 ºC		Vacuum
11	Bitter	12 ºC	No	Foil
12	Bitter	12 ºC		Vacuum
13	Bitter	20 °C	Yes	Foil
14	Bitter	20 °C		Vacuum
15	Bitter	20 °C	No	Foil
16	Bitter	20 °C		Vacuum
1	Milk	Control	Yes	Foil
2	Milk	Control		Vacuum
3	Milk	Control	No	Foil
4	Milk	Control		Vacuum
5	Milk	6 °C	Yes	Foil
6	Milk	6 °C		Vacuum
7	Milk	6 °C	No	Foil
8	Milk	6 °C		Vacuum
9	Milk	12 ºC	Yes	Foil
10	Milk	12 ºC		Vacuum
11	Milk	12 ºC	No	Foil
12	Milk	12 ºC		Vacuum
13	Milk	20 °C	Yes	Foil
14	Milk	20 °C		Vacuum
15	Milk	20 °C	No	Foil
16	Milk	20 °C		Vacuum

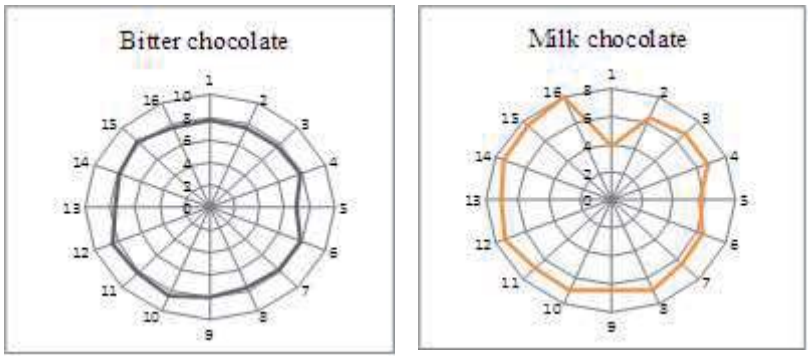
RESULTS AND DISCUSSION

Sensory evaluation of chocolate products

According to the sensory evaluation of retempered and non-retempered bitter chocolate before its packing and storing in different temperature regimes, we can state that there was no visible difference between the individual samples. For both samples, glossiness, fracture, and hardness on bite received the worst evaluation. The sensory evaluation of milk chocolate was characterized by greater differences between individual descriptors. Non-retempered chocolate was characterized by a slightly higher quality than chocolate that had been retempered. Fracture consistency was evaluated as the worst, which may be due to a higher proportion of sugar and milk fat in the chocolate sample. This evaluation was also reflected in the assessment of fracture and colour. Even a strange taste was noticed and the melting of the chocolate component in the mouth was evaluated as worse. Both of these evaluations can again be related to a higher proportion of sugar and fat, and also to the

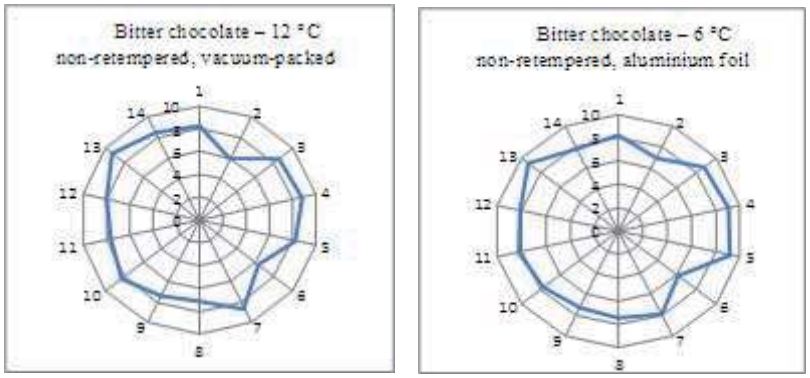
addition of dried milk, their poor dissolution or poor tempering of the chocolate sample. This can then be manifested by the presence of fat bloom, which may affect the appearance and structure of the products (Machálková et al. 2016). Figure 1 shows the overall evaluation of samples of bitter and milk chocolates stored in accordance to the outline shown in (Table 1) (see Methods). In the case of bitter chocolate, the best samples received evaluation higher than 8 points, while in milk chocolate, it was only in the best sample. The other samples of milk chocolate did not exceed the eight-point score. The best evaluation was achieved by the retempered vacuum-packed sample of bitter chocolate stored at 12 ºC. For milk chocolate, the non-retempered sample vacuum-packed in foil and stored at 20 °C turned out to be the most acceptable.

Figure 1 Overall evaluation of bitter and milk chocolate



Bitter chocolate *Milk chocolate*

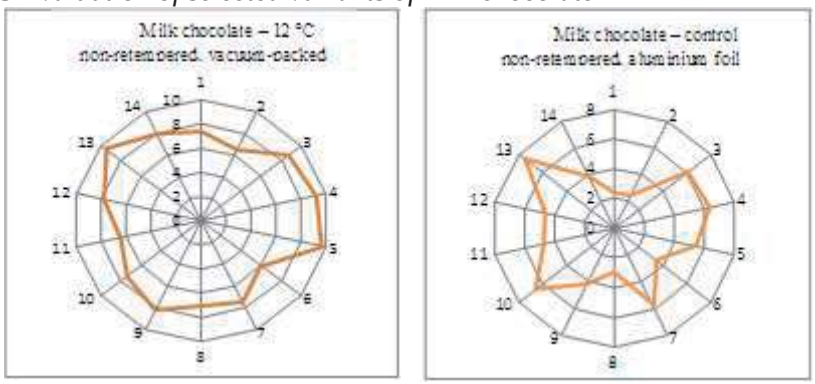
Figure 2 Evaluation of selected variants of bitter chocolate



Bitter chocolate – 12 °C non-retempered, vacuum-packed Bitter chocolate - 6 °C non-retempered, aluminium foil

Legend: 1 - colour; 2 - glossiness; 3 - fat bloom top side; 4 – fat bloom bottom side; 5 - cross section; 6 - fracture; 7 - aroma - overall; 8 - consistency at fracture; 9 - hardness on bite; 10 - adhesion – stickiness on palate; 11 - melting of chocolate component in mouth; 12 – overall taste of product; 13 - strange taste; 14 - overall sample evaluation

Figure 3 Evaluation of selected variants of milk chocolate



Milk chocolate - 12 °C non-retempered, vacuum-packed Milk chocolate - control non-retempered, aluminium foil

Legend: 1 - colour; 2 - glossiness; 3 - fat bloom top side; 4 – fat bloom bottom side; 5 - cross section; 6 - fracture; 7 - aroma - overall; 8 - consistency at fracture; 9 - hardness on bite; 10 - adhesion – stickiness on palate; 11 - melting of chocolate component in mouth; 12 – overall taste of product; 13 - strange taste; 14 - overall sample evaluation

Another part of the sensory evaluation was focused on a detailed assessment of the influence of composition, technology of production, packing, and storage temperatures on the product quality. Due to the large number of variants, only those with the best and worst evaluations for individual types of chocolate are presented in the text. In the case of bitter chocolate, the best evaluation received the product that was non-retempered, vacuum-packed and stored at 12 °C. The worst evaluation received the product stored at 6 °C, non-retempered and packed in aluminium foil (Figure 2). Due to the statistically insignificant differences ($p = 0.05$) between samples of bitter chocolate, the best and worst samples could not be clearly determined. Depending on the number of the worst and best evaluated parameters, non-retempered samples packed in aluminium foil, namely the control sample and the sample stored at 20 °C, received the best evaluation. We consider the worst to be non-retempered samples vacuum-packed and stored at 6 °C and 20 °C. For milk chocolate samples, statistically significant differences were found between the different variants in colour, glossiness, fat bloom on the top side and cross-section of the product, fracture consistency, hardness on bite, in taste and in overall evaluation. The best evaluation received the non-retempered vacuum-packed product stored at 20 °C. The lowest score received the non-retempered control sample packed in aluminium foil (Figure 3). For all samples of milk chocolate, there are lager fluctuations in individual descriptors compared to bitter chocolate. In the case of milk chocolate, this can be caused by a higher sugar and fat content, which is associated with a higher probability of fat and sugar bloom.

Machálková (2016) states that retempering positively affects the quality of chocolate stored at room temperature, which in our case has not been confirmed, as our non-retempered chocolate received the best evaluation. This can be due to a lower portion of cocoa butter in the recipe which has an effect on the product stability and can be enhanced by retempering. Improved stability of non-retempered products can be explained by the presence of more cocoa butter in the stable V form, which is formed during retempering. The stable form of cocoa butter is associated with a higher melting point, improved product resistance to fat bloom, and better sensory properties (Nöbel et al. 2009). Colour, glossiness, shape of chocolate, and its surface texture are among the basic features that characterize the appearance and influence of the consumer's interest in the product (Simonot and Elias 2002). Colour perception can be greatly influenced. These attributes originate from complex interactions of incident light, optical properties, and human perception (Afoakwa 2010). In the case of our evaluation, colour was provably ($p = 0.05$) unfavourably evaluated for the non-retempered products packed in aluminium foil and stored at -18 °C and 6 °C. Machálková et al. (2015), however, states that freezing of the product or storing it at 6 °C results in maintaining its quality and freshness. Prior to consumption or sensory evaluation, the product should be stabilized at 20 °C ± 2 °C (Afoakwa 2010). Formation of micro dew may result in loss of glossiness which occurred significantly ($p = 0.05$) for both storage regimes.

Texture of chocolate products

Hardness of chocolate is influenced not only by the recipe and tempering technology (Afoakwa 2010), but also by the temperature conditions during storage. All samples were tempered to the same temperature prior to their analysis to ensure the objectivity of measurements. Bitter chocolate showed higher hardness (27.28 N) compared to milk chocolate (9.32 N). The representation of individual size fractions of cocoa fat crystals has also played its role here. According to Quast et al. (2013) and Fernandes et al. (2013), a crystalline form V (β) is desirable for chocolate production and dominates in well-tempered chocolate. Retempering also supports this process (Machálková 2016). A favourable effect of retempering on hardness was found in bitter chocolate (28.19 N) in contrast to non-retempered samples (26.35 N). The opposite was found in milk chocolate. Non-retempered samples exhibited a higher hardness (9.39 N) than non-retempered ones (9.25 N). The inconclusiveness of retempering can be justified by the low content of cocoa butter in the product. Chocolate products generally do not tolerate high temperatures during storage. They promote the migration of fat through the matrix of chocolate particles, which leads to recrystallization of fat on the surface (Aguilera et al. 2004, Lohman and Hartel 1994). Fat migration is related to softening of chocolate layer and overall sensory deterioration of products (Svanber et al. 2011). Figure 4 illustrates the effect of different storage regimes on the texture of chocolate products. We can state that the storage temperature did not have any significant effect on the texture of the products and the room temperature did not affect the texture of the products negatively.

Texture measurements of all variants included in the experiment (Figure 5) indicate large differences in the textures of milk and bitter chocolate. Variability within individual groups after three months of storage is not large. With longer storage, larger differences can be expected. It is therefore clear that technology and storage do not play a key role, but the influence of the recipe, especially the content of cocoa butter do. This experience is also confirmed by Nöbel et al. (2009), Afoakwa (2010), and Machálková et al. (2015).

Figure 4 Influence of storage temperature on the texture of bitter and milk chocolate (Vertical columns represent 0.95 confidence intervals, X-axis - storage regime, Y-axis – texture)

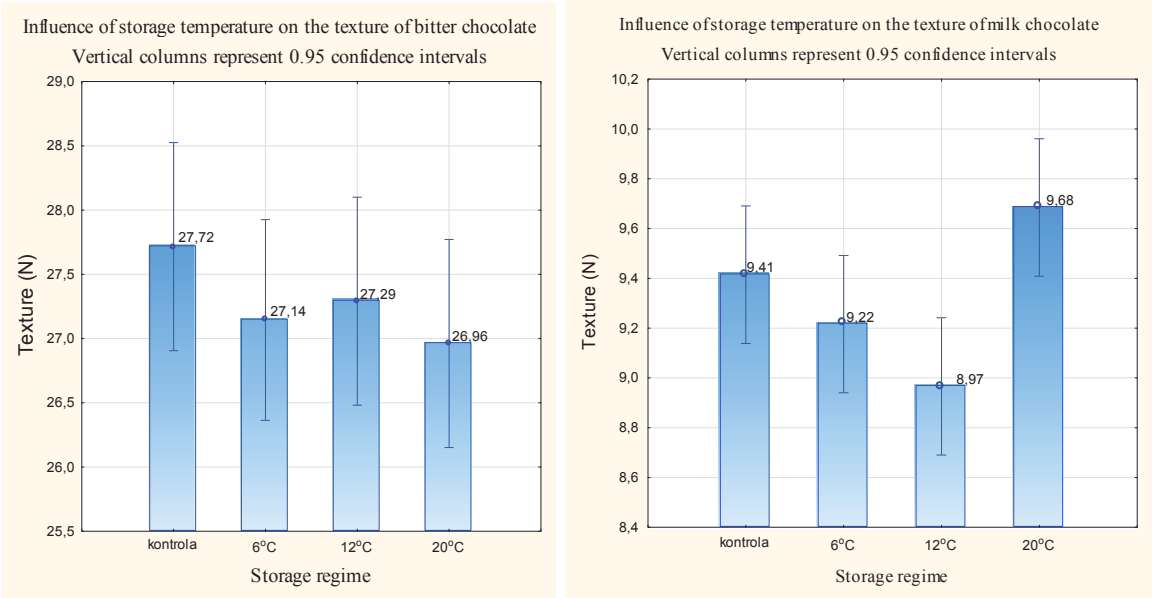
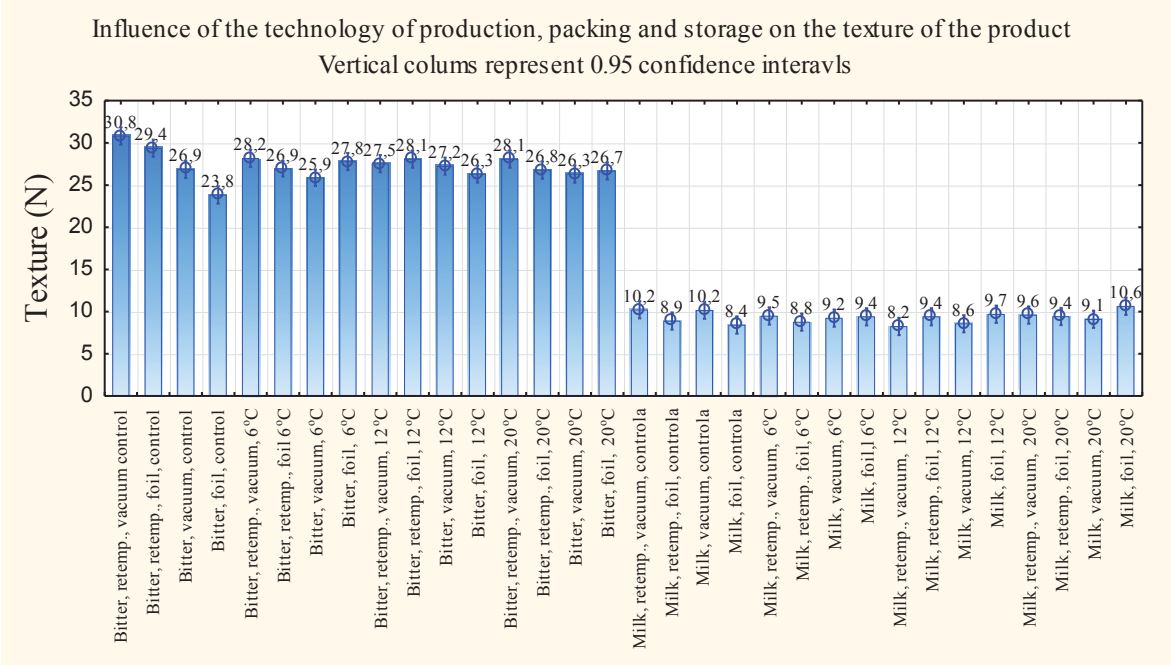


Figure 5 Influence of the technology of production, packing, and storage on the texture of the product (Vertical columns represent 0.95 confidence intervals, X-axis - variants 1 to 16; Y-axis - texture; Bitter chocolate; Milk chocolate)



CONCLUSION

The overall evaluation of the descriptors of sensory analysis after three months of storage has been uniform for bitter chocolate. Milk chocolate showed lesser glossiness, which could be due

to a higher sugar content that could cause the presence of sugar bloom on the product surface. The influence of the technological process was more pronounced on the texture of bitter chocolate, where the better texture occurred in non-tempered samples. The retempering process in this case had a beneficial effect on the hardness of samples because they contained a higher proportion of cocoa butter. In contrast, in terms of the milk chocolate texture, non-retempered samples received higher scores. The impact of packing was more evident in milk chocolate samples, with the best sample being vacuum-packed and the worst sample was packed in aluminium foil. Statistically significant differences were not observed in bitter chocolate. Storage regimes had a greater impact on milk chocolate samples. We have found out that room temperature does not affect them negatively. In contrast, the bitter chocolate samples stored at 20 °C had worst texture, but with very minimal differences between different storage regimes.

Many aspects influence the quality of chocolate products including their rheological, physical, and sensory properties. In this case, the recipe plays a key role, especially the content of cocoa butter.

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EXTRACTION OF FERULIC ACID FROM WHEAT BRAN BY ALKALINE HYDROLYSIS

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Abstract: Wheat bran is a low-cost by-product of the milling industry. Traditionally, it is used as livestock feed, but nowadays intention of wheat flour industry is to find a new value-added applications for a wheat bran. Wheat is a good source of phenolic compounds, from which the ferulic acid is the most abundant one. Ferulic acid exhibits lots of beneficial effects so it is widely used in food, health and cosmetic industries. In this paper, the optimization of extraction process of ferulic acid from wheat bran is presented. Ferulic acid was extracted by alkaline-hydrolysis using NaOH and later purified by precipitating hemicelluloses and glucomannans with ethanol. To optimize the extraction process for laboratory conditions, three initial weights of wheat bran, four concentrations of NaOH and three hydrolysis temperatures were examined. Obtained extracts were analysed by HPLC and amount of ferulic and other phenolic acids were determined. It was found out that only initial weight of ferulic acid had statistically significant effect on ferulic acid yields, however effects of concentration of NaOH and hydrolysis temperature were not so statistically significant. Besides ferulic acid, other phenolic compounds were also found to be present in wheat bran extracts such as sinapic, caffeic and coumaric acid, but they were found only in minor amounts.

Key Words: wheat bran, HPLC, ferulic acid, extraction, food waste, phenolic acids, alkaline hydrolysis

INTRODUCTION

Milling industry produces large volumes of by-products that have not been exploited to their full potential (Ruthes et al. 2017). Of all by-products, wheat bran is the most important one (Pruckler et al. 2014). Wheat bran is a by-product from the roller milling of the wheat grain to produce wheat flour (Apprich et al. 2014). This material represents about 25% of the grain weight. Milling industry generates about 150 million tons of wheat bran per year (Pruckler et al. 2014). Traditionally, it is used as livestock feed (Apprich et al. 2014). Only small amounts of this by-product are sold as commercial bran for food industry. Currently, wheat flour industry intends to find new value-added applications for wheat bran (Pruckler et al. 2014).

The major components of wheat bran are dietary fibre, protein, starch, water and minerals (Apprich et al. 2014). Minor components include phenolic compounds, flavonoids, lignans and phytic acid. Phenolic acids are the most frequent phenolic compounds in wheat. They are derived either from hydroxycinnamic (*p*-coumaric, caffeic, ferulic and sinapic acids), or from hydroxybenzoic (*p*-hydroxybenzoic, protocatechuic, vannilic, syringic and gallic acids) acid (Wang et al. 2013). Ferulic acid (4-hydroxy-3-methoxycinnamic acid) represents the most common phenolic acid located in wheat bran with concentration about 20–1500 mg/100 g (Apprich et al. 2014). Ferulic acid esterifies the C-5 hydroxyl group of arabinose residues in arabinoxylans (Barberousse et al. 2009). Extraction of ferulic acid and other phenolic compounds from agricultural residues is becoming more and more important since nowadays trend is to develop value-added commodities made from by-products (Buranov and Mazza 2009).

Ferulic acid exhibits several physiological benefits such as anti-microbial, anti-oxidants, anti-inflammatory, anti-thrombosis and anti-cancer activities (Qu et al. 2017). Moreover it has beneficial effects on coronary diseases, lowers cholesterol in serum and liver and improves sperm

viability (Ou et al. 2007). That is why ferulic acid shows wide potential of commercial use in food (preservative agent, gel-forming properties, flavour precursor), health (antioxidant, antimicrobial, antiinflammatory, etc.) and cosmetic (photoprotecting agent) industries (Barberousse et al. 2009). Moreover, ferulic acid is favorable, beacuse human body can easily absorb it and metabolize it (Ou et al. 2007).

Ferulic acid is in plant material cross-linked with polysaccharides via ester and ether bonds creating lignin/phenolics-carbohydrate complexes (Buranov and Mazza 2009). Therefore release and purification of ferulic acid from these complexes is challenging. There are two methods that can break the cross-link and release ferulic acid from plant cell walls (Ou et al. 2007). Ferulic acid can be released either enzymatically using feruloyl esterases, or by alkaline-hydrolysis using dilute (0.1–4 M) NaOH solution at 50–70 °C (Buranov and Mazza 2009). Purification of ferulic acid from the hydrolysate is difficult since it contains many contaminating components (waxes, oligomeric hemicelluloses). One of the approaches how to extract ferulic acid from enzymatic solution is the use of activated charcoal. Another way how to purify ferulic acid is to use an anion macroporous resin, as it has high capacity for adsorbing ferulic acid (Ou et al. 2007). However, these purification processes are expensive for industrial use, so an alternative simple and cheaper way how to purifiy ferulic acid from the hydrolysate is needed. Oily substances and hemicelluloses can be precipitated by adding ethanol up to an ethanol concentration of 30% in the hydrolysate. Using this method ferulic acid is dissolved in ethanol, while other components remain insoluble in hydrolysate (Buranov and Mazza 2009).

The aim of this study was to optimize the method for ferulic acid extraction from wheat bran by alkaline hydrolysis and purification of extracted ferulic acid using ethanol precipitation method. Optimization was focused on maximalization of extraction yields of ferulic acid. Optimized factors included initial weight of wheat bran, concentration of sodium hydroxide and hydrolysis temperature. Quantification of ferulic acid was performed by high-performance liquid chromatography (HPLC). Besides ferulic acid, other selected phenolic acids were also analyzed and quantified.

MATERIALS AND METHODS

Characterization of wheat bran samples

Wheat bran was provided by Mlýny J. Voženílek, spol. s r. o. from Předměřice nad Labem in Czech Republic. The bran was obtained from pre-treated wheat grains. It consists mostly of outter tissues and parts of wheat grains from which endosperm was removed. Maximum humidity of the bran is 15% and content of crude fiber is 8%. The bran was stored at room temperature until used.

Extraction process

Extraction process was carried out according to the method described by Buranov and Mazza (2009). To optimize extraction process for our laboratory conditions, three initial weights of wheat bran, four concentrations of sodium hydroxide and three hydrolysis temperatures were tested (Table 1). Total amount of 150 ml of sodium hydroxide was added to wheat bran in Erlenmeyer flask. The flask was kept at constant temperature on the shaker for 4 hours on 200 rpm, ensuring total hydrolysis. After cooling down, the hydrolysate was neutralized by 6 M hydrochloric acid.

Table 1 Optimized factors

Optimized factor	Initial weight of bran [g]	Concentration of NaOH [M]	Temperature of hydrolysis [°C]
1	5	0.5	40
2	10	1	50
3	15	2	60
4	–	3	–

The hemicelluloses and glucomannans were precipitated by adding 95% ethanol. The amount of addend ethanol corresponded to three times of the original volume. The precipitate was separated by filtration under the vacuum with Buchner funnel. Excess ethanol was removed from the filtrate

on rotary vacuum evaporator to approximately one third of initial volume, making sure that concentration of ethanol does not decrease under 30%. This lead to the formation of a brown extract which contained ferulic acid. The extract was finally passed through a 0.45-µm filter before HPLC analysis.

HPLC analysis

The extracts were analysed by HPLC to determine the quantity of ferulic and other phenolic acids. The HPLC system consisted of an Agilent 1260 Infinity apparatus equipped with diode array detector (DAD) with 10 mm absorption cell, autosampler with temperature regulation unit, quaternary pump, column thermostat and degasser. Phenolic acids were separated on Poroshell 120 Agilent C18 column (150 mm × 4.6 mm, particle size 2.7 nm) at 45 °C. The mobile phase consisted of acetonitrile (solvent A) and 2.5% solution of formic acid (v/v) (solvent B). Gradient elution was used for the analysis. The gradient was as follows: 0 min, 5% A; 10 min, 15% A; 30 min, 25% A; 35 min, 30% A; 50 min, 55% A; 55 min, 90% A; 57 min, 100% A and then held for 10 min before returning to the initial conditions. The data were collected by the Agilent 1260 Infinity chromatography data system. Identification of the individual phenolic acids was based on the comparism of retention times and the UV spectra obtained by DAD of unknown peaks to those of reference authentic standards. All samples were run in triplicate. Quantification of individual phenolic acids was performed via calibration curve of standards. Calibration curves for each standard were prepared over a concentration range 0.1– 100 mg/l with eight different concentration levels and triplicate injections at each level.

Statistical analysis

Data analysis and statistical evaluation was realised by Microsoft Excel and XL stat. Analysis of variance (ANOVA) was set to 95% confidence interval.

RESULTS AND DISCUSSION

Optimization of wheat bran initial weight

In the first stage of optimization, initial weight of wheat bran was tested. Other two parameters (concentration of NaOH and temperature of hydrolysis) were chosen according to Buranov and Mazza (2009) – 0.5 M and 50 °C. Weights examined were 5, 10 and 15 g. Yields of ferulic acid for different weights are shown in Table 2.

Table 2 Optimization of initial weight

Initial weight of bran [g]	Yields of ferulic acid [mg/g]
5	2.91 ± 0.15
10	2.75 ± 0.19
15	1.90 ± 0.13

From the results it is obvious that the highest average yields were observed on the 5 g samples – 2.91 mg/g. Further increase of initial weight of bran has led to significant decrease of extraction yields ($p < 0.0001$; $F = 260.1$; $F_{crit} = 3.89$). In addition to changing yields, a change in viscosity was observed. The viscosity of samples increased with growing weight. Samples with 15 g of bran were almost impossible to stir during hydrolysis which affected the effectivity of hydrolysis (the extraction solvent was not able to properly react with the hemicellulose structure). It resulted in significantly lower ferulic acid yields (1.90 mg/g). On the basis of this experiment, it was decided to run other experiments with 10 g of initial weight because the potential industrial use of this process demands the highest initial weight of bran as possible.

Optimization of sodium hydroxide concentration

Next optimized parameter was the concentration of sodium hydroxide. The initial weight of wheat bran was chosen according to previous experiment (10 g) and temperature of hydrolysis was chosen 50 °C according to Buranov and Mazza (2009). Examined concentrations of NaOH were 0.5, 1, 2 and 3 M. Yields of ferulic acid are shown in Table 3.

The highest average yields were obtained using 0.5 M NaOH (2.75 mg/g). Further increase of NaOH concentration did not cause statistically significant change of ferulic acid yields ($p = 0.079$; $F = 2.72$; $F_{crit} = 3.24$). This fact is important for potential industrial production of ferulic acid. It is possible to use low concentrations of hydroxide, which is economically advantageous. Nevertheless, it was noticed that 3 M sample was the easiest to filtrate. This is probably caused by salting out effect – during the neutralization, greater amount of sodium chloride is generated and hemicelluloses become more insoluble. This may lead to more distinct precipitation of hemicelluloses (Sun 2010). This phenomenom did not have effect on ferulic acid yields, but it is a notable finding for production of ferulic acid in industrial scale.

Table 3 Optimization of concentration of sodium hydroxide

Concentration of NaOH [M]	Yields of ferulic acid [mg/g]
0.5	2.75 ± 0.19
1	2.62 ± 0.16
2	2.63 ± 0.14
3	2.71 ± 0.17

Optimization of hydrolysis temperature

During the last experiment, optimal hydrolysis temperature was determined. The initial weight of wheat bran and concentration of NaOH were chosen according to previous experiments (10 g; 0.5 M). Temperatures investigated were 40, 50 and 60 °C. The 40 °C hydrolysate had the lightest colour, wheareas the 60 °C hydrolysate was the darkest one. After precipitation with ethanol, the 40 °C sample had fine agglomerations of precipitated glucomannans and hemicelluloses and the 60 °C sample had thick agglomerations. Yields of ferulic acid are shown in Table 4.

Table 4 Optimization of hydrolysis temperature

Hydrolysis temperature [°C]	Yields of ferulic acid [mg/g]
40	2.67 ± 0.18
50	2.60 ± 0.13
60	2.73 ± 0.16

The highest average yields were obtained when 60 °C extraction temperature was used (2.73 mg/g). Further change of hydrolysis temperature did not cause statistically significant change of ferulic acid yields ($p = 0.88$; $F = 0.13$; $F_{crit} = 3.89$). However, high temperatures may have positive effect on ferulic acid yields obtained by using hot-water extraction of wheat bran. Ferulic acid yields from this treatment are much lower than from alkaline hydrolysis (0.81 mg/g; 95 °C) (Sun et al. 2003).

Quantification of other selected phenolic acids

Besides ferulic acid, other selected phenolic acids were analyzed and quantified in purified hydrolysate. Sinapic, caffeic and coumaric acids were quantified during the optimization of concentration of NaOH and hydolysis temperature. Yields of the determined phenolic acids are shown in Table 5.

Table 5 Quantification of other phenolic acids

	Yields of sinapic acid [mg/g]	Yields of caffeic acid [mg/g]	Yields of coumaric acid [mg/g]
40 °C	0.234 ± 0.011	0.040 ± 0.002	0.065 ± 0.003
50 °C	0.240 ± 0.020	0.038 ± 0.001	0.068 ± 0.004
60 °C	0.140 ± 0.007	< LOD	0.075 ± 0.003
1 M	0.235 ± 0.012	0.017 ± 0.001	0.066 ± 0.003
2 M	0.214 ± 0.016	0.011 ± 0.001	0.063 ± 0.004
3 M	0.224 ± 0.017	< LOD	0.070 ± 0.004

From the results it can be seen that other phenolic acids can be extracted from wheat bran. However, yields of these acids are much lower than yields of ferulic acid. From these selected phenolic compounds sinapic acid is the most abundant one with the maximum average yield of 0.240 mg/g. Caffeic and coumaric acids were found only in trace amounts.

Comparison with other studies

Buranov and Mazza (2009) used alkaline hydrolysis with 0.5 M NaOH and 50 °C to extract ferulic acid from grain processing waste with yields 3.91 mg/g of ferulic acid, which is slightly more than in our work. Coumaric acid yields in our work are comparable to 0.020 mg/g yields published by Buranov and Mazza (2009). Liu et al. (2016) used three-step extraction at room temperature using methanol in the first step and sodium hydroxide in the second and third step. Ferulic acid and coumaric acid yields from all steps were 3.36 mg/g and 0.191 mg/g respectively. Liu et al. (2016) also used steam explosion technique which provided yields several times higher after first and second step of extraction than without this treatment. On the contrary after third step, the yields were lower than without steam explosion treatment. Gunenc et al. (2015) also used three-step extraction at room temperature using methanol in first and sodium hydroxide in second and third step. Ferulic acid yields ranged from 1.58 to 1.73 mg/g and coumaric acid yields ranged from 0.310 to 0.400 mg/g. Sinapic acid was also quantified and its yields ranged from 0.020 to 0.100 mg/g, whereas yields from our experiment ranged from 0.140 to 0.240 mg/g. Caffeic acid yields ranged from 0.030 to 0.100 mg/g, which is comparable to the results of our work. Xie et al. (2010) used enzymatic hydrolysis of wheat bran. After 4 days of fermentation with different species of fungi ferulic acid yields ranged from 0.21 to 1.91 mg/g. Kroon et al. (2000) used enzymatic hydrolysis of wheat bran with *Penicillium funiculosum* and after 24 hours they achieved a yield of 3.30 mg/g of ferulic acid.

CONCLUSION

The method for ferulic acid extraction from wheat bran by alkaline hydrolysis was optimized. Three initial weights of wheat bran, four concentrations of sodium hydroxide and three hydrolysis temperatures were tested.

It was found out that only the wheat bran initial weight has significant effect on ferulic acid yields – increase of initial weight has led to statistically significant decrease of extraction yields. Concentration of NaOH and hydrolysis temperature does not have significant effect on ferulic acid yields – change of NaOH concentration and hydrolysis temperature did not cause statistically significant change of ferulic acid yields.

Except the ferulic acid other phenolic acids (sinapic, caffeic and cumaric acids) were also extracted from the wheat bran. However, yields of these acids were much lower than yields of ferulic acid.

The optimized method has high potential for industrial use, because milling industry produces large volumes of wheat bran that has not been exploited to its full potential. Enzymatic hydrolysis of wheat bran is more specific and provides an environmentally friendly option to alkaline hydrolysis, but it is much more expensive and demanding on working conditions.

ACKNOWLEDGEMENTS

This work was financially supported by the project: Materials Research Centre at FCH BUT Sustainability and Development, REG LO1211, with financial support from the National Programme for Sustainability I (Ministry of Education, Youth and Sports) and by the specific research project FCH-S-17-4695.

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THE QUALITY OF HULLED WHEAT SPECIES IN ORGANIC FARMING

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Abstract: As organic farmers are searching perspective crops for growing, there is possible to choice neglected wheat species and also have a new market and sell opportunities. Concerning wheat, there are landraces so called hulled wheat species (einkorn, emmer wheat, spelt) comprising parts of collections of the world gene banks. Our paper aims at presenting the results of the study and the assessment of spring wheat forms, four einkorn cultivars, eight emmer wheat cultivars, seven spelt wheat cultivars in particular, as compared to modern bread wheat variety. Small-plot trials were established at three different localities within the Czech Republic in 2010 and 2012 in organic farming fields. The results of the trials show that the grains were characterised by a high proportion of protein in grain (up to 18.1%). However, they may be difficult to use for common baking (low Gluten index and sedimentation value). The main advantage was a high share of nutritionally valuable Albumins, Globulins and insoluble rest protein fractions in comparison with modern control varieties of bread wheat.

Key Words: organic farming, hulled wheat, baking quality, protein fractions

INTRODUCTION

Plant genetic resources are considered a unique and non-renewable source with which to enhance the plant genetic base (Dotlacil et al. 2010). The significance of the genetic diversity of low-input organic varieties has increased in the last several years. Genetic diversity resources have to be identified and strategies to enable the use of those diverse varieties in the organic breeding processes have to be developed and implemented (Serpolay et al. 2001).

Wheat (*Triticum aestivum* L.) is one from the most important crops for human diet (Moudry et al. 2013a). Despite a remarkable development in organic farming throughout Europe (Moudry et al. 2013b), there are not enough varieties which have been purposely bred for use within the organic farming system (Stehno et al. 2010). In particular, conventional bred and tested varieties which were reproduced under the organic farming conditions are grown there (Lammerts van Bueren et al. 2002). But there are many references from different authors (Wolfe et al. 2008) being reported lower baking quality of bread wheat within organic farming.

Organic farming can work with a wider diversity of crops (Konvalina et al. 2011). There are many neglected wheat species such as Triticum monococcum, diccocum or spelta which have potential to be grown in organic farming. They are more tolerant to many stress factors (Konvalina et al. 2014), e. g. decreasing quality of arable land (Kopecky et al. 2016). But they can provide grain in high quality (Piergiovanni et al. 1996). Hulled wheat species could be suitable for cultivation in marginal areas, under conditions of low-input or organic farming, where modern soft wheat varieties are unable to develop their full productive potential, because they are selected for favourable pedoclimatic and intensive agronomic conditions (Lacko-Bartosova et al. 2015).

Our article aims at evaluating the grain quality and protein fractions characteristics in a set of 25 varieties of hulled and bread wheat varieties grown in organic farming.

MATERIAL AND METHODS

Used varieties

The landraces of einkorn: Triticum monoccocum 38, Triticum monoccocum 44, No. 8910 and Schwedisches Einkorn. Emmer wheat (Triticum diccocum Schrank ex Schübler): Rudico, Weisser Sommer, May-Emmer, Triticum dicoccon (Brno), Triticum dicoccon (Dagestan), Triticum dicoccon (Palestine), Triticum dicoccon (Tapioszele) and Triticum dicoccum (Tabor). Spelt wheat (Triticum spelta L: Triticum spelta (Ruzyne), Triticum spelta (Tabor 22), Triticum spelta (Tabor 23), VIR St. Petersburg, Spalda bila jarni, Triticum spelta No. 8930 and Triticum spelta (Kew). All the varieties were spring wheat forms and came from the Gene bank of the Crop Research Institute in Prague-Ruzyne. As control were used 4 landraces varieties namely Postoloprtska presivka 6, Rosamova ceska cervena presivka, Cervena perla and Kasticka presivka 203; and 2 modern varieties of bread wheat (*Triticum aestivum L.*) namely Vanek and SW Kadrilj.

Field Trials

Varieties were sown in a randomized, complete block design on the organic certified research area in Ceske Budejovice, Prague Ruzyne and Uhrineves (CZ) during 2010 and 2012. 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. There was no artificial nitrogen application during the growing season. Trial were weeded by harrowing.

Laboratory analysis

The baking quality analysis in grain were tested after the dehulling of the grains by The International Association for Cereal Chemistry (ICC) methods: crude protein content (ICC 105/2); index of sedimentation - SDS test (ICC 151); wet gluten content (ICC 106/2), gluten index (ICC 155) and baking experiment (Lachman et al 2012). Gluten content was measured by ELISA Technologies. Protein fractions were measured according the methodology developed by Osborn (1907) with modifications (Lookhart and Bean 1995).

Statistical analysis

Data were processed by the Statistica 9.0 (StatSoft. Inc., USA) programme. The comparison of varieties and their division into statistically different categories were provided by the Turkey HSD test.

RESULTS AND DISCUSSION

From the technological point of view, there is an important protein content and its quality. In case of hulled wheat species, there was high protein content. Regarding the tested collection of varieties, the average protein content in grain achieved 15.59% in einkorn, 16.04% – emmer wheat, 15.74% – spelt wheat, 13.21% – land races of bread wheat (Table 1). Regarding the modern varieties, the average protein content in grain achieved 12.79%. In the group of hulled wheat species, there was also high wet gluten content (36.5%; 38.0%; 42.2%) in comparison with control varieties of bread wheat (30,9%). Gluten index was very low in einkorn and emmer varieties with15. SDS test and Zeleny test was in case of diploid (einkorn) and tetraploid (emmer) at the half level of other species (Table 1). On the other hand, in case of hexaploid spelt wheat there was baking quality higher (Gluten index – 36) and Zeleny test 34 ml – this level is comparable to the group of bread wheat varieties.

Table 1 The baking quality characteristics of different wheat species

Species	Protein content (%)	Wet gluten content (%)	Gluten index	SDS test (ml)	Zeleny test (ml)
Einkorn	15.59^{b}	36.45^{bc}	14.39^{a}	29.04^{a}	14.07^{a}
Emmer	16.04^{b}	38.04^{c}	15.43^{a}	33.15^{a}	15.81^{a}
Spelt	15.74^{b}	42.26^{d}	35.68^{b}	57.35^{b}	34.35^{b}
Landraces of bread wheat	13.21^{a}	33.11^{ab}	41.46^{c}	56.65^{b}	35.96^{b}
Control varieties of bread wheat	12.79^{a}	30.87^{a}	68.75^{d}	65.38^{c}	42.36^{c}

Legend: Within column values followed by the same letter are not significantly different at P < 0.05 (Tukey HSD test).

From the nutritional point of view, there is an important composition of protein fractions (glutenins and gliadins) (Shewry et al. 2000). For human nutrition are important protoplasmatic fractions "albumins and globulins" and also insoluble rest (fractions which are close to protoplasmatic ones). In case of einkorn and emmer there was lower gluten content – but concentration is too high for people with the celiatic disease. On the other hand, there was better protein fractions composition in case of einkorn and emmer. Einkorn had Albumins, Globulins and Insoluble rest fractions more than 59% in comparison with bread wheat (Table 2). Very simmilar composition had also emmer and some varieties of spelt. High content of protoplasmatic fractions is good from nutritional point of view, however, It has influence on the negative way to technological quality.

Table 2 Protein fractions content of different wheat species

Species	Gluten content (mg/100g)	Albumins + Globulins (%)	Gliadins (%)	Glutenins (%)	Insoluble rest (%)
Einkorn	153.45^{a}	26.67^{c}	27.93^{a}	27.64^{a}	17.76^{d}
Emmer	194.86^{b}	28.67^{b}	30.06^{b}	27.70^{a}	13.67^{c}
Spelt	248.17^{c}	27.04^{b}	31.03^{bc}	29.95^{b}	12.12^{b}
Landraces of bread wheat	265.96^{c}	26.30^{b}	32.17^{c}	30.59^{b}	10.92^{b}
Control varieties of bread wheat	248.52^{c}	21.38^{a}	34.67^{d}	36.96^{c}	6.99^{a}

Legend: Within column values followed by the same letter are not significantly different at P < 0.05 (Tukey HSD test).

CONCLUSIONS

The hulled wheat species are supposed to be grown in organic farming, because of in this conditions can provide more stable and high yield in comparison with modern bread wheat varieties. Hulled wheat species had a high protein contend and wet gluten content. There were differences between species – einkorn and emmer were not suitable for „clasical" baking processing. But there is potential for other products eq. wheat rice (einkorn) or pasta (emmer). Spelt will be possible to use in "clasical" baking industry, but the best solution will be use grain in the mixture with bread wheat. The most important advantage of hulled wheat species is nutritional value of grain from the point of view of protein fractions composition – hulled wheat species had in average more albumins and globulins and insoluble rest fractions than modern varieties of bread wheat. On the other hand there is lower gliadin and glutenins content – it is possitive from nutritional point of view, but not from technological.

ACKNOWLEDGEMENTS

Our work was supported by the Ministry of Agriculture of the Czech Republic – NAZV, Grant No. QJ1310072.

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EFFECT OF SELECTED OILS ADDITION IN DIET ON FATTY ACIDS CONTENT IN LIVER TISSUE OF RATS

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Abstract: The aim of this experiment was to evaluate the effect of selected oils addition in diet on fatty acids content in liver tissue of model animals (rats). Animals were divided into four groups and fed daily *ad libidum* with basic feed mixture enriched of 6% selected oils. Palm oil (control group), fish oil and *Schizochytrium alga* oil – DHA (test groups), safflower oil (negative control) were used. At the end of the experiment liver tissue of rats was removed. Fatty acids representation in liver tissue was determined by gas chromatography. It was concluded that composition of fatty acids in the diet correlates with their deposition in liver tissues of rats and may have an effect on regulation of chronic inflammatory diseases.

Key Words: fatty acids, liver tissue, rats, gas chromatography

INTRODUCTION

Representation and content of fatty acids in human nutrition is very important. It is possible to distinguish several fatty acids groups – saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). PUFA group is divided (according to location of the first double bond on carbon) to n-3 and n-6 series. This series are characterized by different physiological effects (Erdman et al. 2012) and are essential components of the metabolically active tissues such as liver (Noolen et al. 2014).

Several studies have demonstrated potential health benefits of substituting SFA with unsaturated fatty acids, particularly oleic acid (18:1, MUFA) and PUFA n-3 serie. Thus, reducing consumption of foods rich in SFA and increasing consumption of foods containing oleic acid or PUFA n-3 serie is likely to reduce the incidence of metabolic disease (Kennedy et al. 2009).

The starting compounds of PUFA n-3 and n-6 series are α-linolenic acid (18:3n-3) and linoleic acid (18:2n-6), both metabolized by the same set of enzymes (elongase and desaturase) to long-chain PUFA. The most important metabolites of PUFA n-6 and n-3 series are arachidonic acid (20:4n-6), eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3) (Grofová 2010). The final metabolites of PUFA n-6 and n-3 series are eicosanoids (prostaglandins, leukotrienes and tromboxanes). These metabolites play important roles in regulation of inflammatory response (Noolen et al. 2014). The ratio of PUFA n-6 : n-3 under 5 : 1 or lower is recommended in human nutrition (Kouba and Mourot 2011). A diet with a high ratio of PUFA n-6 : n-3, in conjunction with a genetic factors, can be associated with an increasing prevalence of chronic inflammatory diseases such as cardiovascular disease. Eicosanoids generated from arachidonic acid have a pro-inflammatory effect (platelet aggregation, vascular wall diminution). Eicosanoids generated from eicosapentaenoic acid and docosahexaenoic acid can elicit anti-inflammatory and cardioprotective effects, reduce the risk of autoimmune disease and cancer (Komprda 2003, McDaniel et al. 2011).

This experiment was focused on determination of fatty acids content in liver tissue of rats. The aim of this experiment was to test the hypothesis regarding of the effect of selected oils addition in diet of rats on fatty acids level in model animals and apply received knowledge in human nutrition.

MATERIAL AND METHODS

Tested samples description

Forty-eight adult male rats of the laboratory strain Wistar Albino (produced by Faculty of Medicine, Masaryk University, Brno, Czech Republic) were used. Animals were randomly divided into four groups and housed in the plastic boxes (53.5 x 32.5 x 30.5 cm) per four animals. Room was maintained at 23 ± 1 °C, humidity 60% and 12/12 h light/dark cycle. The animals were fed daily *ad libidum* with basic feed mixture, pelletized complete chow for mice and rats (Biokron, Blučina, Czech Republic) and had free access to drinking water. Feed mixture was composed of wheat, oat, wheat sprouts, soybean meal, extruded soybean, maize, dried milk, dried whey, dried yeast, grounded limestone, monocalcium phosphate, salt, L-lysine hydrochloride, premix of vitamins and minerals. The diet was formed by admixing of 6% selected oil to the chow: palm oil (control group), fish oil and *Schizochytrium alga* oil – DHA (test groups), safflower oil (negative control). Fatty acids content in used oils is presented in Table 1. Feed consumption was measured daily. Animals were weighed in weekly intervals. At the end of the experiment (after 10 weeks) liver tissue of rats was removed.

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.

Table 1 Fatty acids content in used oils (in % of total fatty acids)

Fatty acid	Designation	Palm oil	DHA oil	Fish oil	Safflower oil
Lauric	12:0	0.3			
Myristic	14:0	0.8	4.4	4.6	0.4
Pentadecanoic	15:0	-	7.6	9.6	7.4
Palmitic	16:0	39.4	14.6	15.3	8.3
Palmitoleic	16:1n-7	0.4	0.9	8.1	0.6
Heptadecanoic	17:0	-	0.3	0.6	0.3
Stearic	18:0	4.8	1.7	3.1	3.1
Oleic	18:1n-9	43.8	15.5	25.1	14.2
Vaccenic	18:1n-7	1.3	-	-	-
Linoleic	18:2n-6	8.4	5.9	9.5	61.7
Linolenic n-6	18:3n-6	0.1	0.3	0.4	0.7
Linolenic n-3	18:3n-3	0.3	0.4	1.4	0.4
Eicosenoic	20:1n-9	0.3	-	-	-
Eicosadienoic	20:2n-6	-	0.1	0.4	0.2
Homo-Gamma-Linolenic	20:3n-6	-	0.7	0.2	0.2
Arachidonic	20:4n-6	-	0.7	0.8	0.5
EPA	20:5n-3	-	0.9	8.5	0.5
DTA	22:4n-6	-	13.0	0.3	0.3
DPA	22:5n-3	-	0.6	1.1	0.3
DHA	22:6n-3	-	32.3	11.2	1.4

Fatty acids determination

Liver tissue was analyzed according to the protocol described in the paper of Komprda et al. 2013. Liver samples were lyophilized (lyophilizer Christ Alpha 1–2 LD) under following conditions: main drying at -45 °C/24 hours, final drying at -50 °C/3 hours.

For total lipid extraction mixture hexan/2-propanol in ratio 3 : 2 (v/v; HIP 1.15 ml) and in ratio 7 : 2 (v/v; HIP 2, 10 ml) was used. A sample (approx. 5 g) was homogenized for 2 minutes (homogenizer DIAX 900 Heidolph) and left in ultrasonic bath for 15 minutes (PS10000, Notus-Powersonic). After sample filtration, aqueous solution of sodium sulphate (24 ml, 0,5 mol/l) was added to the filtrate and the sample was shaken in a separating funnel (3 min). The upper organic phase with HIP 1 (extraction) was combined with the organic phase HIP 2 (repeated extraction). The solvent mixture with HIP was evaporated on a rotary vacuum evaporator (IKA RV 05-ST) at temperature of 40 °C and at 50 rpm/min. The solvent remainder was blown under a nitrogen atmosphere to a constant weight. The total lipid content was determined gravimetrically.

The sample with extracted total lipids (40–60 mg) was mixed with 3 ml Pentadecanoic acid (15 : 0) in isooctane (concentration of 1 mg/ml) and 3 ml sodium methanolate (1.15 g metallic sodium/100 ml methanol) in the boiling flask. The mixture was heated in water bath (K 10 E 1 Medingen) at 65 °C for 15 minutes under reflux. Methanolic solution of boron trifluoride (3 ml, concentration of 14%) was added and was mixture was heated again for 5 minutes at 65 °C. The boiling flask was cooled to room temperature and 2 ml isooctane and 5 ml of saturated solution of sodium chloride were added. Mixture was shaken for 10 minutes (Shaker GFL 3005). Upper organic phase (1 ml) was used for chromatographic analysis.

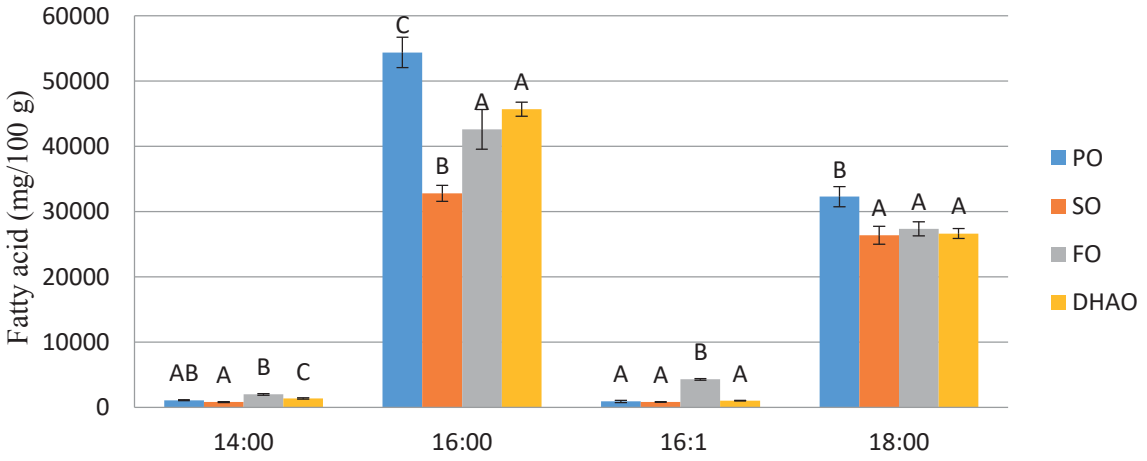
Fatty acid methyl esters were identified by gas chromatograph Fisons GC 8000 series with capillary column DB-23, 60 m x 0.25 mm x 0.25 µm (Agilent Technologies, USA), flame ionization detector and autosampler HT300A. The carrier gas was used nitrogen. Temperature program: 140 °C/1 min., gradient 5 °C/ min. to 200 ˚C/1 min, gradient 3 ˚C/min. to 240 °C held for 15 min., injector temperature 250 °C, detector temperature 260 °C. Separation conditions: flow rate of 1.5 ml/min, pressure of 200 kPa, split ratio of 20 : 1.

The measured data were statistically evaluated in Microsoft Excel 2010 and Statistica 12 programmes by one-way analysis of the variance ratio test, including Tukey's post-hoc test ($p < 0.05$).

RESULTS AND DISCUSSION

The experiment was focused on the effect of selected oils addition in diet on fatty acids content in liver tissue of rats. High content of palmitic (16:0) and stearic (18:0) acids and low content of myristic (14:0) and palmitoleic acids (16:1) were detected in all groups. The control group (palm oil) is characterized by significantly high content of palmitic and stearic acids compared with other test groups (fish oil, DHA oil) and negative control group (safflower oil). The representation of SFA and MUFA in liver is shown in Figure 1.

Figure 1 SFA and MUFA content in the liver of rats

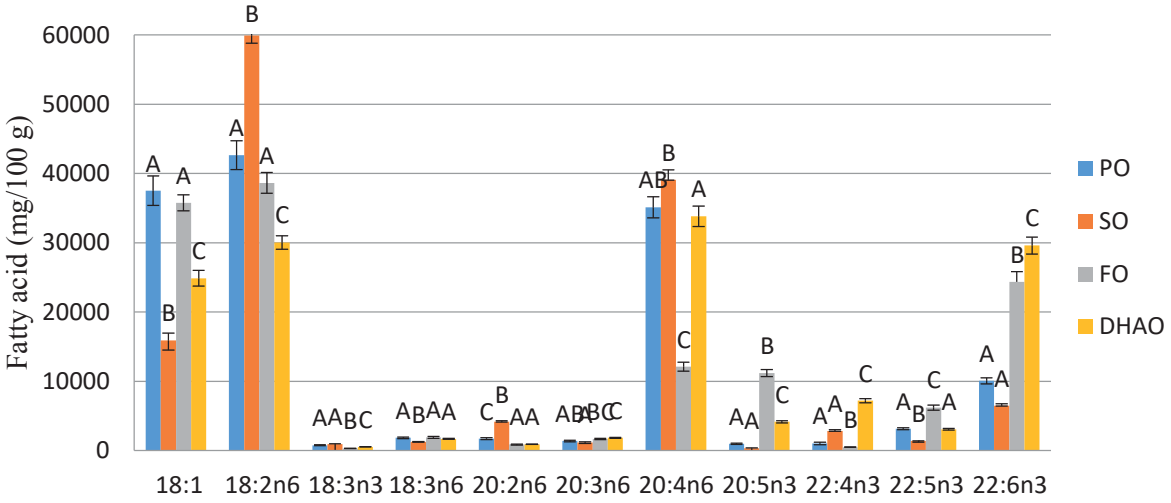


Legend: PO- Palm Oil, SO – Safflower Oil, FO – Fish Oil, DHAO – DHA Oil; $^{A-C}$ means with different letters within a given trait differ at $P < 0.05$

PUFA composition of liver samples correlates with fatty acids composition of selected oils added to chow (Figure 2). The increase of PUFA n-6 serie (linoleic acid, 18:2n-6; arachidonic acid,

20:4n-6) was expected in a group with diet enriched of safflower oil compared with control group (palm oil).

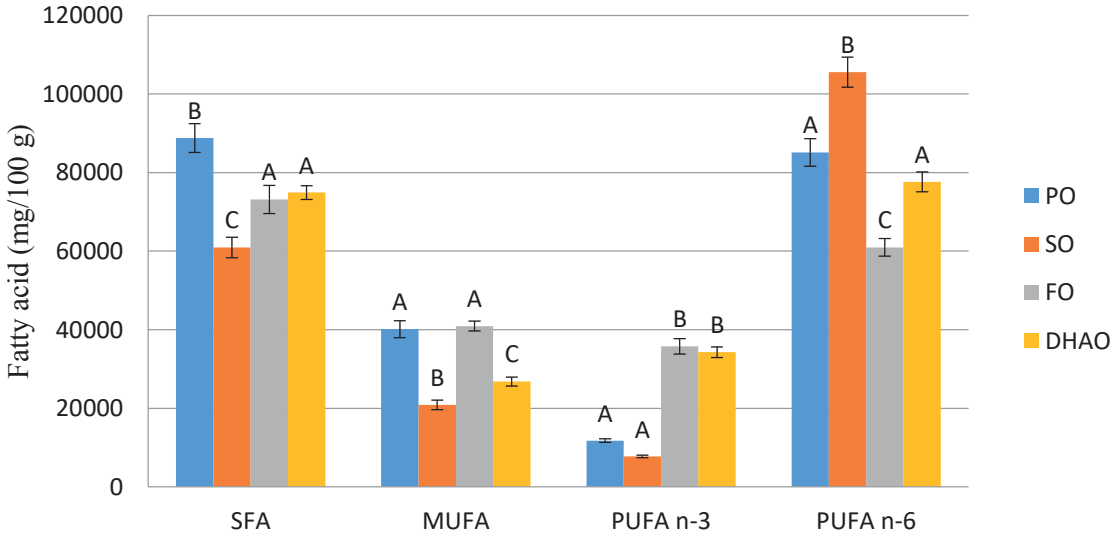
Figure 2 PUFA content in the liver of rats



Legend: PO- Palm Oil, SO – Safflower Oil, FO – Fish Oil, DHAO – DHA Oil; $^{A-C}$ means with different letters within a given trait differ at P < 0.05

High intake of safflower oil in diet increased content of PUFA n-6 in liver tissue. Diet with addition of fish oil and DHA oil had an positive effect on increasing PUFA n-3 in tested tissue. The representation of fatty acids groups detected in liver is shown in Figure 3. The increased intake of food containing PUFA n-3 and restriction of SFA, trans-fatty acids and consumption of PUFA n-6 have been the right approach leading to our health improvement (Grofová 2010). The total recommended daily intake of fats is set at 30%, PUFA group should be represented by 7% (Komprda 2003).

Figure 3 Content of SFA, MUFA and PUFA group in the liver of rats

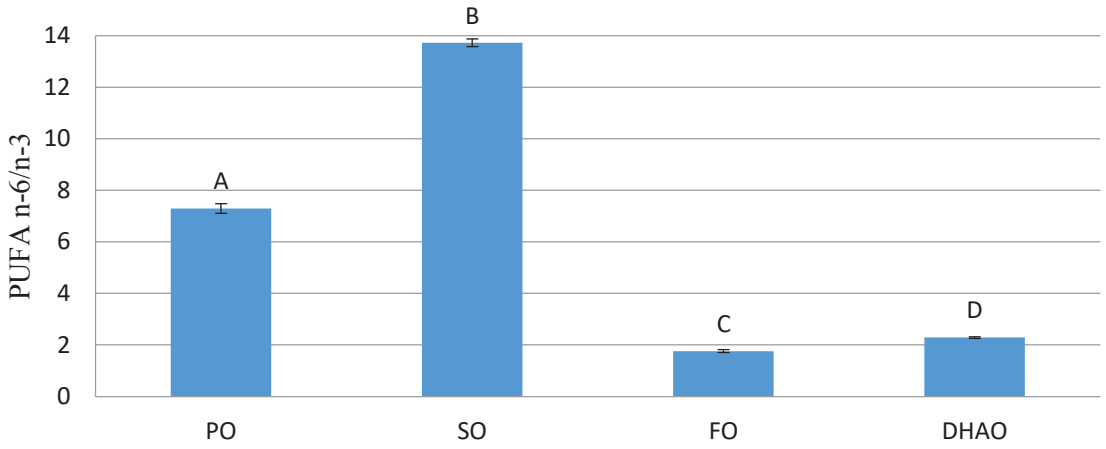


Legend: PO- Palm Oil, SO – Safflower Oil, FO – Fish Oil, DHAO – DHA Oil; $^{A-C}$ means with different letters within a given trait differ at P < 0.05

The highest value of the ratio of PUFA n-6 : n-3 was observed in the negative control group (safflower oil) than in the test groups (fish oil, DHA oil) and control group (palm oil). This result was expected due to the high content of linoleic acid (18:2n-6) in safflower oil. Significant reduction in the ratio of PUFA n-6 : n-3 in the diet enriched of fish oil and DHA oil (rich sources of PUFA n-3) was measured in comparison to control group with palm oil. The ratio of PUFA n-6 : n-3 in the diet

enriched of safflower oil was significantly increased compared to other groups (Figure 4). Ratio of PUFA n-6 : n-3 should be optimally from 1 : 1 to 4 : 1 in human diet (Kohout 2010), but it is significantly higher (15-16.7 : 1) in economic developed countries with the consequence of increased risk of chronic degenerative diseases (Simopolous 2008).

Figure 4 The ratio of n-6 : n-3 fatty acids in the liver of rats



Legend: PO- Palm Oil, SO – Safflower Oil, FO – Fish Oil, DHAO – DHA Oil; $^{A-C}$ means with different letters within a given trait differ at P < 0.05

The qualitative and quantitative representation of fatty acids depends on the diet and on the nutrient conversion, which is affected by a number of factors like metabolism, age, seasons, temperature, length of light and the chemical form in which the nutrients are administered (Rozíková et al. 2012).

CONCLUSION

It can be concluded based on our results that, composition of fatty acids in the diet correlates with their deposition in liver tissues of rats. Deposition of linoleic acid (18:2n-6) in the liver was increased significantly ($P < 0.05$) under the diet enriched of safflower oil rich in linoleic acid, compared to the control group with palm oil. Deposition of DHA (22:6n-3) in the liver increased significantly ($P < 0.05$) under the diet enriched of fish oil and DHA oil, therefore diet rich in PUFA n-3 serie compared to control group (palm oil). Increased amount of PUFA n-3 serie in the diet affects desirable increase of these fatty acids levels in liver tissues. Therefore, the ratio of PUFA n-6 : n-3 can approach almost to an optimum and so reduce the risk of cardio-vascular disease due to favorable physiological effects.

ACKNOWLEDGEMENTS

The research was financially supported by the Internal Grant Agency of the Mendel University in Brno (project No. TP 3/2017). The results and outcomes were prepared using instrumentation funded from the Research and Development for Innovations Operational Programme, project CZ.1.05/4.1.00/04.0135 Teaching and Research Facilities for Biotechnological Disciplines and Extension of Infrastructure.

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DYNAMICS OF CHANGES IN THE CONTENT OF SELECTED ANTHOCYANINS DURING THE PROCESSING OF GRAIN OF THE SCORPION WHEAT VARIETY

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Abstract: The aim of this study was to monitor the dynamics of the changes in the content of selected anthocyanins in the processing of the blue-grain Scorpion wheat variety for food. Nine selected anthocyanins were monitored by high performance liquid chromatography and their content determined in wholemeal flour, bran, and soft flour. To determine the content of individual anthocyanins and their degradation rates during grain processing to the final product, wholemeal flour was used to bake products. The dominant anthocyanin was found to be delphinidin-3-O-rutinoside in wholemeal flour (12.75 μg/g) and in bran (43.69 μg/g). The content of the most common anthocyanins in wholemeal flour, including the dominant delphinidin-3-O-rutinoside, dropped quickly to 2.59 μg/g during processing to wholemeal bakery products.

Key Words: Scorpion wheat grain, blue aleurone, anthocyanins, flour, bran, wholemeal bakery products

INTRODUCTION

Common wheat (*Triticum aestivum* L.) includes in addition to conventional colouring of caryopsis also genotypes with increased content of natural dyes in different anatomical layers of the grain. These are colour pigments of the anthocyanin group (caryopses with purple pericarp and blue aleurone) and carotenoids (caryopses with yellow endosperm). The most represented anthocyanins include delphinidin-3-glycoside and delphinidin-3-rutinoside in blue-grained wheat (Trojan et al. 2010, Knievel et al. 2009).

The blue colouration of aleurone is due to the Ba genes (blue aleurone). A well-known donor is the California UC66049 line that carries the Ba1 gene on the 4B chromosome or the Thatcher Blue carrying the Ba2 gene on chromosome 4A. The blue colouration of the wheat grain was also found in the independent taxon *T. aestivum var. tschermakianum Mansf.*, on the basis of which the winter variety Scorpion was bred. However, the gene causing the blue colouration of grain in this variety has not yet been localized. This variety was bred in the Crop Research Institute (CRI) Prague-Ruzyně and Agrotest fyto, s.r.o., Kroměříž. The Scorpion variety is currently widely used for crossbreeding with common wheat varieties to increase yield. It is also possible to increase the colour pigment content by combining the blue aleurone and purple pericarp genes (Martinek 2016). The occurrence of wheat with very dark grain colouration and detection of a wide range of 26 different anthocyanins was confirmed by Garg et al. in 2016.

The above colour pigments show antioxidant activity, therefore the caryopses of colour wheat varieties with increased content of these natural dyes appear to be suitable for the production of so-called functional foods (Trojan et al. 2010). These are foods with not only nutritional value but also with beneficial effects on the health of consumers, which has been scientifically documented (Chabinová et al. 2011). It has been demonstrated that anthocyanin-type antioxidants contained in the diet have preventive action against arthritis, atherosclerosis, inflammatory processes, and some types of cancer (Lachman et al. 2003). They reduce the risk of cardiovascular diseases, protect the organism from oxidative stress and DNA from damage, prevent aggregation of thrombocytes,

lipoprotein oxidation, etc. (Hrnčířová et al. 2011). For this reason, coloured wheat is becoming more and more important and has recently been of interest to both scientists and food producers.

However, it has been found that anthocyanins are relatively unstable compounds and more easily undergo degradation in food processing and storage (Jing et al. 2007). Up to now, however, there is only a limited amount of information describing it (Patras et al. 2010).

MATERIAL AND METHODS

Description of tested samples

We have used caryopses of coloured wheat of the Scorpion variety with blue aleurone, harvested in full maturity. The samples were ground on the Chopin Moulin CD1 mill. Three fractions of grist, namely flour, semolina and bran, were obtained. Wholemeal flour was obtained by destruction of the whole grain on the Perten 120 laboratory mill. Products baked from wholemeal flour, according to the baking experiment, was dried and ground, as well.

Baking experiment - Rapid Mix Test

The baking experiment recipe was adapted to the conditions of the laboratory of the Institute of Food Technology. The recipe consisted of 500 grams of wholemeal flour, 25 g of yeast, 7.5 g of salt, 5 g of sugar, 5 g of sunflower fat and 300 ml of water. Of all the ingredients dough was prepared on the Zelmer PROFI Fenomen dough maker at 1,200 rpm. It was followed by maturing the dough in the proofer at 32 °C temperature and 80% ± 5% relative humidity for 20 minutes. After removal from the proofer, the dough was rolled and left to rest for 10 minutes and then divided into desired pieces of 80 g, which took three minutes. This was followed by the formation of pieces of dough and rising in a proofer for 25 minutes under the same conditions as when it matured. Before baking, the dough was sprinkled with water and baked at 230 °C to 240 °C for 20 minutes. The oven was steamed with 50 ml of water prior to baking. The pieces were baked in a laboratory oven with proofer from the Polish producer ZBPP Sp. Zo. o., Bydgoszcz.

Extraction and high performance liquid chromatography (HPLC)

The content of selected anthocyanins was determined in flour, bran, wholemeal flour and wholemeal bakery products by extraction and high performance liquid chromatography.

For the extraction of anthocyanins in the samples, a solution of methanol and 4M HCl in a ratio of 85 : 15 was used. The extraction was performed four times on each sample using Vortex MS2 Minishaker IKA® (5 s) and Orbital Shaker PSU-10i, BioSan (400 rpm, 30 min). To accelerate the sedimentation of the extracted sample, the Hettich Universal 32 R centrifuge (4,000 rpm, 10 min) was used. After extraction, the samples were evaporated in a stream of nitrogen using the Sampler Concentrator, Miulab, NDK 200-2, and stored in the dark at -18 °C until the subsequent chromatographic analysis.

To identify the selected anthocyanins, Agilent 1 100 liquid chromatograph (Agilent Technologies, Germany), Kinetex-Biphenyl 100A column (150 x 4.6 mm, particle size 5 µm) was used. The mobile phase composition was methanol and 6% formic acid. A UV-Vis detector was used to detect the substances being separated. Separation conditions included gradient separation, flow rate 1 ml/min, injection volume of standards and samples 10 µl, separation duration 28 minutes, wavelength 525 nm, and separation temperature 25 °C.

The chemicals and standards used were purchased from Sigma-Aldrich s.r.o. (USA). As standards, nine standard solutions of anthocyanins were selected, namely myrtillin chloride, delphinidin-3-O-rutinoside, kuromanin chloride, petunidin-3-0-glucoside chloride, keracyanin chloride, malvidin-3-0-glucoside chloride, peonidin-glucoside chloride (Peo G), callistephin chloride, and peonidin-3-O-rutinoside chloride (Peo R).

All tested samples were analyzed three times. The measured data were statistically evaluated in Microsoft Excel 2010 and Statistica 12 programmes by one-way analysis of the variance ratio test, including Tukey's post-hoc test ($p < 0.05$).

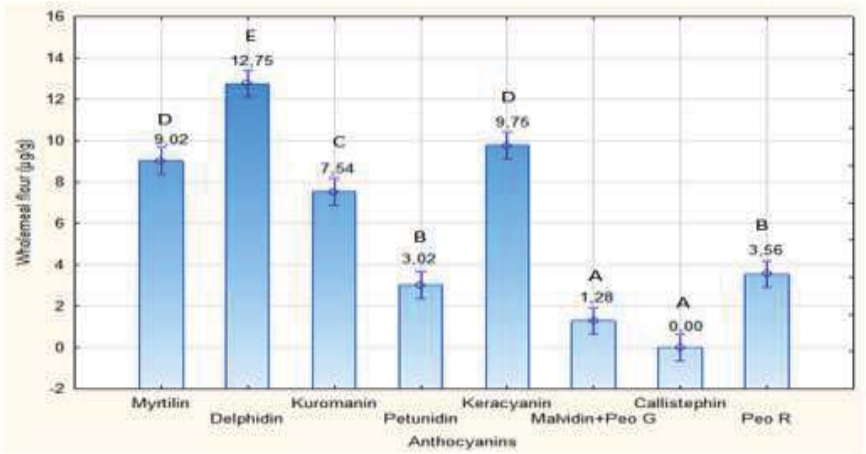
RESULTS AND DISCUSSION

Content of selected anthocyanins in individual fractions of grain

Anthocyanins in the caryopses of the blue-grain Scorpion wheat variety are found in the aleurone layer, which is located between the husk layers (bran) and the endosperm. By destroying the whole grain during grinding, the anthocyanins are transferred to the flour. Eight of the nine monitored anthocyanins were identified in the wholemeal flour samples. Delphinidin-3-O-rutinoside (delphinidin) was found to be the dominant anthocyanin, which corresponds to already published results by Abdel-Aal et al. 2008 and Ficco et al. 2014. Furthermore, high contents of keracyanin chloride (keracyanin) and myrtillin chloride (myrtillin) were also found (Figure 1).

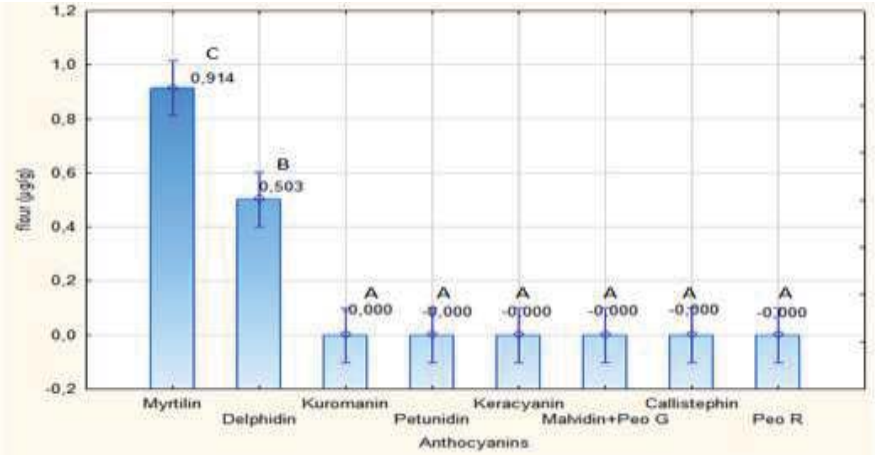
When grinding grain for soft flour, only a part of the aleurone gets into this fraction, and it is also evident in the content of anthocyanins, which is steeply decreasing (Figure 2). Low concentrations of only two anthocyanins from a total of nine, namely myrtillin chloride (myrtillin) and delphinidin-3-O-rutinoside (delphinidin) were detected in the soft flour.

Figure 1 Content of selected anthocyanins in wholemeal flour (Scorpion variety)



Legend: Vertical columns represent 0.95 confidence intervals. The averages of the different variants do not differ significantly (p > 0.95) if the same upper index is indicated.

Figure 2 Content of selected anthocyanins in flour



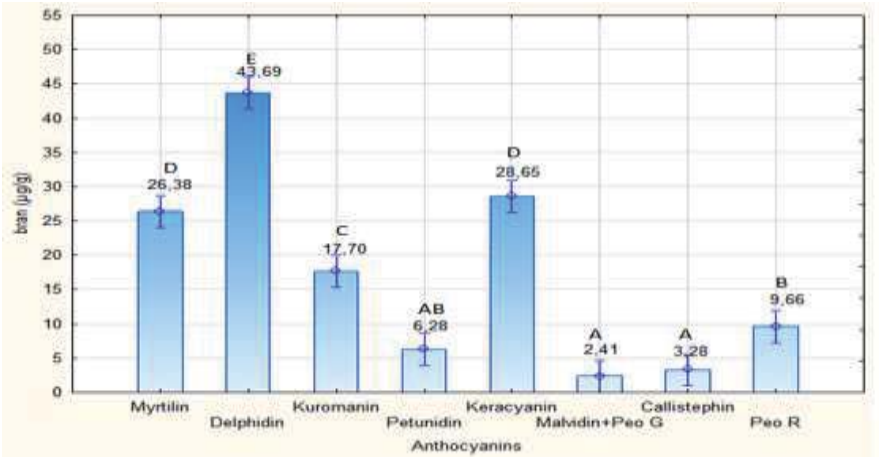
Legend: Vertical columns represent 0.95 confidence intervals. The averages of the different variants do not differ significantly (p > 0.95) if the same upper index is indicated.

Aleurone layer is part of the husk layers of grain (Prugar et al. 2008). Therefore, the anthocyanin content in bran was considerably higher (Figure 3). We can say that the process of grain grinding affects the content of anthocyanins in individual fractions. We must respect it in the production of bakery products for which we want to increase the share of anthocyanins.

Due to the presence of high proportion of anthocyanins in outer grain layers, colour wheat varieties can be used in the form of wholemeal flour or flour enriched with bran particles to produce coloured bakery products (Syed Jaafar et al. 2013, Li et al. 2015). From a baking technology point

of view, however, the husk layers have a rather negative impact. They affect the quality and workability of dough and often the appearance of the finished product as well. They often reduce the volume of bakery products (Machálková et al. 2017) and therefore they are being separated during production of flour (Kučerová 2004).

Figure 3 Content of selected anthocyanins in bran (Scorpion variety)

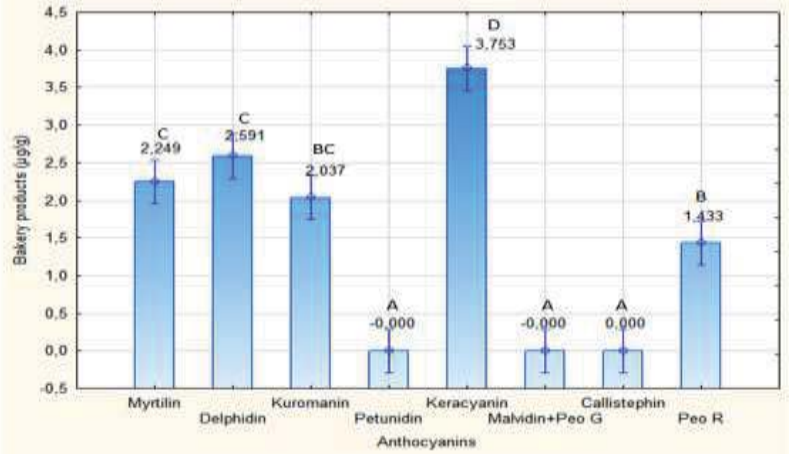


Legend: Vertical columns represent 0.95 confidence intervals. The averages of the different variants do not differ significantly (p > 0.95) if the same upper index is indicated.

Content of selected anthocyanins in wholemeal bakery products

When wholemeal flour was processed into a final bakery product, the content of the monitored anthocyanins dropped significantly. The content of the most represented anthocyanins in wholemeal flour, including the dominant delphinidin-3-O-rutinoside (delphinidin), declined rapidly. Keracyanin chloride (keracyanin) and peonidin-3-O-rutinoside chloride (Peo R) were degraded to almost a third of their original content. Other of the monitored anthocyanins were completely degraded (Figure 4). Figure 5 shows the comparison of the content of selected anthocyanins in the mill products and in the final bakery product.

Figure 4 Content of selected anthocyanins in wholemeal bakery products

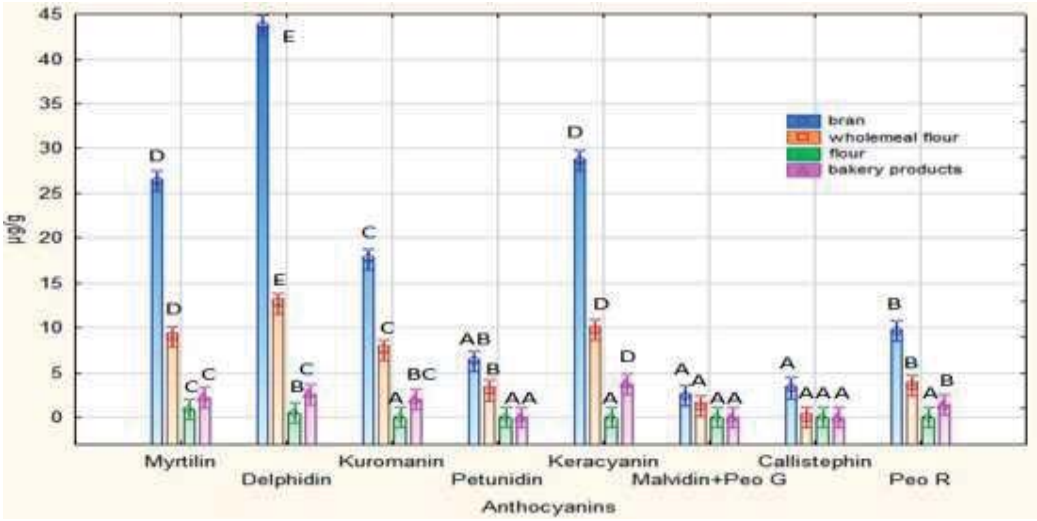


Legend: Vertical columns represent 0.95 confidence intervals. The averages of the different variants do not differ significantly (p > 0.95) if the same upper index is indicated.

According to Giusti et al. (2003), the total content of anthocyanins in the final bakery product is influenced by a number of factors. The main role is played primarily by the heat treatment of the raw material. Bartl et al. (2015) state that the loss of anthocyanin content in bread made from wholemeal flour of blue-grain wheat varieties depends on the baking conditions. It is higher at a baking temperature of 180 °C for 31 minutes (degradation of anthocyanin content by 40.8%) versus baking at 240 °C for 21 minutes (degradation of the anthocyanin content by 7.1%). Critical is thus the length of exposure to higher temperature. The stability of anthocyanins also depends on the intrinsic properties of the product, the chemical structure and concentration of anthocyanins present, pH,

storage conditions, effect of light, oxygen, and presence of enzymes, proteins, and metal ions (Patras et al. 2010).

Figure 5 Content of selected anthocyanins in milling and bakery products



Legend: Vertical columns represent 0.95 confidence intervals. The averages of the different variants do not differ significantly (p > 0.95) if the same upper index is indicated.

CONCLUSION

Anthocyanins have been found to be primarily located in husk layers of caryopses. In the blue-grain Scorpion variety, they are mainly in the aleurone layer, which is concentrated mainly in bran in the standard milling process. The dominant anthocyanin was found to be delphinidin-3-O-rutinoside, which can be considered as more important than other. In order to preserve the part of the anthocyanins in the bakery products, it is preferable to use wholemeal flour in the formulated composition, or to add bran to the mixture. The addition of bran particles, which exhibit the highest content of these colour pigments, must respect possible technological difficulties that are reflected in the quality of the final product. Anthocyanins are, however, unstable compounds that are easily degraded during food processing and changes in their contents take place. The partial or complete degradation of individual anthocyanins is mainly due to thermal processing, as confirmed by our results. According to Bartl et al. study (2015) the length of exposure to higher temperature is critical. Therefore, it is more suitable to operate at higher temperature for a shorter period of time for the highest content of anthocyanins.

ACKNOWLEDGEMENTS

The research was financially supported by the Internal Grant Agency FA project No. IP 38/2017.

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PLANT BIOLOGY

OPTIMALIZATION OF DNA ISOLATION PROCESS IN FRESHWATER MICROALGAE USING HOMOGENIZER

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Abstract: Last decades microalgae are gaining much interest due to high-valued biomolecules content. Increasing number of genetic studies and modifications require quality input material and short time analysis at the same time. Appropriate DNA extraction is one of the crucial and time limiting steps. Therefore in this study we set up optimum parameters for automatic homogenizer Precellys® 24 Evolution (using bead mills technology) for DNA extraction from selected species of green microalgae. Speed parameter 4 500 rpm, 6 800 rpm and 10 000 rpm was tested to obtain the best ratio quality/quantity/purity of DNA material. There were 3 protocols set up due to different sensitivity of microalgae cell walls on bead mills power, otherwise universal protocol needs to be a compromise between quality and quantity.

Key Words: green microalgae, DNA isolation, homogenizer

INRODUCTION

Microalgae are abounding in exceptional ability to adapt to extreme conditions. It means they can quickly reprogram their growth, metabolism and physiology (Weber et al. 2007). Some of mechanisms able to manage these changes may be induced by epigenetic aberrations in genetic code. People started to realize these special algal features and exponential growth of publications in Science Direct database confirms that high-valued products obtained from microalgae became hot topic during last decade. Especially genetic engineering became necessary for achieving new and economically viable products (Enzing et al. 2012) and has made significant progress towards biofuel production (Dunahay et al. 1992, Roessler et al. 1994), pharmaceutical (Soetaert et Vandamme 2010), feed (Yaakob et al. 2014) and food industries (Johanningmeier et Fischer 2010). And the basis of all genetic analysis is a quality input DNA material.

Nowadays, isolation of DNA is well managed and established process, ordinarily simplified by commercial kits. Anyway, obsolete sample extraction technique, like mortar and pestle, are still being used (Bhau et al. 2016). Even though it is established and well proved extraction, on the other hand, it is time consuming process with considerable losses of sample and therefore there is an effort to replace this technique for equipment much more effective. One of the options for grinding samples prior to DNA analysis is automatic homogenizer based on bead beating technology. There have been many protocols created for DNA extraction from different plant (Ramos et al. 2014) and animal tissues and microorganisms (Lopez et al. 2017) available, anyway there are still predicaments you can face in terms of green microalgae material.

While plant or animal cells are part of tissues and organs and these higher structures are further protected for example by cuticle, waxes, hair etc., microalgae are unicellular microorganisms, which need protection of the single cell against environment (Skaloud et al. 2013). In this regard, microalgae are protected by rigid, thick cell wall, which can be difficult to disrupt and extract (Kim et al. 2016). In this study we decided to optimize parameters of extraction in automatic homogenizer with the aim

to obtain optimal quality and quantity DNA material from microalgae for sensitive downstream applications including PCR, qPCR, genotyping, sequencing and more.

MATERIAL AND METHODS

Microalgae species and cultivation

For this study, five species of microalgae from different family were chosen (see Table 1). Algae samples were purchased from UTEX Culture Collection of Algae (Austin, USA) and subsequently re-cultivated in our laboratory of Plant Metabolomics and Epigenetics in Mendel University in Brno (Brno, Czech Republic). Inorganic liquid Tris Acetate Phosphate (TAP) medium, containing only inorganic salts and trace elements for algal growth (Purkayastha et al. 2017), was used.

Cultivation proceeded in Erlenmayer flasks, under sterile and well defined conditions:

- light: 70 µmol m^2/s
- temperature: 23 °C
- photoperiod: 12 h light/12 h dark

Figure 1. Classification of selected green microalgae species

Empire	Eukaryota				
Kingdom	Plantae				
Subkingdom	Viridiplantae				
Phylum	Chlorophyta				
Subphylum	Chlorophytina				
Class	Trebouxiophyceae			Chlorophyceae	
Order	Trebouxiophyceae	Chlorellales		Sphaeropleales	
Family	Coccomyxaceae	Chlorellaceae		Scenedesmaceae	
Genus	Coccomyxa	Chlorella	Parachlorella	Scenedesmus	
Species	*Coccomyxa subellipsoidea*	*Chlorella vulgaris*	*Parachlorella kessleri*	*Scenedesmus obliquus*	*Scenedesmus quadricauda*

DNA extraction

Many different physical parameters can affect DNA extraction step: liquid nitrogen treatment, bead mills type/size, rotor speed, number of cycles and also DNA isolation method. There are many variations of DNA isolation kits, specialized at animal/plant tissues with different kit patents. In this study we used PowerPlant® Pro DNA Isolation Kit (Quiagen, Germany).

Green algal cells were harvested by pipetting algal stock solution into 2 ml tubes, centrifuged (13 000 rpm/1 min) and culturing medium was removed. Glass beads (0.5 mm in diameter) were added to the weighed algal fresh sample and tubes were transferred into liquid nitrogen for a minute to disrupt cell walls. Algal cells were mixed with lysis buffer, phenolic separation solution, precipitation solution and RNase A solution from PowerPlant® DNA isolation kit. Tubes with samples were inserted into automatic homogenizer Precellys® 24 Evolution. This equipment is based on high-speed rotation, which is able to disrupt cell walls from high variety of samples. Multi-directional movement (3D motion) of sample holder transmits high level of energy to the beads inside each tube, and can grind up to 24 samples at one time. This ensures equal homogenization for all processed samples. The speed can range from 4 000 up to 10 000 rpm and whole process takes proportionally seconds (30 s up to 90 s), where also number of cycles can be set (up to 3). Another advantage of this equipment is also avoiding cross contamination comparing mortar and pestle. We were monitoring three different homogenizer speed set up: 4 500 rpm, 6 800 rpm and 10 000 rpm at a constant 20 s extraction time in 2 cycles. After homogenization, DNA isolation further proceeded according to kit manual. Samples were centrifuged at 13 000 x g for 10 minutes. Avoiding pellet, supernatants were transferred to a clean collection tube and treated with ethanol. DNA was captured on a spin filter and washed two times. Finally 75 µl of 10 mM Tris (pH 8.0) was loaded onto filter, incubated 2 minutes at room temperature and centrifuged 30 seconds at 10 000 × g. DNA material flowed through and was stored frozen (-20 °C). Microalgae samples were tested in triplicates.

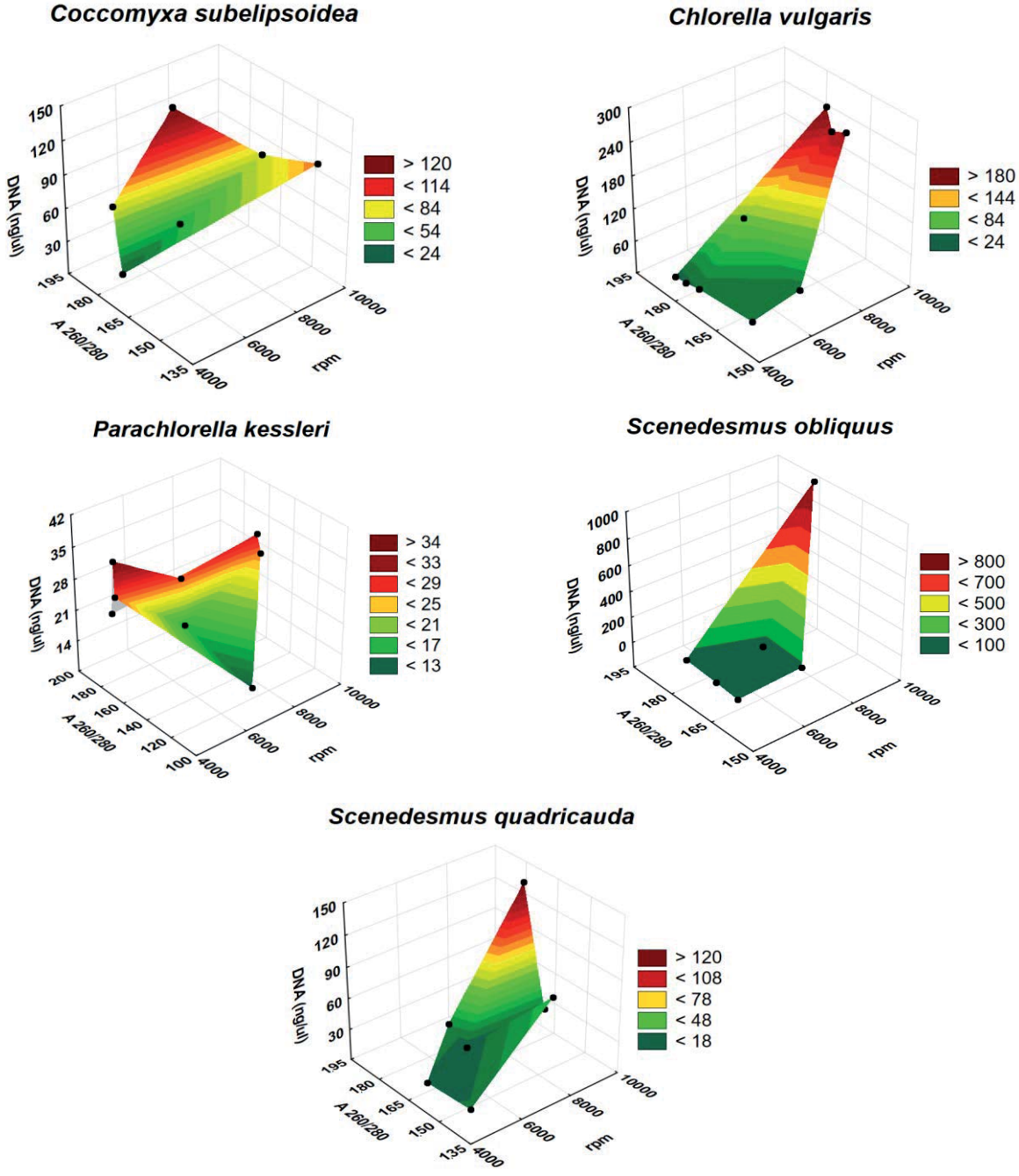
DNA quality

After DNA isolation, the concentration and purity of obtained material was determined by Tecan Infinite® 200 PRO spectrophotometer using NanoQuant plate, and calculated from absorbance ratio A 260/280. The integrity of obtained DNA was verified by agarose gel electrophoresis. DNA samples were loaded directly onto an agarose gel of viscosity 0.8% which was made in TAE and was colored with ethidium bromide dye. The electrophoretic process was running 1.5 h using 60 V.

RESULTS AND DISCUSSION

Quality and quantity of isolated DNA

Figure 2. 3D histogram shows a plot of DNA concentration vs. rpm and vs. A 260/280 ratio



Legend: DNA (ng/µl) - concentration of DNA (ng/µl), A 260/280 - absorbance purity ratio, rpm - homogenization speed; greens colour symbolize lower DNA concentration and red colours increasing DNA concentration

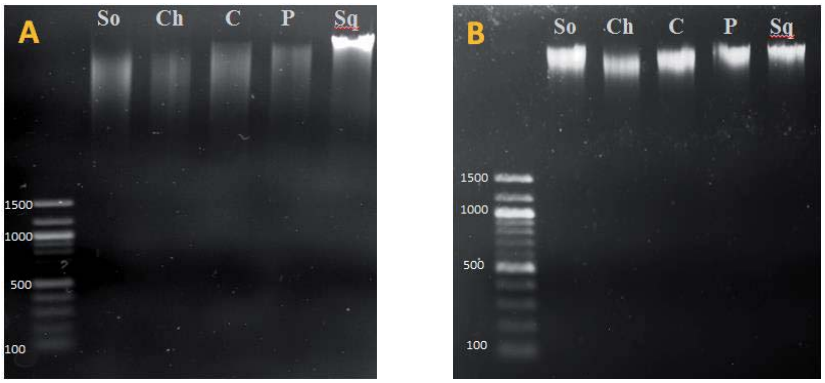
Testing three different homogenizer speed modes (rpm), DNA concentration ranged between 20–900 ng/µl within five different algae species. Considering the highest yield of DNA material obtained, we got the best results from the highest speed mode (10 000 rpm) in *Scenedesmus obliquus* (946.4 ± 14.8 ng/ul), Chlorella vulgaris (236.1 ± 37.4 ng/µl) and *Scenedesmus quadricauda* (130.0 ± 4.9 ng/µl). The higher speed, the more efficient cell damage and DNA release. However, exceptions were *Coccomyxa subelipsoidea* with the best obtained DNA yield (141.8 ± 12.3 ng/µl), using speed 6 800 rpm and *Parachlorella kessleri*, where we obtained the highest DNA concentration (34.6 ± 4.4 ng / µl) using the lowest speed 4 500 rpm. This could be caused by huge variety and different cell wall characteristics in each microalga species, where disruption and extraction methods can largely differ (Praveenkumar et al. 2015).

A 260/280 purity value of measured samples fluctuated between 1.4–1.9. Value of ~ 1.8 is generally accepted as pure for DNA material, therefore values suitable for further analysis were set up to 1.7–1.9. Inaccurate ratios may indicate contamination by residual phenols, guanidine or other reagent used in protocol (Wilfinger et al. 1997). In case of *Coccomyxa* and *Parachlorella*, the higher speed, the worse purity was obtained. In case of *Chlorella*, and both *Scenedesmus* species the purity had an opposite trend. The lower speed, the purity of DNA was getting worse.

Integrity of DNA material

The goal was not only to get the best yield, but also to get intact DNA material and therefore samples with the highest DNA concentration were subsequently run on gel electrophoresis to verify DNA integrity. Gel electrophoresis in the Figure 3A showed the integrity of the DNA in each microalgae. Four of five samples were not acceptable.

Figure 3 Agarose gel electrophoresis of A: the highest yields protocols in different microalgae, B: optimized protocols in different microalgae



Legend: So - Scenedesmus obliquus, Ch - Chlorella vulgaris, C - Coccomyxa subelipsoidea, P - Parachlorella kessleri, Sq - Scenedesmus quadricauda; 100, 500, 1000, 1500 - DNA marker in base pairs

Limiting step was to break microalgae cell wall and at the same time obtain intact gDNA. Only *Scenedesmus quadricauda* appears to endure such a bead mills power in regards to be able to keep DNA integrity. *Scenedesmus quadricauda* has probably the strongest cell wall and the highest speed 10 000 rpm did not affect the DNA integrity with the best yield at the same time.

Table 1 Optimal speed settings for DNA quality and quantity

	Speed (rpm)	Yield (ng/µl)	Purity (A 260/280)	DNA integrity
Coccomyxa subelipsoidea	4 500	58.60	1.82	↑
Chlorella vulgaris	4 500	26.20	1.85	↑
Parachlorella kessleri	6 800	26.90	1.76	↑
Scenedesmus obligus	4 500	42.00	1.75	↑
Scenedesmus quadricauda	10 000	130.00	1.81	↑

Therefore for the rest of microalgae we needed to find a compromise between quality and quantity and we chose samples with the best ratio yield : quality : purity (see Figure 3B) and according this we set up 3 different protocols for obtaining suitable, full length genome DNA and passable DNA concentration and purity (see Table 1). DNA marker with upper most band of 1 500 bp is proving there is no shearing in this area.

CONCLUSION

In the present work we carried out with the objective of optimizing an automatic homogenizer extraction method of genomic DNA in five species of fresh green microalgae. Speed parameter was tested in regards to concentration, purity and integrity of DNA material. This article points on high variability in microalgae species. The highest rotor speed (10 000 rpm) led to highest yields only in three species (*Chlorella vulgaris, Scenedesmus obliquus* and *Scenedesmus quadricauda*) with the fact, only DNA of *Scenedesmus quadricauda* stayed intact after homogenization. The highest DNA concentration of *Coccomyxa subelipsoidea* was obtained with 6800 rpm and of *Parachlorella kessleri* with 4 500 rpm speed mode also with unsatisfactory DNA integrity. With the optimized protocols we obtained lower yields but better quality of DNA material and integrity. A protocol of 4 500 rpm × 20 s × 2 cycles gave the best results for *Coccomyxa subelipsoidea, Chlorella vulgaris and Scenedesmus obliquus* homogenization, 6 800 rpm × 20 s × 2 cycles was the best option for *Parachlorella kessleri* specie and finally 10 000 rpm × 20 s × 2 cycles has showed as the best set up for *Scenedesmus quadricauda*. It is impossible to focus extraction protocol for each microalgae species separately, anyway it necessary to take into account algal morphology before extraction. In this study we set optimum extraction parameters for selected species of green microalgae to obtain high quality DNA material for further research.

ACKNOWLEDGEMENTS

The research was financially supported by the Internal Grand Agency of Mendel University in Brno IP 2/2017 and CEITEC 2020 (LQ1601) with financial support from the Ministry of Education, Youth and Sports of the Czech Republic under the National Sustainability Programme II.

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USAGE OF UV IRRADIATION FOR NUCLEUS DESTRUCTION OF *PETUNIA HYBRIDA*

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Abstract: Production costs of F1 seeds of *Petunia hybrida* can be decreased by using sterile component. Cytoplasmic male sterility (CMS), located in mitochondria, is transferred either by back crossing or by protoplast fusion. Asymmetric protoplast fusion represents the most effective way. Protoplast nucleus of one component (donor of CMS) must be destroyed by UV radiation. Protoplast cytoplasm of the second component must by destroyed by metabolic inhibitors. Asymmetrically fused protoplast possesses CMS from donor and rest of the features of fertile component. The aim of this experiment was to determine the amount of UV radiation (in seconds) needed for destruction of protoplast nucleus. Optimal UV radiation, based on results from 3 different *Petunia hybrida* genotypes is 510 µW/cm^2 for a duration of 300 seconds.

Key Words: Petunia hybrida, protoplast, cytoplasmic male sterility, UV radiation

INTRODUCTION

Petunia hybrida is among the most popular annual ornamental plants worldwide, so many breeding and production companies put a strong emphasis on reducing production costs and introducing new varieties with improved features, esthetic or growing performance (Anderson 2007). Majority of current assortment are F1 hybrids or varieties multiplied by cuttings (Gerats and Strommer 2009). Production costs of F1 seeds are high due to manual emasculation and pollination of maternal component with a pollen collected from paternal component (Sink 1984). Promising way how to decrease the costs is, besides production relocation to developing countries, inducting cytoplasmic male sterility (CMS) to the maternal component, so functional pollen is not produced. Sterile component does not require emasculation that must occur otherwise every day and represents about 40% of all labor costs.

Transfer of CMS into maternal component is possible via 2 methods, back crossing and protoplast fusion. Back crossing method is used in breeding programs for more than 50 years, but the main drawbacks are: long duration and impossibility to apply it to vegetative components. To transfer CMS by back crossing takes at least 5 or 6 generations, when donor of CMS must be crossed with a fertile component (Gaus 2002).

Second, more promising method, is protoplast fusion, that is used primarily by researches rather than professional plant breeders. This method is cheap, quick and can be used also to transfer CMS to vegetative component. Protoplast fusion can be symmetrical or asymmetrical, but the 4 main stages are the same: isolation of protoplasts of different genotypes, protoplast fusion, protoplast cultivation and plant regeneration (Bhojwani and Dantu 2013).

Principle of the symmetrical fusion is, that nuclear and extra-nuclear content of both components (donor of CMS as well as fertile component we want to transfer to sterile) are fused together. There is a possibility that a fusion occurs also among 2 protoplasts of donor CMS or among 2 protoplasts of fertile component. To ensure that a correct fusion happened (1 protoplast of CMS donor is fused with 1 protoplast of fertile component) the plants must be further grown until they have at least 1 flower. Usage of chemical dyes to visually distinguish the components is also possible (Shankar et al. 2013). With asymmetrical fusion, it is not necessary because cybrids have a combination of 1 protoplast of CMS donor and 1 protoplast of a fertile component. Protoplasts of a CMS donor must be irradiated

by UV radiation so the nucleus is destroyed (Staxén et al. 1992). Protoplasts of fertile component must be treated with metabolic inhibitors, to remove cytoplasm so just nucleus remains. Crucial is to determine the optimal amount of UV radiation and concentration of metabolic inhibitor for the specific species and genotype.

MATERIAL AND METHODS

Characterization of experimental design and methods

The aim of this research was to determine the optimal UV irradiation needed for *Petunia hybrida* nucleus destruction. This is a necessary phase of an ongoing research focusing on usage of asymmetrical protoplast fusion in breeding programs of *Petunia hybrida*. The goal is to use this method to transfer CMS into the fertile component.

In the conducted experiment 3 different genotypes were used, each of them repeated 3 times. The genotypes M–1, M–012, M–033 have back crossed CMS and are used in production of commercial F1 hybrids. This material was provided by Czech biotechnological company Černý-BioPro, Prague.

Protoplast isolation was performed according to the protocol by Meyer et al. 2009 (Meyer et al. 2009) with modified preplasmolytic and degradation enzymatic solution. As a preplasmolytic solution was used 0.6 M mannitol and 0.05 mM MES. The degradation enzymatic solution contained: 1.5% cellulase Onuzaka R10 (Serva), 0.5% Macerozyme R10 (Serva), 0.6 M mannitol, 0.05 M MES, 3 mM CaCl2 and 1 mM KCl.

Leaves were picked in the afternoon from the donor plants grown in a greenhouse, placed into zip lock bag and stored in the fridge with a temperature of 4–6 °C. After 12 hours, leaves were bleached 3 times in commercial bleaching solution SAVO 30 %, epidermis on the bottom side was removed by scalpel. 2 grams of leaves were placed into the Petri dish with 5 ml of preplasmolytic solution, left for 1 hour in darkness, temperature 24 °C. Afterwards the preplasmolytic solution was removed and replaced by degradation enzymatic solution. Petri dish was placed for 2 hours into thermostat with a constant temperature of 26 °C. Every 15 minutes the Petri dish was shaken gently for 3 minutes so the protoplasts can be removed easier from the leaf tissue. Afterwards the solution was filtered through filtration tissue. Protoplasts were rinsed out 3 times in the washing solution in centrifuge (6 min, 740 rpm). The yield and vitality of protoplasts for each genotype were tested.

Before UV irradiation protoplasts were diluted to concentration 100 000 protoplasts/1 ml. 3.5 ml of the protoplast suspension were given into each of the 5 plastic Petri dishes. For the UV irradiation was used UV lamp GV17 with a wavelength 253.7 nm and power 510 μW/cm^2. The tested length of the irradiation was 0, 180, 300, 420 and 600 seconds.

Petri dishes with treated protoplasts were placed into the thermostat with a constant temperature of 25 °C. After 10 days, the protoplasts were examined with inversion microscope. The optimal length of UV radiation was, when the nucleus of 100% of protoplasts was destroyed and the cells were not dividing. The same examination was preformed also 30 days after UV irradiation.

RESULTS AND DISCUSSION

Irradiated protoplasts were examined using inversion microscope. The aim was to determine what UV irradiation dosage destroys 100 % protoplast nuclei. In table 1 are listed results of 3 repetitions per genotype obtained after 10 days after UV irradiation for duration of 0 sec (Control), 180 sec, 300 sec, 420 sec and 600 sec.

Table 1 Protoplasts 10 days after UV irradiation

	0 sec	180 sec	300 sec	420 sec	600 sec
Genotype M–1	LLL	LLL	DDD	DDD	DDD
Genotype M–021	LLL	LLL	DDD	DDD	DDD
Genotype M–033	LLL	LLL	DDD	DDD	DDD

Legend: L – live protoplasts, D – dead protoplasts; 3 letters per cell represent 3 repetitions for every genotype and UV dosage

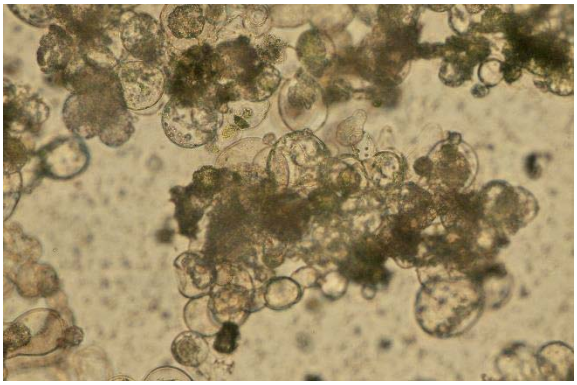
10 days after UV irradiation of a Control (0 sec) the calluses started to form from the fused protoplasts. That means, that the nucleus was not destroyed (Figure 1A). Protoplasts treated with a UV irradiation for 180 seconds (Figure 1B) formed smaller calluses in comparison to Control, cell division occurred. This means that this dosage was not sufficient to destroy the nucleus. Protoplasts treated with UV radiation of 510 μW/cm^2 for 300 seconds (Figure 1C) started with a cell division, increased their length, but the cell division was not finished, since the formation of nucleus in the newly formed cell did not happen. This means that UV radiation destroyed the protoplast nucleus and this dosage is sufficient and optimal. UV irradiation of 420 seconds (Figure 1D) led to the same results as for 300 seconds. The cell division started, size of protoplasts increased and nucleus of new cell was not formed. 600 seconds of UV irradiation (Figure 1E) led to complete destruction not only of the nucleus but the whole protoplast. Cellular content was dissolved to the cultivation medium.

30 days after UV irradiation were the results like 10 days after irradiation. Calluses of Control and dosage 180 sec were formed. For 300 seconds and more of UV radiation the nucleus did not form in the new cell, which means that this duration was sufficient to destroy all protoplast nuclei.

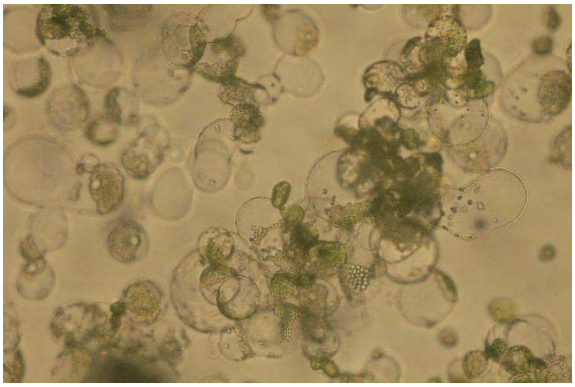
Results were statistically evaluated with program Statistica 12. It was statistically significant ($p = 0.05$) that amount of UV irradiation needed for nucleus destruction is not determined by genotype. Due to character of the experiment and binomial distribution of values (1 – live = protoplast nucleus not destroyed by UV irradiation and 0 – dead = protoplast nucleus destroyed, calluses are not forming) sign test was used. On $p = 0.05$ 180 seconds of UV irradiation is not sufficient and nucleus is not destroyed. On $p = 0.05$ dosage of 300 seconds, 420 seconds and 600 seconds is sufficient but optimal is 300 seconds. The nucleus is destroyed but other cellular content is still vital and able to form callus after asymmetric fusion with a protoplast with nucleus and without cytoplasm.

Figure 1 Protoplasts 10 days after UV irradiation treatment – genotype M–1

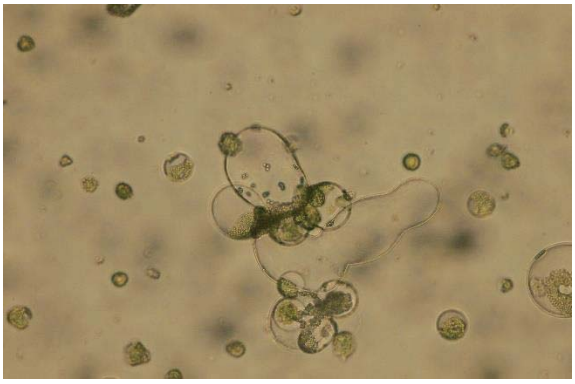
A) Control (0 seconds)



B) 180 seconds

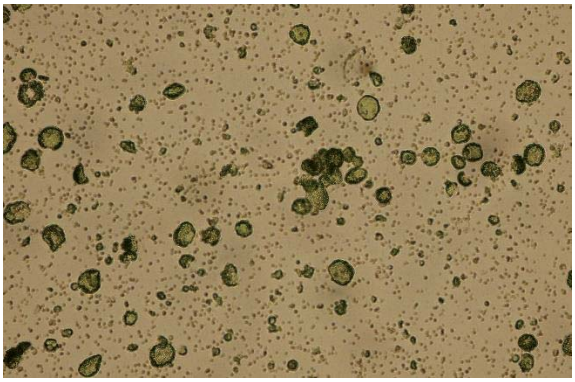


C) 300 seconds



D) 420 seconds

E) 600 seconds



Symmetric protoplast fusion has a big disadvantage, percentage of correctly fused protoplasts that form callus is very low (Sink 1984). Therefore, asymmetric fusion is more effective way. It is used in breeding for resistance (Bhojwani and Dantu 2013), for interspecific crossing (Guangmin et al. 1998) for higher yield and higher content of proteins etc. In scientific research focusing on Petunias, asymmetric fusion was used to cross incompatible species Petunia and Calibrachoa (Meyer et al. 2009) and for introduction of transformed chloroplasts from Tabaco to Petunia (Sigeno et al. 2009). In horticulture praxis and commercial ornamental plant breeding asymmetric fusion is not used yet (Gerats and Strommer 2009). To destroy the nucleus multiple methods can be used, for example X or Gamma rays, but most frequently is used UV radiation (Lakshmanan et al. 2013). UV radiation is in comparison to the other methods easier to use as Lakshmanan et al. (2013) states. This is the reason why UV radiation was used in the conducted experiment. Crucial is to determine the optimal amount of UV irradiation – voltage and duration. Sigeno et at. (2009) used in the experiment duration of UV irradiation of 20 minutes. This is caused by lower voltage. In our experiment, where 510 $\mu W/cm^2$ was used was optimal UV irradiation from 300 seconds. Irradiation of 10 minutes with 510 $\mu W/cm^2$ caused destruction of not only the nucleus but also of other cellular content, that was dissolved to the medium.

CONCLUSION

Asymmetric fusion brings new opportunities to the flower breeding. This method allows creation of new varieties with unique features that would be not possible to achieve with classical methods due to incompatibility, post fertilization barriers or due to high costs and extensive length of the breeding programme. Cytoplasmic male sterility allows to decrease F1 seed production costs because labour intensive emasculation of maternal component is not needed. Asymmetric protoplast fusion is more effective than back crossing that is used nowadays. Back crossing requires at least 5 or 6 generations to transfer CMS to maternal component and can´t be used for vegetative multiplied component. According to this experiment the optimal amount of UV irradiation to destroy *Petunia hybrida* nucleus is 510 $\mu W/cm^2$ for a duration of 300 seconds. Longer irradiation tends to destroy other cellular content as well, lower dosage does not destroy the nucleus.

ACKNOWLEDGEMENTS

The research was financially supported by IGA Využití asymetrické fúze protoplastů ve šlechtitelských programech u Petunia hybrida (IGA-ZF/2017-AP006).

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COMPLEX GENOME REARRANGEMENTS IN AN ARABIDOPSIS T-DNA LINE

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Abstract: In recent years, an increasing number of studies demonstrated that many Arabidopsis T-DNA lines might carry massive genome changes as a direct consequence of the transfer DNA (T-DNA) integration event. In this work, we report an analysis of a segregating T-DNA mutant line (SAIL_218_G01), which contains such genomic rearrangements. The initial PCR analyses did not yield any homozygous plants, suggesting putative gametophyte lethality. However, this result is likely caused by chromosomal translocations, which were clearly identified in several heterozygous plants by a pollen viability assay. Surprisingly, we identified plants that carried the T-DNA and still produced 100% viable pollen. For reciprocal balanced translocations, these plants should be then homozygous for the T-DNA insertion. Yet, the offspring of some of these plants still segregated the T-DNA, suggesting more complex genome rearrangements in the studied Arabidopsis mutant line.

Key Words: chromosome translocation, genome rearrangement, T-DNA, Arabidopsis

INTRODUCTION

T-DNA insertional mutagenesis is a crucial technique in gene function studies and therefore plays an important role in Arabidopsis research (O'Malley and Ecker 2010). As early as 1997 there were more than 20 000 Arabidopsis T-DNA transformants and approximately 4 000 mutants have been already identified (Azpiroz-Leehan and Feldmann 1997). Simultaneously with advances in successful mutant identification, first analyses of several T-DNA integration sites revealed massive rearrangements of chromosomal DNA including deletions and translocations in various plant species including tobacco (Ohba et al. 1995), rice (Takano et al. 1997) and Arabidopsis (Nacry et al. 1998). The complete sequence of Arabidopsis genome (Arabidopsis Genome Initiative 2000) together with public collections of T-DNA insertional mutants allowed for a successful functional characterization of numerous genes. However, a recent analysis of tens of T-DNA mutant lines from public collections revealed that about 20% of analysed lines carried chromosomal rearrangements (Clark and Krysan 2010). Importantly, the authors suggested that a simple pollen viability assay was able to identify all but one chromosome translocations in studied heterozygous plants. In extreme cases, a T-DNA integration may trigger a cascade of incorrect repairs of several double-stranded breaks on two chromosomes and subsequently produce translocation, inversion, duplication and deletion (Hu et al. 2017). In this work, we present analyses of a T-DNA line that likely carries similar global rearrangements of the genome.

MATERIAL AND METHODS

Plant material

The seeds of SAIL_218_G01 Arabidopsis T-DNA segregating line were surface sterilized with sodium hypochlorite and germinated on ½ Murashige-Skoog medium containing 0.7% agar. After two weeks, the seedlings were transferred to soil and grown under long-day conditions (16 hours light at 21 °C, 8 hours dark at 19 °C; 65% humidity). After another two weeks, a third rosette leaf was excised, frozen in liquid nitrogen and used for DNA extraction.

DNA extraction

The frozen plant material was homogenized for 1 minute using 2 mm glass beads and the genomic DNA was isolated according to a standard DNA extraction protocol (Edwards et al. 1991).

PCR analyses

The sequences of oligonucleotides for PCR reactions were designed using iSect Tools (http://signal.salk.edu/tdnaprimers.2.html). The gene-specific primers were 218_G01LP (5'-TAATTGGCAAAGACGAAGACG) and 218_G01RP (5'-GGTTCAGAGATGATGCTGAGG). The corresponding PCR cycle included a denaturation step (30 s, 95 °C), an annealing step (40 s, 55 °C) and an extension step (100 s, 72 °C). The PCR cycle was repeated 35 times. The T-DNA insertion was detected using 218_G01RP and the T-DNA specific oligonucleotide LB3 (5'-TAGCATCTGAATTTCATAACCAATCTCGATACAC). The corresponding PCR cycle included a denaturation step (30 s, 95 °C), an annealing step (35 s, 55 °C) and an extension step (80 s, 72 °C). The PCR cycle was repeated 30 times.

Pollen viability assay

The pollen viability was determined using the Alexander method (Alexander 1969). The staining solution contained 0.01% (w/v) malachite green, 0.05% (w/v) acid fuchsin, 0.005% (w/v) orange G, 2% (v/v) acetic acid, 5% (w/v) chloral hydrate, 25% (v/v) glycerol, 5% (v/v) phenol and 10% (v/v) ethanol in double-distilled water. The dissected anthers were treated with 20 µL of staining solution for 5 minutes at room temperature. The sample was examined under the Olympus BX61 upright light microscope.

RESULTS

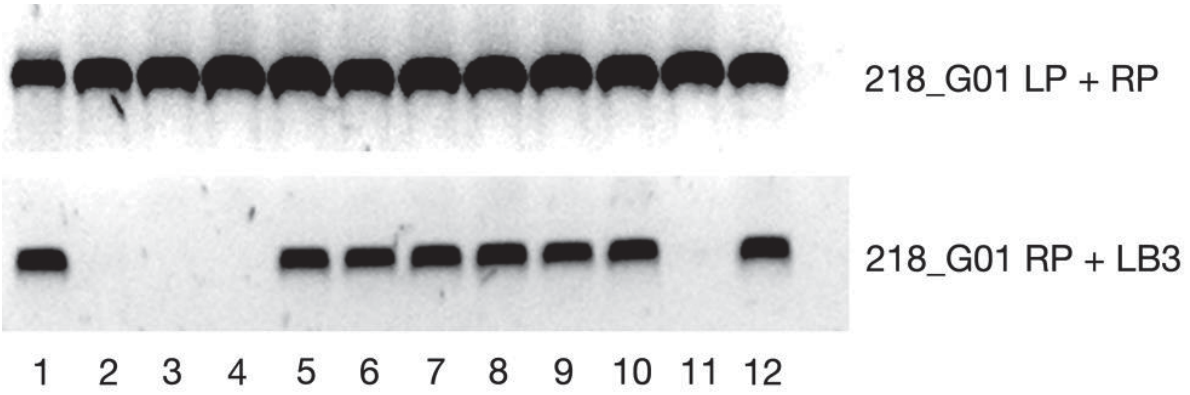
Analysis of the SAIL_218_G01 segregating population

Twelve seedlings from the SAIL_218_G01 segregating T-DNA line were soil planted and grown under long-day conditions. Subsequently, the corresponding genomic DNA was isolated and analysed by PCR using gene-specific oligonucleotides. By design, the PCR with gene-specific primers (218_G01LP and 218_G01RP) should amplify a 900 bp product in case of wild-type and heterozygous plants and should not amplify any products in plants homozygous for the T-DNA insertion. Unexpectedly, in the analysed segregating population we did not identify any homozygous plants (Figure 1). However, the PCR with the T-DNA insertion-specific primers (218_G01RP and LB3) yielded a 500 bp PCR product in 8 out of 12 plants, indicating that the T-DNA segregated according to Mendelian ratios (Figure 1).

Pollen viability assay

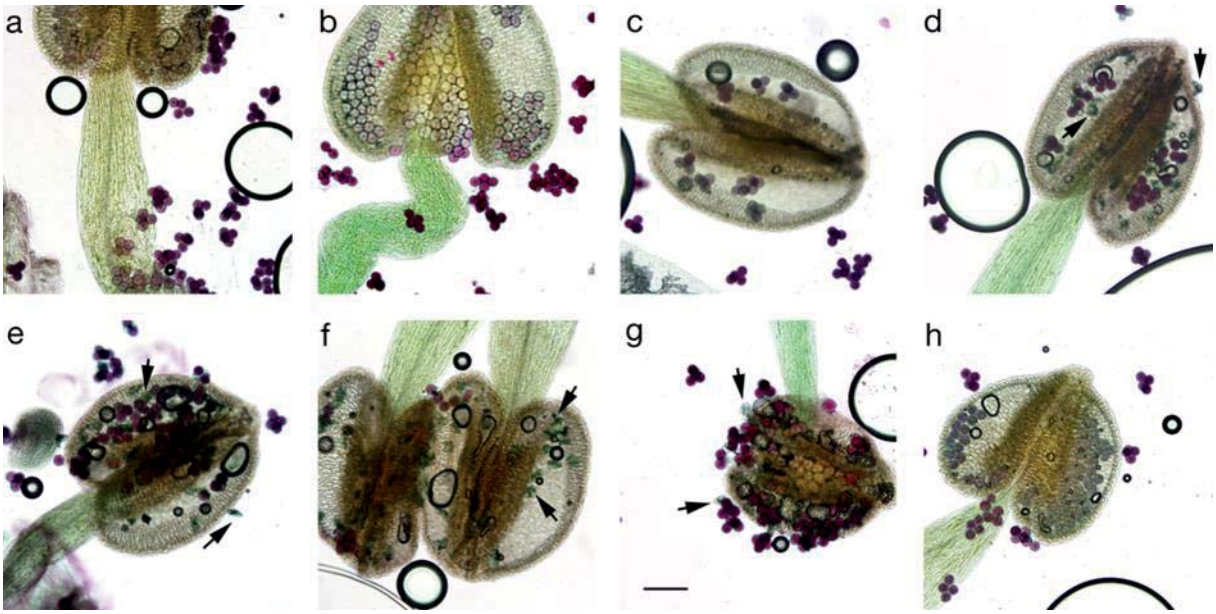
The unusual segregating ratios observed by the PCR analyses might be caused by gametophyte lethality caused by the T-DNA insertion. Therefore, we have analysed pollen viability in twelve studied plants using a simple staining solution (Alexander 1969). In this technique, viable pollen stains crimson red while aborted pollen stains green. Among studied plants, those that did not contain the T-DNA insertion are likely wild-type plants (plants 2, 3, 4 and 11) and produced 100% viable pollen (Figure 2, panel b). Surprisingly, three plants that were identified as putative heterozygotes by PCR analyses showed 100% viable pollen (plants 1, 6 and 12; Figure 2, panels a, c and h), while half of the pollen grains from the remaining putative heterozygotes was found aborted (Figure 2, panels d–g). Such phenomenon is often a consequence of T-DNA-induced chromosomal translocations (Clark and Krysan 2010). Briefly, plants that are heterozygous for a reciprocal translocation caused by a single T-DNA insertion are predicted to produce approximately 50% non-viable pollen. By contrast, homozygous T-DNA lines carrying a reciprocal translocation are genetically balanced and therefore likely produce 100% viable pollen (Curtis et al. 2009). Therefore, the three identified plants carrying 100% viable pollen are likely homozygous plants misidentified by the initial PCR analyses.

Figure 1 PCR analysis of the segregating population.



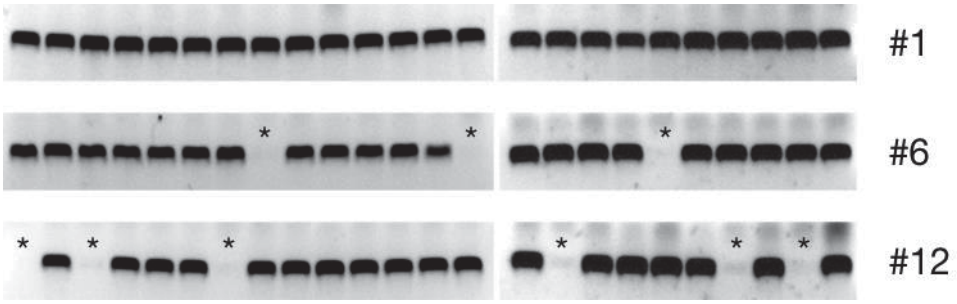
Legend: 218_G01 LP + RP = Gene-specific PCR products, 218_G01RP + LB3 = T-DNA-specific PCR products.

Figure 2 Pollen viability assay.



Legend: (a) plant 1 – a putative homozygote with 100% viable pollen, (b) plant 4 – a wild type plant, same phenotype observed in plants 2, 3 and 11, (c) plant 6 – a putative homozygote, 100% viable pollen, (d–g) plants 7, 8, 9 and 10, respectively – heterozygotes with aborted pollen, (h) plant 12 – a putative homozygote, 100% viable pollen. Arrows highlight aborted pollen. Scale bar = 80 µm.

Figure 3 PCR analyses of the offspring from putative homozygous plants.



Legend: #1 – a true homozygote, no segregation of the T-DNA insertion, #6 and #12 – heterozygous plants, asterisks (*) indicate plants without the T-DNA.

PCR analysis of the offspring

Subsequently, we analysed the T-DNA segregation in the offspring from plants 1, 6 and 12, which produced 100% viable pollen. Importantly, only the offspring from the plant 1 did not show any segregation of the T-DNA insertion, suggesting that the plant 1 was indeed a homozygous line

(Figure 3). Both plants 6 and 12 presented segregation of the insertion, ruling out their homozygosity (Figure 3).

DISCUSSION

Large public collections of T-DNA insertional mutant lines (e.g., Nottingham Arabidopsis Stock Centre, http://arabidopsis.info/) together with resources for their identification (e.g., T-DNA Express, http://signal.salk.edu/cgi-bin/tdnaexpress) and characterization (e.g., iSect Tools, http://signal.salk.edu/isects.html) form an indispensable toolkit for plant biologists. However, a recent thorough characterization of several of these lines revealed that up to one quarter of them might carry large genome rearrangements, mostly in a form of reciprocal chromosomal translocations (Clark and Krysan 2010). In this work, we identified such rearrangements in the SAIL_218_G01 T-DNA line. During the initial PCR characterization of this line, we could not identify a homozygous line for the T-DNA insertion. However, a subsequent pollen viability assay revealed that the initial PCR results were somehow misleading. This technique revealed differences in lines that were supposedly homogenous according to the PCR analyses, suggesting that the gene-specific primers anneal likely on different loci than expected. Importantly, the pollen viability assay identified two distinct phenotypes in this apparently homogenous population. Three plants carrying the T-DNA insertion and showing 100% viable pollen might be putative homozygotes for the T-DNA insertion as their gametes were balanced and therefore viable. On the contrary, five out of 12 plants showed approximately 50% aborted pollen, indicating that these gametes were not genetically balanced and the corresponding plants were likely heterozygous for the T-DNA insertion. However, the subsequent PCR analysis of the offspring from the three allegedly homozygous plants revealed that two of them still segregated the T-DNA insertion. Our results demonstrated that even the pollen viability assay was not sufficient to fully characterize the complexity of the genome rearrangements found in the SAIL_218_G01 T-DNA line. Similar inconsistent pollen viability results were previously reported (Clark and Krysan 2010), even though they likely represent a minority within the T-DNA lines carrying the chromosomal translocations. In our case, the misleading initial PCR and the pollen viability assay point to a more complex genome rearrangements that in addition to chromosome translocation may include inversions, duplications and/or deletions.

CONCLUSION

This work gives an example of an Arabidopsis T-DNA line, which was obtained from public collections and carries complex genome rearrangements. We show that pollen viability assay is an important tool to identify these chromosomal changes and should be considered by the Arabidopsis research community for easy detection of such genome rearrangements in the T-DNA-segregating populations.

ACKNOWLEDGEMENTS

This research was supported by the National Program for Sustainability II by the Ministry of Education, Youths and Sports of the Czech Republic (CEITEC 2020, LQ1601) and by the Erasmus+ program by the Ministry of Education, Science and Sport of the Slovak Republic.

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IN VITRO INDUCTED TETRAPLOID *PETUNIA HYBRIDA* OF A RED COLOR

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Abstract: Petunia hybrida is one of the most important annuals. Niche of the assortment represent a group of tetraploid varieties that are interesting due to their giant flower. However, these are only old open pollinated varieties. Creation of homozygous tetraploid lines is a prerequisite for making F1 varieties. To do so, two homozygous lines used in commercial production of F1 varieties were used. 10 days old seedlings grown on MS medium with 8 g/l Agar and 20 g/l sucrose were treated with a solution of 30 µM oryzalin for 24 hours in in vitro conditions. The plants were washed and transplanted to the same medium. Later, they were grown in the greenhouse. Cytometrically, ploidy was detected. The yield of tetraploids was more than 50%. Octoploid plants were not found. Tetraploid red flowering plants with genotype GGGG and gggg were obtained. Flower size increased by at least 30% for polyploidy.

Key Words: Petunia hybrida, tetraploids, oryzalin, in vitro, red flower

INTRODUCTION

Petunia is one of the world's most important annuals (Anderson 2007). A separate group in the assortments are the varieties of *Petunia h. superbissima*, that were popular at the beginning of the 20th century. Plants are robust with a flower size up to 16 cm (Reimann-Philipp 1969). After the onset of hybrid varieties in the 1950s, varieties in superbissima type almost disappeared from the offer of seed companies (Gertas and Strommer 2009).

Superbissima varieties are tetraploid (2n = 28) (Matsuda 1933). These varieties emerged as spontaneous tetraploid plants within commercial OP (Open Pollinated) variety (Sink 1984). This process finished by the onset of F1 hybrids (Maatsch and Nolting 1968). Therefore, in the assortment of tetraploid varieties we cannot find the color shades that have been breed after 1950, for example red, salmon or yellow.

The method of enriching the assortment of tetraploid varieties for these shades is artificial tetraploid induction from diploids. As a material for this induction, a highly homozygous breeding material is used as a breeding line in the formation of diploid hybrid varieties. Biotechnological methods - induction of tetraploids in in vitro conditions are both effective and profitable (Murphy 2007). The obtained materials could be used to produce F1 tetraploid varieties. The most popular would be large-flowered red-blooming varieties. The parental component of such varieties would have to be homozygous for the dominant gene G, which causes large-scale growth (Plickert 1936). It is located on the V. chromosome (Cornu et al. 1980). The heterozygous hybrid GGgg will be large. A similar crossbreeding system is used in commercial production of large flowering F1 varieties.

MATERIAL AND METHODS

Characterization of experimental design and methods

Seeds of two red-flowering homozygous lines used for commercial production of F1 diploid varieties were used to induce polyploidy. This material was provided by the company Černý-BioPro, Prague, Czech Republic. Large-flowered component 63 is homozygous with GG genotype, bright red. The small-flowered component 017 is homozygous with the genotype gg, bright red.

The seeds were sterilized with 30% commercial bleach solution Savo for 20 minutes and then washed 3 times with sterile distilled water. Sown into 250 ml Erlenmeyer flasks with 50 ml MS medium (Murashige and Skoog 1962), hardened with 8 g/l Agar, and added 20 g/l sucrose. Ten-days old germ plants were treated for 24 hours with an aqueous solution of 30 µM oryzalin. The plants were then washed 2 times with sterile distilled water and passaged on the same medium. Later, 100 plants were transferred from each genotype to greenhouse conditions. For twenty randomly selected plants from each component the ploidy was tested. This was detected cyto-flowmetrically on a Partec PA II instrument with a mercury UV discharge lamp in the Laboratory of Flow Cytometry at the Academy of Sciences of the Czech Republic Průhonice (Otto 1990, Doležel et al. 1994).

Plants were planted in 14 cm diameter pots and grown under standard conditions. In July 2016, the size of 30 flowers was measured on each plant. The plants were described based on the descriptor used at the breeding site (Černý 1974). Tetraploid plants were transferred to in vitro genobank (Šedivá 2009) to prevent the possible loss of these genotypes or virus infection. The results were evaluated by calculating the confidence interval (p = 0.05) for the group of diploid and tetraploid plants, for both components separately. Software Statistics 12 was used for the calculation. The aim of this experiment was to confirm the possibility of inducing polyploidy by oryzalin in vitro in *Petunia hybrida* seeds. Another goal was to create tetraploid line materials of red color and evaluate the predicted tetraploid flower enlargement.

RESULTS AND DISCUSSION

For material 63, 10 tetraploids, 8 diploids and 2 mixoploids were found. For material 017, 13 tetraploids and 7 diploids were found. The arithmetic average of blossom diameter for diploid material 63 was 69 mm and for tetraploids 90 mm, which is statistically significant (p = 0.05) 30% increase. The diploid material 017 was 54 mm and tetraploids 72 mm, which is statistically significant (p = 0.05) 34% increase.

Table 1 Comparison of flower size (mm) among 4n (Group 1) and 2n (Group 2) plants

	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
Component 63	90.013	69.242	161.275	539	0.00	301	240	1.503	1.469	1.047	0.713
Component 017	72.022	53.633	216.349	718	0.00	510	210	1.103	0.855	1.663	0.000

Note: T-test for independent samples; p statistics p = 0.05; measures in mm; computed in STATISTICS 12.

Creation of tetraploid F1 hybrid varieties has so far been unsuccessful. The main problem was the acquirement of homozygous tetraploid lines (Reimann-Philipp 1969). These are obtained by diploid hybrids by self-pollination and following selection. In general, 6 cycles are sufficient to obtain the material later to use to produce F1 hybrids. However, for tetraploids, segregation ratios are more complex and more cycles would be required to obtain a homozygous line. The situation is further complicated by the fact that the combination of the GGGG allele, which must be represented in the paternal component for the creation of a large-flowered variety, is semi-lethal (Reimann-Philipp 1962).

Seeking such a genotype in segregating offspring after self-pollination is highly impossible. A more viable option is induction of tetraploids from diploid homozygous components. Lot of researchers focused on Petunia polyploidization (Comai 2005). In majority of experiments, the mitotic poison colchicine was used. In our conducted experiment, we used oryzalin as a polyploidizing agent, which is significantly less toxic to humans. For many crops, it has been successfully used in in vitro polyploidization protocols (Grace and Andersson 2006, Greplová et al. 2009).

Our results confirm that it is well-suited to *Petunia hybrida*. The results show that more than 50% of the plants were tetraploid. This is much higher yield of tetraploids than with colchicine. When using colchicine, lot of plants are octoploid, which means 8n (Levan 1939). We have not trapped such a plant. In only two cases, the plant was marked as a mixoploid, i.e. it was a chimeric plant that had 2n and 4n.

We believe that polyploidization performed per our protocol takes place at optimum time. The oryzalin solution influences the entire vegetation peak, which is small and compact at the time.

Meristematic cells after polyploidization grow further and form a polyploid plant. The effect of oryzaline is not as drastic as colchicine, and therefore plants with higher ploidy than tetraploids do not appear. We also did not notice deformed plants often described by the authors (Ning et al. 2009). Optimal conditions in in vitro cultures allow the treated germ plants to continue growing even after partial intoxication with the polyploidizing agent. When applied in field conditions, a part of the created polyploid plants die (Seidel 1962).

For component 63, we have been able to obtain plants with a GGGG genotype that will allow us to create a large-flowered tetraploid hybrid. Tetraploidized material 017 will be used as the maternal component. The GGgg hybrids will be large-flowered because the G allele for the velocity is dominant. We further confirmed that induced tetraploid has a larger flower, at least by 30%. The size of 90 mm and 72 mm does not reach the size of OP tetraploid varieties. We think that the size of the tetraploid- induced flower is strongly dependent on the used genetic material. We will focus on that in the further research.

We managed to obtain tetraploid of a red color, which does not exist in the assortment of tetraploid OP varieties (Gerats and Strommer 2009). Naturally, via selecting spontaneous tetraploids from OP variety, this variety could not be created because the first variety of this color, Fire Chief, launched to the market in 1950s, was F1 hybrid (Sink 1984). If a spontaneous tetraploid was selected from such a variety, it would be practically unusable for its high heterozygosity. Thus, diploid induction is practically the only viable possibility to enrich a group of tetraploid varieties with a red flower color. The induction of tetraploids under in vitro conditions could be the foundation of breeding programs leading to the breeding of a completely new F1 tetraploid varieties.

Figure 1 Red flowers of diploid and tetraploid plants

A) Component 017 - diploid

B) Component 017 - tetraploid

C) Component 63 - diploid

D) Component 63 - tetraploid

CONCLUSION

Creating of new ornamental flower varieties is a highly competitive and globalized activity. Many Petunia varieties are protected by patents. A successful strategy to come up with something new is to use modern biotechnological methods to breed almost forgotten groups. This strategy links with the program of production F1 polyploid varieties. In vitro polyploidization can create tetraploid lines that can be used in the breeding programs. This method also allows to extend the colour spectrum of the superbissima group. As a result, there should be balanced varieties with a giant flower. The induction of tetraploids from the homozygous diploid material solves the basic problem with breeding parental components. The used protocol is fast, has low financial demands and high reliability.

ACKNOWLEDGEMENTS

The research was financially supported by the breeding company Černý-BioPro Ltd., Prague, Czech Republic.

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EFFECT OF DIFFERENT PHYTOHORMONES ON GROWTH AND DEVELOPMENT OF MICROPROPAGATED *CANNABIS SATIVA* L.

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Abstract: Cannabis sativa L. is a fast-growing annual herbaceous plant which is cultivated due to its multi-purpose applications. The aim of this work was to adapt the cultivation protocol for micropropagation of two industrial hemp varieties Bialobrzeskie and Monoica. Endogenously applied different concentrations of phytohormones were tested in order to observe the highest shoot proliferation and root formation. The shoot tips were taken from 10–18 days old plantlets. Hemp plants grown on the medium with thidiazuron and gibberellic acid had the longest stems, on media with 1-naphthalene acetic acid and thidiazuron the highest fresh weight and on media enriched with 1-naphthalene acetic acid and 6-benzylaminopurine or on culture medium without phytohormones highest numbers of nodes. However, the highest vitality of the plantlets of both genotypes and the highest number of roots were observed on medium when phytohormones were not present or on medium containing meta-topolin.

Key Words: hemp, meta-topolin, micropropagation, shoot tip culture, thidiazuron

INTRODUCTION

Cannabis sativa L. is a fast-growing annual herbaceous plant, which belongs to the family Cannabaceae. The cannabis plants are predominantly dioecious, with pistillate plants bearing only female flowers and staminate plants developing only male flowers, but it can also be monoecious (Small 2015). It is a short-day flowering plant, with staminate plants usually taller and less robust than pistillate plants. Cannabis is indigenous to central Asia and Middle East (Andre et al. 2016). It has been cultivated throughout recorded history due to its multi-purpose applications. However, recently it became a subject of interest of many researches and it regained merited attention (Galasso et al. 2016). Known for its high content of phytochemicals, but as well as an important source of both cellulosic and woody fibers, cannabis plants have employment in different industrial sectors. In the pharmaceutical industry it is widely used due to its high content of cannabinoids, terpenes and phenolic compounds. Cannabinoids represent the most studied group of compounds, mainly due to their wide application on human health. The most notable cannabinoid is the phytocannabinoid, tetrahydrocannabinol (THC), the primary psychoactive compound in cannabis (Lambert et al. 2005). However, it is very important to emphasize that *Cannabis sativa* is grown depending on the usage of its products. Based on the THC content, cannabis can be used for medicinal or recreational purpose (content higher than 0.2% THC) and as industrial hemp (content up to 0.2% THC). Industrial hemp has variety of applications e.g. as a source of fiber for textile industry, for production of seed oil, plastics, biofuel, but as well seeds can be used as animal and bird feed (Small 2015). Being a fast-growing plant and due to high biomass production hemp can be used in the phytoremediation processes in industrially polluted regions for removing considerable quantities of heavy metals from the soil (Shi et al. 2012, Ahmad et al. 2016).

The maintenance of clonal fidelity is an important issue in developing a secure and stable *in vitro* clonal repository of elite *C. sativa* germplasm. *In vitro* culture techniques provide an important means of plant propagation, but as well can be a useful tool to study physiological, morphological and molecular changes during plant development. However, multiplication and rooting can still be a bottleneck for hemp cultivation *in vitro*. Although, few researches were published on cultivation of cannabis in *in vitro* conditions, it is still challenging to adapt protocol for micropropagation based on different genotypes used (Wang et al. 2009, Chaohua et al. 2016, Lata et al. 2009, 2016). Moreover, it is known that success of *in vitro* plants cultivation strongly depends on the genotype, explant type, and phytohormones used in the culture medium (Delporte et al. 2016). Different phytohormones were tested in order to improve protocol for micropropagation of cannabis e.g. thidiazuron (TDZ) and 1-naphthalene acetic acid (NAA) for shoot proliferation and indole-3-butyric acid (IBA) and NAA for rooting (Wang et al. 2009), while Lata et al. (2016) in their work tested meta-topolin (mT), for shoot and root formation from nodal segments.

The aim of this work was to optimize the cultivation protocol for micropropagation of two industrial hemp varieties Bialobrzeskie and Monoica. Moreover, different phytohormones in different concentrations were tested in order to improve shoot proliferation and root formation.

MATERIAL AND METHODS

Plant material

Two monoecious varieties of industrial hemp: Bialobrzeskie and Monoica, both obtained from Agritec Plant Research, Ltd. in Sumperk, Czech Republic were selected for this study. Bialobrzeskie is a Polish variety registered in 1968 while Monoica is a Hungarian variety, registered in 2006 (Bjelková 2011). In order to obtain plantlets for *in vitro* multiplication, seeds of above mentioned varieties were used and placed on the nutritional medium. For surface sterilization, hemp seeds were firstly thoroughly washed with few drops of detergent under running tap water. Furthermore, seeds were surface sterilized with 0.2% of mercury chloride for 13 minutes and afterwards rinsed three times with sterile distilled water to remove traces of sterilizing agents. The sterilized seeds were germinated on half-strength MS medium (Murashige and Skoog 1962) supplemented with 10 g/l sucrose and 6.5 g/l agar. The pH value of the media was adjusted to 5.8 before autoclaving at 121°C, 100 kPa, for 20 min. The seeds were maintained in a cultivation room under 18/6 light dark cycle at 24 ± 2 °C (Wang et al. 2009).

Plantlets multiplication, elongation and rooting

The shoot tips (~2 cm in length) were taken from 10–18 days old plantlets obtained from seeds. Eight variants of MS multiplication media, mainly differing in phytohormones and their concentrations, were tested (Table 1). The phytohormones tested were: cytokinins - 6-benzylaminopurine (BAP), thidiazuron (TDZ) or meta-topolin (mT); auxins - 1-naphthalene acetic acid (NAA) or indole-3-acetic acid (IAA) and gibberellins in a form of gibberellic acid (GA_3). Regarding the carbon source, 30 g/l sucrose was used in variants 1–7 of MS multiplication medium variants while only in the variant 8 was added 15 g/l sucrose. As gelling agents of the media, agar in concentrations of 6.5, 7, and 8 g/l and phytagel in concentration of 3 g/l (variant 4) were tested. Moreover, media variants 6–8 were supplemented with 1 g/l activated charcoal. The pH value of the media was adjusted to 5.7–5.8 before autoclaving at 121°C, 100 kPa, for 20 min. Thermolabile components of the medium i.e. phytohormones were filter sterilized (Whatman Puradisc 25, AS 0.2 μm) and added separately to cooled autoclaved medium. The shoot tips placed on nutritional media were maintained in a cultivation room under 18/6 light dark cycle at 24 ± 2 °C. After two weeks of cultivation period, plantlets were subsequently transferred on the fresh medium of the same composition while only at the variant 7 was added 2.5 mg/l GA_3 (Lata et al. 2009). Growth and development of plantlets was monitored for four weeks interval where photos of the plantlets appearance were taken periodically with Canon EOS 60D digital camera. However, evaluation of the experiments was done at the end of the fourth week. Evaluated parameters were fresh weight, shoot length, number of nodes, callus appearance and plantlets vitality. Furthermore, after one month on multiplication medium plantlets were transferred to the rooting medium. Rooting medium was prepared according to the protocol previously published

by Lata et al. (2009). Briefly, rooting medium contained MS basal salts full concentration, 30 g/l sucrose, 0.5 g/l activated charcoal, 8 g/l agar and 0.5 mg/l of indole-3-butyric acid (IBA). The pH value of the media was adjusted to 5.7 before autoclaving at 121°C, 100 kPa, for 20 min. The plantlets were placed on rooting media and were maintained in a cultivation room under 18/6 light dark cycle at 24 ± 2 °C. After one month on rooting media the root appearance was evaluated.

Table 1 Composition of MS multiplication media

Variants of MS medium	Concentrations of phytohormones			Gelling agents	Supplements
	Auxins	Cytokinins	Gibberellins		
1	0.1 mg/l NAA	0.1 mg/l TDZ	-	6.5 g/l agar	-
2	0.1 mg/l NAA	0.4 mg/l BAP	-	6.5 g/l agar	-
3	0.1 mg/l IAA	0.4 mg/l BAP	-	6.5 g/l agar	-
4	0.1 mg/l NAA	0.1 mg/l TDZ	-	3 g/l phytagel	-
5	0.1 mg/l NAA	0.1 mg/l BAP	-	6.5 g/l agar	-
6	-	0.5 mg/l mT	-	8 g/l agar	1 g/l activated charcoal
7	-	0.1 mg/l TDZ	2.5 mg/l GA_3	8 g/l agar	1 g/l activated charcoal
8	-	-	-	7 g/l agar	1 g/l activated charcoal

Legend: MS – Murashige and Skoog (1962), NAA – 1-naphthalene acetic acid, TDZ – thidiazuron, BAP – 6-benzylaminopurine, IAA – indole-3-acetic acid, mT – meta-topolin, GA_3 – gibberellic acid.

Experimental design and statistical analysis of data

The effects of different phytohormones in different concentrations were tested on two hemp varieties Bialobrzeskie and Monoica grown *in vitro*. Five shoot tips were placed equidistant to each other in one cultivation jar. At the end of the multiplication period (one month), altogether three cultivation jars (i.e. 15 numbered hemp plantlets) were sampled. All statistical analyses were performed using one-way analysis of variance (ANOVA; effects of hemp variety and cultivation media variants were considered fixed); pairwise contrasts were tested using LSD test and differences among treatments were considered significant at $P < 0.05$.

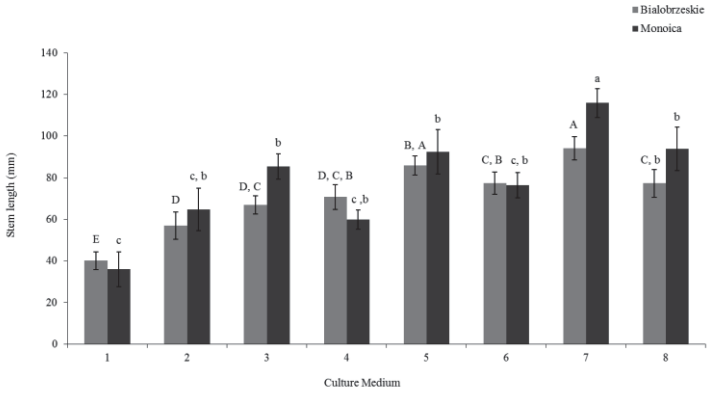
RESULTS AND DISCUSSION

Shoot tips of two hemp varieties Bialobrzeskie and Monoica were placed on eight different culture media in order to observe on which media shoot proliferation and root formation will be the highest. Evaluated parameters were fresh weight, shoot length and number of nodes (Figure 1–3). In addition, callus and root appearance and plantlets vitality were also evaluated but data are not shown.

In both varieties the longest stem was observed when grown on media supplemented with TDZ and GA_3 (medium 7) while shortest stem was recorded on the media with TDZ and NAA (medium 1). However, when IAA and BAP applied (medium 3), significantly longer stems were observed in variety Monoica in comparison with Bialobrzeskie variety (Figure 1). To obtain new stem segments for micropropagation, stem length is one of the critical parameters. However, it is difficult to estimate to which extent longer stems could be a genotype related trait and when different combinations of phytohormones start to play the role. It is most likely a combination of factors, but at least in the case of medium 3, the different, variety-given sensitivity to phytohormones was observed. IAA, as a naturally strong auxin (promotes cell elongation) did not seem to have a significant effect on Bialobrzeskie variety. However, as expected with GA_3 application in combination with TDZ, plantlets grown on medium 7 had the longest stem. It is well known that after application of GA_3 an increase in both cell elongation and cell division occurs during stem growth (Sun 2010). Moreover, culture medium enriched with NAA and BAP (medium 5) in both varieties tested

was comparable with medium 7. Similar results were obtained in experiments with certain species of bonsai (Rahimi et al. 2013).

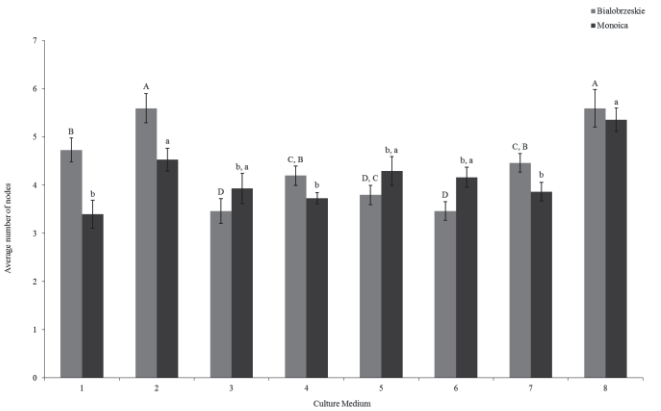
Figure 1 Effect of different culture media on stem length of Bialobrzeskie and Monoica hemp variety



Legend: Capital letters represent statistical differences among different culture media in Bialobrzeskie variety while lower case letters represent statistical differences among different culture media in Monoica variety. Error bars show standard deviation, and letters indicate significant differences (P < 0.05).

Genotype-related variability can also be considered in average number of nodes formed on plantlets grown on various culture media, where the highest rate for both genotypes was observed in growth medium enriched with NAA and BAP or on culture medium without phytohormones, respectively (medium 2 and 8) (Figure 2).

Figure 2 Effect of different culture media on average number of nodes of Bialobrzeskie and Monoica hemp variety

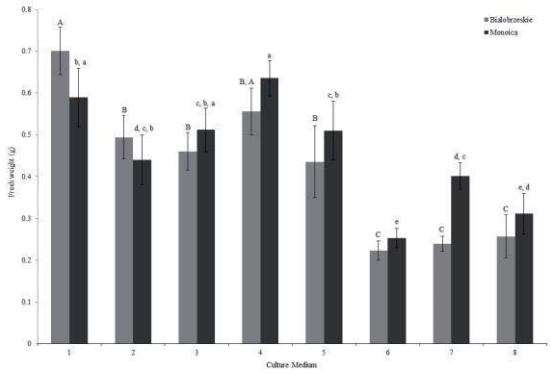


Legend: Capital letters represent statistical differences among different culture media in Bialobrzeskie variety while lower case letters represent statistical differences among different culture media in Monoica variety. Error bars show standard deviation, and letters indicate significant differences (P < 0.05).

More nodes formed may form a better basis for axillary proliferation, thus easier multiplication of plant tissues (Alvarez et al. 2006). Especially higher concentration of BAP and low concentration of NAA are known to be beneficial for shoot-bud differentiation and the similar effect was observed in the study with groundnut (Venkatachalam and Jayabalan 1997). Interestingly, unlike to our results, a significant effect of TDZ on formation of axillary buds and shoots in cannabis plantlets was recorded (Lata et al. 2009, Wang et al. 2009). However, in their studies high-content THC variety was used. Furthermore, in both varieties the highest fresh weight was recorded when culture medium was enriched with NAA and TDZ (medium 1 and 4) while the lowest fresh weight was measured in the medium with mT (medium 6) (Figure 3). However, even that highest fresh weight was recorded on medium with NAA and TDZ, the presence of NAA strongly influenced the callus formation and different general shoot architecture (data not shown). The combined effect of endogenous applied auxins and cytokinins caused the formation of callus in a study on soybean hypocotyls (Liu et al. 1997) or only auxins on *in vitro* cultures of apple (Welander and Snygg 1987).

On the other hand, the significantly highest fresh weight for both genotypes was observed in media where TDZ was present. This cytokinin is known to induce axillary proliferation in lower concentrations, but may inhibit shoot elongation, which is confirmed by the stem length data (Figure 1). Also in high concentration, it may cause the formation of callus or fasciated shoots which could lead to higher fresh weight (Huetteman and Preece 1992). Moreover, the plants with thicker stems, short shoots and generally more sturdy constitution were present in culture media 1 and 4. However, stem-widening effect of TDZ was recorded in a study when cannabis plants were grown *in vitro* (Wang et al. 2009).

Figure 3 Effect of different culture media on fresh weight of Bialobrzeskie and Monoica hemp variety



Legend: Capital letters represent statistical differences among different culture media in Bialobrzeskie variety while lower case letters represent statistical differences among different culture media in Monoica variety. Error bars show standard deviation, and letters indicate significant differences (P < 0.05).

The same TDZ effect was observed in our experiments (data not shown). However, stem length, number of nodes and fresh weight does not necessarily correlate with the vitality of the plantlets grown *in vitro*. Even though hemp plants grown on the medium with TDZ and GA_3 had the longest stems, on media with NAA and TDZ the highest fresh weight and on media enriched with NAA and BAP or on culture medium without phytohormones the highest numbers of nodes, the effect on plantlets vitality was variable. The most vital plantlets of both genotypes with the highest numbers of roots were observed on medium when phytohormones where not present (medium 8) or on medium containing mT (medium 6). Good results regarding the general shoot formation of *Cannabis sativa* L. plantlets *in vitro* using mT were observed in a study published by Lata et al. (2016), even though the mentioned research group used in their study high-content THC variety, which may have a slightly different response to mT than industrial varieties used in our study.

CONCLUSION

This research shows that for the micropropagation of two hemp varieties Bialobrzeskie and Monoica, the culture media supplemented with mT, but without other phytohormones produced the best overall appearance of the plantlets. Sustainability of plant culture *in vitro* is necessary for any further modifications of the experimental conditions, so the medium with mT will be used in the future research focused on phytoremediation potential of the *Cannabis sativa* L. in *in vitro* conditions.

ACKNOWLEDGEMENTS

This research was financially supported by the IGA FA MENDELU No. TP 5/2017. The authors gratefully acknowledge Ing. Marie Bjelková, Ph.D. from Agritec Plant Research, Ltd. in Sumperk for providing plant material.

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DETERMINATION OF THE CONTENT OF PIGMENTS IN SEEDS

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Abstract: The contents of chlorophyll *a*, chlorophyll *b*, β-carotene, lutein and anthocyanins were measured spectrophotometrically in extracts from seeds of wheat and hemp varieties. Within varieties of wheat variety Citrus has the second highest total chlorophyll content and the highest carotenoid content, but the lowest content of anthocyanins. Other measured varieties of wheat were Novosibirskaya 67, PS Karkulka, Rebell and Skorpion. Two hemp varieties were measured: Bialobrzeskie, which had higher total chlorophyll and carotenoid content, but was lower in anthocyanins than Monoica variety. No correlation was observed between the germination rate and the content of pigments, especially chlorophylls due to low number of studied varieties, but it has been verified that this easy, quick and low cost spectrophotometric method can be used for the determination of pigments, especially chlorophylls and carotenoids in seeds.

Key Words: anthocyanins, carotenoids, chlorophylls, germination, spectrophotometry

INTRODUCTION

The seeds contain 3 main types of storage reserves – polysaccharides (starch), proteins and lipids in different ratios. In relatively minor quantities, phytin is present, which is an important source of phosphate and mineral elements to the seed. Other constituents present within seeds are pigments such as anthocyanins (proanthocyanidins), carotenoids and chlorophylls. Colored pigments most often accumulate in the inner layers or aleurone layer of the testa (seed coat) (Bewley et al. 2013).

It was found that chlorophylls and carotenoids are also present in appreciable quantities in embryo axis and cotyledons of physiologically mature seeds. Content of chlorophylls and the ratio of carotenoid to chlorophyll content (Car/Chl) can be an indicator of seed tolerance to stress factors, because high content of chlorophyll reduces seed tolerance to abiotic stresses and an increased Car/Chl ratio means higher seed tolerance, on the contrary. The carotenoids in seeds are mainly lutein and β-carotene and play an important role in protecting seeds against oxidative stress as antioxidants (Smolikova et al. 2011).

Determination of the content of chlorophylls and carotenoids in seeds can be done by different methods. The expensive and much more time-consuming high-performance liquid chromatography (HPLC) method was used to measure chlorophyll in canola seed (Ward et al. 1994) and carotenoids in soybean seeds (Monma et al. 1994). Another method based on the measurement of fluorescence of chlorophyll *a* in seed coat is a highly sensitive and rapid method, which is a new technology applicable for characterisation of seed quality in seed industry (Jalink et al. 1998, 1999, Kenanoglu et al. 2013, 2016). Recently, a simple method of spectrophotometric measurement of chlorophylls and carotenoids in extracts from seeds was modified (Bulda et al. 2008, Smolikova et al. 2011).

The aim of this work was to determinate the content of chlorophylls, carotenoids and anthocyanins in seeds of two varieties of hemp and five varieties of wheat using the spectrophotometric method (Bulda et al. 2008, Smolikova et al. 2011, Varga et al. 2013). Then, the standard laboratory tests for germination of the varieties were performed and subsequently, values of germination rate were compared with the measured content of chlorophylls, carotenoids and anthocyanins.

MATERIAL AND METHODS

Plant material

In this study, seeds of two industrial hemp varieties (*Cannabis sativa* L.) Bialobrzeskie and Monoica obtained from Agritec Plant Research, Ltd. in Sumperk and five varieties of wheat (*Triticum aestivum* L.) harvested in 2016 - Citrus, PS Karkulka, Novosibirskaya 67, Rebell and Skorpion from the Agricultural Research Institute (Agrotest Fyto, Ltd.) in Kromeriz were used.

Extraction and spectrophotometric measurement of pigments

Samples of seeds (500 mg) were ground in a laboratory mill (Analysette 3 SPARTAN, Fritsch) approximately for 5 minutes. The homogenate was mixed and ground in the mortar with 2 ml of petroleum ether (PE 60–80 °C, pure) and tetrahydrofuran (THF, p.a.) mixture in ratio 1:1. Then, 3 ml of PE was added and grinding continued. The sample was rinsed and ground in the mortar 2 times with 3 ml of PE : THF (ratio 4 : 1) mixture. The homogenate was filtered through syringe nylon filters (polyamide) with a pore size of 0.45 µm. The obtained filtrate was used to measure β-carotene, lutein, chlorophyll *a* and *b* with the spectrophotometer Spectronic 20 Genesys (Thermo Scientific). The pigment content was calculated from equations presented by Bulda et al. (2008), where the total content of chlorophylls was calculated as the sum of chlorophyll *a* and chlorophyll *b*, and the content of carotenoids was the sum of β-carotene and lutein.

For the determination of anthocyanins, 3 grams of seeds were needed, and were ground the same as for analysis of chlorophylls and carotenoids. Extraction of anthocyanins was performed according to Varga et al. (2013). The obtained filtrate was used to measure the total content of anthocyanins with a Sunrise spectrophotometer (Tecan). The total anthocyanin content (TAC) was calculated from equations and expressed as cyanidin-3-glucoside according to Varga et al. (2013).

Standard laboratory germination test

Standard laboratory germination tests were performed in four replicates of 50 seeds each. The seeds of each replicate were laid between moistened filter papers (Whatman, Grade 1) in Petri dishes. Germination tests were carried out at 20 °C in the dark (Thermostat, TS 606/2). Normal seedlings with developed shoot and roots, abnormal seedling and dead seeds were evaluated after 7 days for hemp and after 8 days for wheat according to UKZUZ (2014).

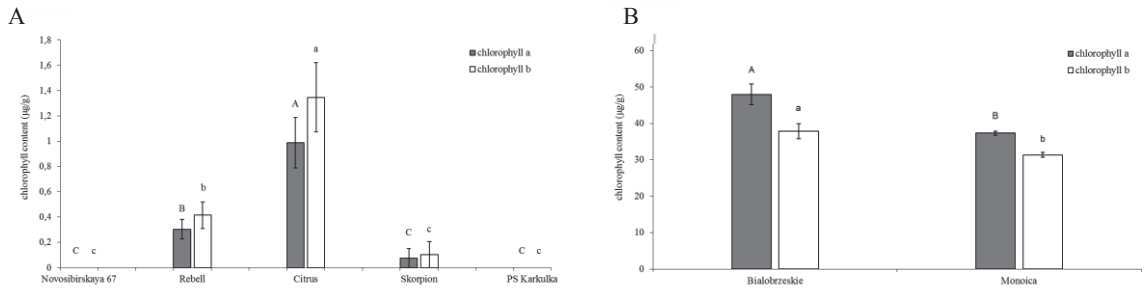
Statistical analysis of data

Data of all the parameters were evaluated in Microsoft Excel software, statistical differences were tested by one-way ANOVA „F“ test at the level of significance $\alpha = 0.05$.

RESULTS AND DISCUSSION

It was found out, that the content of chlorophyll *a,* chlorophyll *b,* the total content of chlorophylls, β-carotene, lutein, carotenoids and the total contents of anthocyanins differed significantly between plant species as well as within varieties of wheat and hemp.

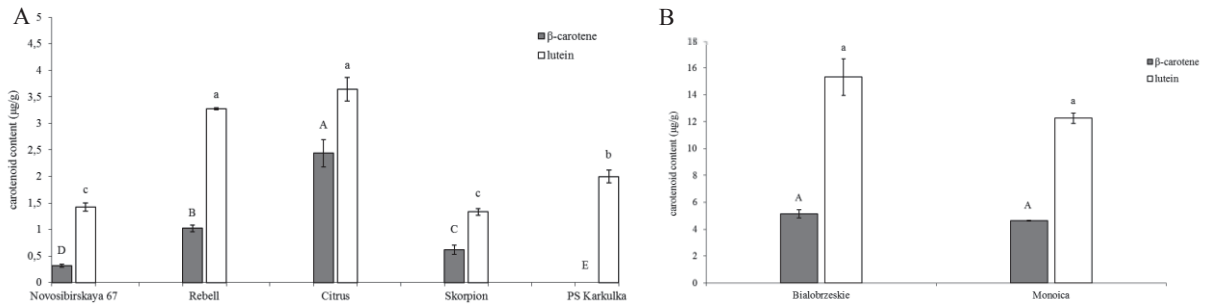
Figure 1 Comparison of the content of chlorophyll a and chlorophyll b (µg/g dry weight) in seeds of wheat (A) and hemp (B) varieties



Legend: Capital letters above each column represent statistical differences between varieties for chlorophyll a, while lower case letter stand for chlorophyll b.

In two varieties of wheat - PS Karkulka and Novosibirskaya 67, none of the chlorophylls was detected. The varieties Rebell and Skorpion had low total content of chlorophylls (0.72 µg/g, and 0.18 µg/g resp.) and variety Citrus contained the highest amount of total chlorophylls (2.33 µg/g, of which chlorophyll *a* was 0.99 µg/g and chlorophyll *b* was 1.35 µg/g) (Figure 1A). In contrast to wheat, varieties of hemp contained significantly higher amounts of chlorophylls. The variety Bialobrzeskie contained 85.81 µg/g of total chlorophylls (47.98 µg/g of chlorophyll *a* and 37.84 µg/g of chlorophyll *b*), while the variety Monoica slightly lower content - 68.68 µg/g of total chlorophylls (37.32 µg/g of chlorophyll *a* and 31.34 µg/g of chlorophyll *b*) (Figure 1B). The determination of the total content of chlorophylls by spectrophotometric method was performed also in other plant species and was also significantly different, ranging from 51.73 µg/g in extracts from seeds of carrot to 0.35 µg/g in extracts from seeds of soybean (Bulda et al. 2008). Another method, which is based on the measurement of fluorescence signal of chlorophyll *a* in the seed coat, determined both low and high chlorophyll *a* content in seeds of cabbage and tomato (Jalink et al. 1998, 1999).

Figure 2 Comparison of the content of β-carotene and lutein (µg/g dry weight) in seeds of wheat (A) and hemp (B) varieties

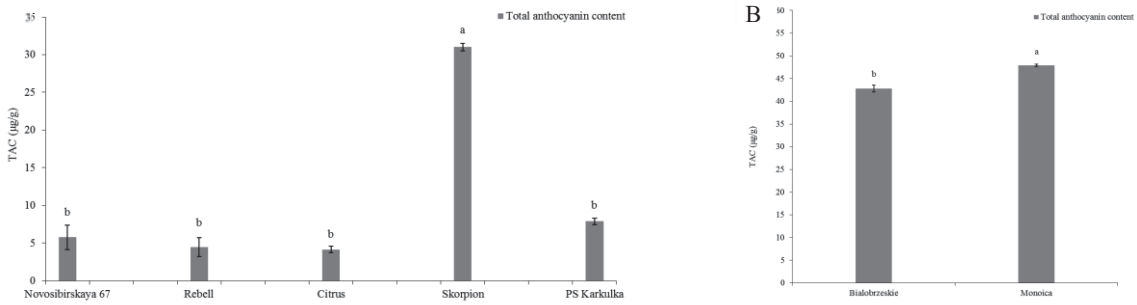


Legend: Capital letters above each column represent statistical differences between varieties for β-carotene, while lower case letter stand for lutein.

The content of carotenoids in the range from 1.74 µg/g to 6.08 µg/g of dry weight (Figure 2A) differed within species more than the content of total chlorophylls. In wheat the highest content of carotenoids was measured in seeds of variety Citrus (6.08 µg/g, of which 2.44 µg/g was β-carotene and 3.65 µg/g lutein). In seeds of variety PS Karkulka, only 2.00 µg/g of lutein was detected, β-carotene was not detected. The detailed results for all varieties are displayed in Table 1. The Citrus variety of wheat belongs to varieties characterized by yellow endosperm, which causes the increased content of carotenoids (Paznocht et al. 2018), as confirmed by this study. As well as the total content of chlorophylls, the content of carotenoids is significantly higher in hemp varieties. The variety Bialobrzeskie contained 20.44 µg/g of carotenoids, (of which 5.14 µg/g was β-carotene and 15.30 µg/g lutein). A little less carotenoids were detected in variety Monoica – 16.90 µg/g of carotenoids (Figure 2B). The determination of the content of carotenoids by spectrophotometric method was performed also in other plant species and differed also significantly from 16.86 µg/g in extracts from seeds of rapeseed to 0.86 µg/g in extracts from seeds of soybean (Bulda et al. 2008). Lutein was detected as the major seed carotenoid (Figure 2) as confirmed by this and other studies (Howitt and Pogson 2006, Smolikova et al. 2011).

The total anthocyanin content (TAC) expressed as the cyanidin-3-glucoside was the highest in seeds of variety Skorpion (31.00 µg/g). A much lower content of anthocyanins in all other varieties ranged from 7.88 µg/g (PS Karkulka) to 4.16 µg/g in Citrus (Figure 3A, Table 1). The variety Skorpion belongs to varieties characterized by blue aleurone layer, which causes the increased content of anthocyanins. This is similar for the variety PS Karkulka, which is characterized by purple pericarp. Both varieties are called "coloured" wheat, which are characterized by an increased content of anthocyanins. The content of anthocyanins is not only dependent on the genotype, but is considerably influenced by the environment as described by other studies (Varga et al. 2013, Garg et al. 2016). The variety Rebell is a standard genotype of wheat with red grain and the variety Novosibirskaya 67 is characterized by white grain (Martinek et al. 2013). The variety of hemp Bialobrzeskie had lower total content of anthocyanins of 42.82 µg/g than variety Monoica with 47.90 µg/g (Figure 3B).

Figure 3 Comparison of the total content of anthocyanins in seeds of wheat (A) and hemp (B) varieties



Legend: Letters above each column represent statistical differences between varieties TAC – the total content of anthocyanins expressed as µg/g cyanidin-3-glucoside.

The standard laboratory tests of germination were performed for all of these varieties according to UKZUZ (2014). The determination of germination rate as a seed quality parameter was important for comparison with measured values of pigments.

Within varieties of wheat the measured germination rate ranged between 80 and 95% (Novosibirskaya 67 and Rebell resp., Table 1). In Figure 4, the correlation of germination rate to the total content of chlorophyll *a* and *b* is shown. From the low R^2 value it could be concluded, that no correlation between these two parameters was observed. This fact can be most probably caused by the low number of varieties tested and/or by the fact that some seeds were infected.

Figure 4 Effect of the total content of chlorophylls on the standard laboratory germination in wheat varieties

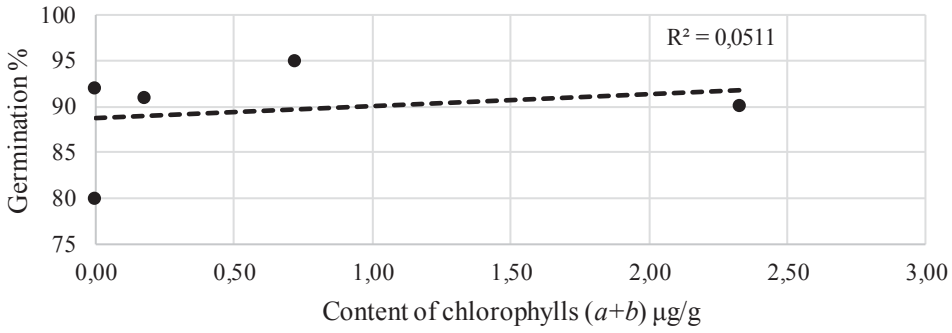


Table 1 Content of pigments (µg/g dry weight) and the standard laboratory germination (%) as a seed quality parameters of wheat and hemp varieties

Variety	Chl *a*	Chl *b*	Chl (*a*+*b*)	β-car	lutein	Car	TAC	Car/Chl ratio	Germination
Citrus	0.99	1.35	2.33	2.44	3.65	6.08	4.16	2.8	90 ± 1 b*
PS Karkulka	nd	nd	nd	nd	2.00	2.00	7.88	nd	92 ± 1.5 ab
Novosibirskaya 67	nd	nd	nd	0.32	1.42	1.74	5.75	nd	80 ± 3 bc
Rebell	0.30	0.41	0.72	1.02	3.28	4.30	4.45	6.6	95 ± 1.5 ab
Skorpion	0.08	0.10	0.18	0.62	1.33	1.95	31.00	1.2	91 ± 2 b
Bialobrzeskie	47.98	37.84	85.81	5.14	15.30	20.44	42.82	0.2	84 ± 3.56 A
Monoica	37.32	31.36	68.68	4.65	12.25	16.90	47.90	0.2	62 ± 1.71 B

*Legend: Data for germination are reported as the mean value ± standard error. Capital letters represent statistical differences between varieties of hemp, while lower case letters stand for differences between wheat varieties. Chl – chlorophyll, β-car – β-caroten, Car – carotenoids, TAC – the total anthocyanin content, inf. – infection, nd – not detected, * – infection.*

The hemp variety Bialobrezskie had a germination rate of 84%, which was much higher than 62% for variety Monoica (Table 1).

Some other studies, based on the measurement of fluorescence of chlorophyll *a* in the seed coat show negative correlation with germination rate for cabbage, tomato, soybean and pepper (Jalink et al. 1998, 1999, Cicero et al. 2009, Kenanoglu et al. 2013, 2016).

CONCLUSION

In this study, spectrophotometric measurement of the content of pigments, namely chlorophyll *a*, chlorophyll *b*, β-carotene, lutein and anthocyanins for varieties of wheat and hemp was performed. The content of the individual pigments within the species was compared and consequently, the influence on standard laboratory germination test, which is the seed quality parameter, was monitored. No correlation due to low number of studied varieties was observed. In future studies, the content of pigments and germination rate will be determined for other plant species and a larger set of varieties, possibly supported with the extended test of seed vitality.

ACKNOWLEDGEMENTS

This research was financially supported by the IGA FA MENDELU No. IP 5/2017. Acknowledgments belong to Ing. Marie Bjelková, Ph.D. from the Agritec Plant Research, Ltd. in Sumperk and Ing. Petr Martinek, CSc. from the Agricultural Research Institute (Agrotest Fyto, Ltd.) in Kromeriz, for providing seeds.

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OBJECTIVE EVALUATION OF SEED GERMINATION BY PROTEOMICS AND PRINCIPAL COMPONENT ANALYSIS

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Abstract: Seed germination is a complicated phase of plant's life which ensures the propagation and survival of plants over a period of unfavourable environmental conditions. The timing of metabolism reactivation and the progress of seed germination is a result of multiple factors and, if successful, leads to the seedling establishment. These processes that contribute to seed germination are relatively flexible and can (to some extent) adapt to fluctuations in the environment. This makes the whole system extremely variable and challenging. Here, we demonstrate that the multivariate analysis is an excellent tool to rapidly assess germination homogeneity and evaluate variables in the seed omics experiments. Germination of two contrasting plant species was analysed by shotgun LC-MS analysis resulting in an extensive amount of data. The principal component analysis (PCA) provided a fast preview of the system heterogeneity, including the level of biological variability.

Key Words: seed proteome, mass spectrometry, biological variability

INTRODUCTION

At present, five of ten world's top crops are propagated by seeds, namely rice, sorghum, soybeans, wheat and maize. The agricultural production in Europe is even more in favour of generatively propagated plants, including cereals, oilseeds, sugar beet, vegetables, legumes and grasses, and significantly outweighs the production of vegetatively propagated plants. Seeds are crucial for plant propagation and its utilization in agriculture (food production, crop breeding, storage of genetic resources), as well as in other industrial sectors. Further, the Food and Agriculture Organization (FAO) claims that 60% energy intake of the world's population is provided by only five cereal crops (rice, wheat, maize, millet and sorghum) and the food production should increase by 60% by 2050 to support the expected population growth.

The biological diversity serves as a way for populations to adapt to changing environments. With more variation, it is more likely that some individuals in a population will be more suited for the environment. Thanks to the epigenetic control, these variations can be found even within the population with the same genetic code. In plants, the sessile way of life does not allow to escape the unfavourable environmental conditions and plants have to be much more flexible in their development than mobile organisms. The seed germination represents highly important developmental stage which has to be controlled carefully, otherwise, the plant will not survive. Its progress could be affected by many factors, such as the process of seed maturation and desiccation on mother plant, seed viability, dormancy, number and quality of the storage compounds, robustness of the seed coats, seed size and different environmental factors (Bewley et al. 2012).

The proteins stored in seeds during the late phase of seed maturation and newly synthesized proteins provide an essential tool to comprehend complex processes of germination. Proteomics is one of the fastest developing scientific fields. During last decades, highly sensitive, fast and accurate instruments were developed, as well as sophisticated software for the data processing. However, the proteomic analysis is still limited by several factors, including the system variability which, if not considered, could result in misleading data interpretation (Shweiki et al. 2017). Here, we demonstrate the benefits of a modified principal component analysis of seed proteomics data and its performance in the identification of biologically significant changes.

MATERIAL AND METHODS

Plant material and experimental design

Seeds of two contrasting plant species, *Arabidopsis thaliana* (ecotype Columbia) and eggplant (*Solanum melongena*; var. Nero), were chosen for the analysis of the seed proteome dynamics during germination and the analysis of the effect of hydrogen peroxide (H_2O_2) on seed germination, respectively. Undressed eggplant seeds were purchased from commercial seed provider Moravoseed CZ a.s., *Arabidopsis* seeds were provided from the private stock of the Department of Molecular Biology and Radiobiology. The experimental setup was following:

1) 30 mg of *Arabidopsis* seeds were placed on Petri plate with filter paper (Whatman) and imbibed in 3.5 ml of distilled water at 20 °C. Samples were collected in three time-points: 6 hours after imbibition (HAI), 24 HAI and 48 HAI; and stored at -80 °C. The experiment was carried out in two biological replicates with differing seed generations.

2) 50 eggplant seeds were placed on Petri plate with filter paper (Whatman) and imbibed in 3.5 ml of (i) distilled water or (ii) 50mM H_2O_2 at 25 °C and 29 °C. The number of the first germinated seeds was calculated in the third day after imbibition (DAI). In order to minimize the effect of biological variability, only fully germinated individuals were collected and stored at -80 °C. The experiment was carried out in two biological replicates with the same seed stock.

LC-MS proteome profiling and data processing

Seeds were homogenized (RetschMill MM400) and the proteins were extracted using TCA/acetone/phenol method and analysed using a gel-free shotgun protocol based on nanoHPLC and MS/MS (Cerna et al. 2017, Novák et al. 2015). The extracted proteins were digested with immobilized trypsin (Promega), desalted, dried and dissolved in 0.5% (v/v) formic acid in 5% (v/v) acetonitrile. The samples were analysed in at least two technical replicates by nanoflow C18 reverse-phase liquid chromatography using a 15cm 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. The acquired spectra were extracted and processed as described previously (e.g. Baldrianová et al. 2015) by Data Analysis 4.1 and the resulting MGF files were searched against Arabidopsis (TAIR 10) and eggplant (SME_r2.5.1) protein databases by Sequest HT and Mascot 2.4 (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) and processed with Proteome Discoverer. Calculations and visualization of principal component analysis (PCA) were done in Origin 2016 (www.originlab.com).

RESULTS AND DISCUSSION

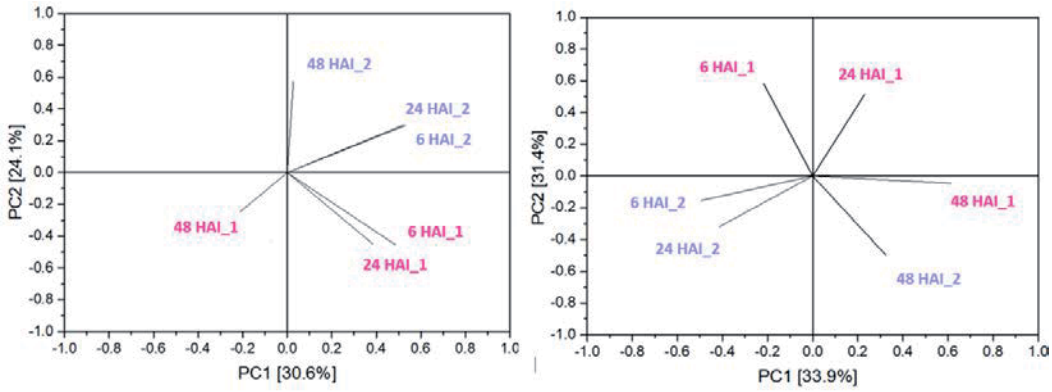
PCA is a statistical method for extracting relevant information from huge datasets. Briefly, PCA finds new variables (principal components; PC) that are linear functions of those in the original dataset, that successively maximize variance and that are uncorrelated with each other (Jolliffe and Cadima 2016). Here, we demonstrate that adequately filtered and normalized data from a label-free proteome profiling can be processed by PCA to evaluate biological variability in seed germination by the clustering of individual components according to their mutual similarity. First, the proteomic data generated by Proteome Discoverer were normalized to the total spectral matches (PSMs) to compensate minor variations in sample concentrations (Černý et al. 2013). In seeds, the majority of proteome is formed by several high-abundant proteins. Next, to compensate this disproportionality, relative abundances were calculated for each protein by dividing individual normalized PSMs values by the respective protein's PSMs average in all measured samples. This second normalization step significantly improved the output of PCA analyses. Next, PSMs-based quantification of low-abundant proteins is not reliable. To further increase significance of the PCA output, only proteins detected in all samples with more than two peptides and 10 PSMs were considered for the analysis.

Reduction of PCA input dataset provides a more precise overview about the *Arabidopsis* proteome dynamics during seed germination

Arabidopsis thaliana has tiny seeds that germinate fairly quickly and uniformly (100% of seeds germinate within 48 hours). *A. thaliana* is primarily self pollinating plant with the estimated out-crossing rate below 0.3% and the genetic variability within the seed stock is thus very low. Here, to observe biological variability, we analysed two stocks of seeds that originated from 2014 and 2016 harvests, respectively. The proteomic data were processed by a standard PCA analysis and by our modified method with a two-step normalization (Figure 1). The elimination of a low-confident quantitative data significantly improved the PCA output and we were able to illustrate similarities in the germination time-dependence.

Figure 1 PCA analysis describing the seed proteome dynamics of two independent Arabidopsis generations during seed germination

A) PCA based on 1,200 proteins *B) PCA based on 240 abundant proteins*



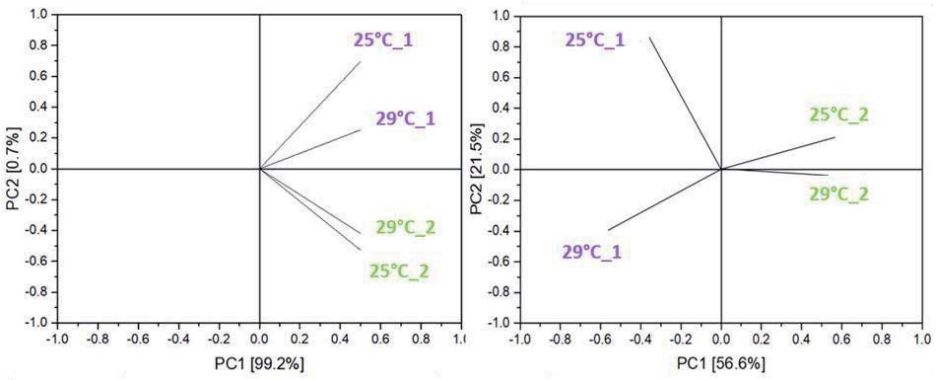
We did not find any significant difference in the visible seed germination but the proteomic analysis and the PCA revealed that the early stages of germination are different (Figure 1B). The PCA clearly separates the germination progress (PC1) and the biological variability (PC2). However, at 48 HAI the fully established seedlings from different stocks are becoming similar. These results illustrate the complexity of biological systems which in the same experimental setup reach a similar developmental state but do not follow the same time progress.

PCA of adequately normalized proteomic data provides information about the effect of biological variability and temperature during eggplant seed germination

Eggplant is a typical example of slowly and nonuniformly germinating plant species with strict temperature requirements for germination. Under optimal conditions, the first seeds may start to germinate within 3 DAI (days after imbibition) but the last of the viable seeds may take up to 10 days to germinate. Temperature is a major stimulant in the eggplant germination. Here, the significant differences in the visible germination are clearly evident. We performed the proteomic analysis of seeds germinated at 25 °C and 29 °C to see if it is possible to discriminate these differences at the molecular level. Only fully and uniformly germinated seeds were collected for the analysis and the PCA was based on 245 most abundant proteins. Figure 2A and 2B show the comparison of PCA results obtained after a one-step and a two-step normalization, respectively. It is clearly seen that PCA based on a one-step normalization could generate misleading data and here, it shows that all samples are nearly identical (clustered only by PC2 with extremely low level of significance). In contrast, the second normalization of individual proteins provided a more realistic data layout that correlated well with the seed morphology. The biological variability is separated by more significant PC1 and its effect is larger than that of temperature clustered by PC2. Further, the PCA shows a different effect of temperature between two biological replicates which correlates with the results observed at the physiological level. Since the same storage and cultivation conditions were maintained, it seems that there is some unknown factor which delays the seed germination. It is likely that this factor could be actually the biological variability caused by the seed origin.

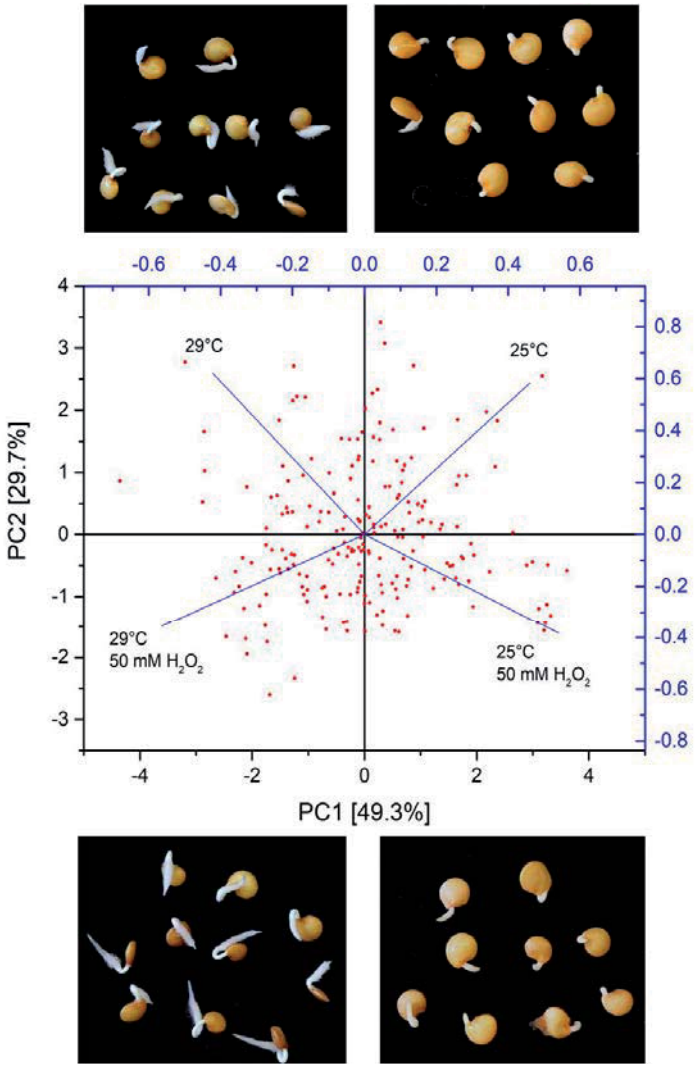
Figure 2 PCA analysis describing the effect of temperature and biological variability of germinating eggplant seeds

A) One-step normalization *B) Two-step normalization*



Optimized PCA of two-component experimental system

Figure 3 PCA separates effects of two different germination stimulants in eggplant seed proteome



Phytohormones have a significant impact on plant proteome (Černý et al. 2016). Due to the huge heterogeneity and long period of the seed germination, eggplant seems to be an excellent model for the testing of different plant growth regulators improving seed germination. Hydrogen peroxide (H_2O_2) appears to be one of such stimulants whose effect, however, is species-specific. Thus, simultaneously with the temperature experiment, we set an experiment with H_2O_2 to examine

its effect on eggplant seed germination. However, the newly introduced factor makes the system even more variable. To clearly illustrate the clustering of the samples due to the individual treatments, we excluded the effect of biological variability by employing PCA on a single biological replicate using most abundant proteins filtering and two-step data normalization. Figure 3 shows an ideal example of the PCA data layout. The PC1 clusters samples by the effect of temperature, while the PC2 separates the effect of H_2O_2. Here, PCA provides a beneficial preview that the samples differ between each other, even when the analyzed individuals look identically (mock- and H_2O_2-treated seeds germinated at 25 °C).

CONCLUSION

Proteomics offers an insight into the molecular mechanism regulating early phases of seed germination. However, the study of such a complicated process is hindered by multiple factors, including a natural variability of living systems. We employed an optimized PCA analysis to examine the level of biological variability and the effect of different treatments in two independent systems: (i) tiny and fast germinating seeds of *Arabidopsis thaliana* and (ii) slowly and non-uniformly germinating eggplant (*Solanum melongena*) seeds. We show that the two-step data normalization and a reduction of the PCA input to the most abundant proteins highlights the correlations between seed proteome and the germination progress. Further, this analysis of molecular data provides a fast and comprehensive preview of the biological system, including biological variability and effect(s) of stimulants, even when the morphology of the analysed individuals is seemingly similar.

ACKNOWLEDGEMENTS

The results of this research have been acquired within grant TE02000177 (TACR), IP 15/2017 (Internal Grant Agency of Faculty of AgriSciences, Mendel University in Brno) and LQ1601 (CEITEC 2020) project with financial contribution made by the Ministry of Education, Youths and Sports of the Czech Republic within special support paid from the National Programme for Sustainability II funds. Hana Habánová - Brno Ph.D. Talent Scholarship Holder - funded by the Brno City Municipality.

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ANTIOXIDANT RESPONSE OF *ARABIDOPSIS THALIANA* TO ZnSe-NANOPARTIPLES, SELENIUM AND ZINC IONS

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Abstract: The impact of zinc selenide nanoparticles (ZnSe-NPs) on plants is still unknown. The intention of this work was to compare phytotoxicity of ZnSe-NPs and selenium and zinc ions in 100, 250 and 500 µM concentrations. Young seedlings of *Arabidopsis thaliana* (Columbia (Col-0) ecotype) was used as an ecotoxicological model. 250 and 500 µM concentrations were extremely phytotoxic and inhibited the growth. Only the lowest concentrations were used for next analysis. ZnSe-NPs treatment had no visible impact on the growth but led to increased antioxidant response. More antioxidant related genes were upregulated than suppressed. Concurrently, there were higher productions of secondary metabolites which are often synthesis during abiotic stress.

Key words: Arabidopsis thaliana, nanoparticles, phytotoxicity, selenium, zinc

INTRODUCTION

Abiotic stress factors have a negative impact on plant growth, yields and quality of plant's products. One of the biggest global problems is heavy metals (Gielen et al. 2016, Tang et al. 2014). However during the last decades, plants have come into contact with a new stress factor – nanoparticles. Nanotechnology is one of the fastest growing industries. NPs have gained considerable importance because of wide variety of applications - in biomedical, optical, and electronic fields (Rico et al. 2013, Kaveh et al. 2013). Due to its large production and use, their release into the environment is inevitable (Ma et al. 2014). They have difficult predictable mechanisms of toxicity compared with ions. Their phytotoxicity depends on the type of nanomaterial, particle size, specific surface area, concentration and the plants (Ma et al. 2014). NPs have a broad effects resulting from interaction with the plant (Ma et al. 2014, Gielen et al. 2016, Tang et al. 2014, Kaveh et al. 2013). Both positive and negative effects have been presented in the literature (Landa et al. 2012, Jia et al. 1999, Lopez-Moreno et al. 2010, Kumar et al. 2013). Kumar et al. (2013) found that 24 nm size gold NPs at 10 and 80 µg/ml concentrations have significantly induced growth and yield enhancement in *Arabidopsis thaliana*. Landa et al. (2012) pointed to potential environmental risks of ZnO on plants (Landa et al. 2012). TiO_2 NPs negatively affected water transport and transpiration in corn plants (Asli and Neumann, 2009). Many studies have been done on NPs phytotoxicity but some areas are still unclear. ZnSe-NPs have become more popular due to semiconductor properties. They have many potential applications (Zhu et al. 2000). But their plant's toxicity is still unstudied. The objective of this work was to compare phytotoxicity of ZnSe-NPs and selenium and zinc ions in young seedlings of *Arabidopsis thaliana (Columbia (Col-0) ecotype)*. Attention was focused on the antioxidant response, including the biosynthesis of the antioxidant secondary metabolites and their associated enzymes.

MATERIAL AND METHODS

SeZn-NPs and medium

The impact of SeZn-NPs, selenium and zinc ions were tested by cultivating *Arabidopsis thaliana* in the presence of 100, 250 and 500 µM SeZn-NPs and zinc or 10, 25 and 50 µM selenium added to the nutrient medium. Zinc was used in the form zinc sulfate heptahydrate, selenium as a sodium selenite. SeZn-NPs were synthesized by the same method reported previously by Moulick et al. 2015 (Moulick et al. 2015) . The germination media was constituted by standard 0.5 strength Murashige and Skoog (MS) salt supplemented with vitamins and solidified with 0.7% agar. The pH of media was adjusted to 5.7 with 1 M sodium hydroxide. SeZn-NPs solution or sterilized stock solutions of selenium or zinc were added to sterilized media at a final concentration. Germination medium without NPs was used as control.

Plant Species and Culture Condition

The seeds of *Arabidopsis thaliana*, ecotype Columbia, were rinsed with 50% ethanol, followed by sterilization in 50% SAVO, and washing in autoclaved distilled water at least three times. Seeds were germinated under sterile conditions 48 hours at 4 °C. Sterilized seeds were incubated in the cultivated plate at 23 °C and illuminated at 130 µmol m^2/s. with a 16 h light/8 h dark photoperiod. After 4 weeks, plants were removed from the medium immersed in liquid nitrogen and stored at -80 °C until the next processing.

Preparation of methanol extracts

The plants were divided into two parts; roots and leafs. The weighing of fresh tissue was homogenized in 1 ml 80% methanol. Then the samples were centrifuged at 12,000 × g for 10 min at room temperature.

Estimation of total antioxidant capacity

The total antioxidant capacity of extracts was investigated by phosphomolybdenum assay, according to the methods (Alam et al. 2013, Kumaran and Karunakaran, 2007). 100 µl of 50 µg/mL extract was dissolved in 1 ml of the reagent solution (0.6 mol/l sulphuric acid, 28 mol/l sodium phosphate and 4 mmol/l ammonium molybdate solutions), and then the mixture was incubated for 90 min at 95 °C. Rutin was used as the standard. When the samples were cooled to room temperature, the absorbance of the mixture was measured at 695 nm against a blank, which contained the reagent solution and solvent. The total antioxidant capacity was expressed as the equivalent to millimol of trolox per 1 g of fresh weight (mmol trolox/1g FW).

Determination of polyphenols

The total phenolic contents was detected by FC assay, based on the reduction of a phosphowolframate–phosphomolybdate complex by phenolic compounds (Vinson et al. 1998). 100 µl of the extract samples (or standard) and 50 µl of Folin reagent were mixed with 600 µl of water. After reaction for 1 min, 150 µl of 20% sodium carbonate solution and 190 µl of water were added to complete 1ml volume. The absorbance of reaction was measured at 760 nm after 2 h of incubation at room temperature and darkness. Gallic acid was used as the standard.

Determination of flavonoids

The flavonoids were determined by colorimetric method (Jia et al. 1999). 100 µl of the extracts was mixed with 400 µl of water and 30 µl 5% sodium nitrite solution. After 5 min reaction, 30 µl of 10% aluminium chloride hexahydrate was added to the mixture. Subsequent to 5 min, the addition of 200 µl sodium hydroxide was followed. In 15 min, the absorbance of mixture was measured at 510 nm. Quercetin was used as the standard.

RNA isolation

RNA was isolated from roots and leafs of *Arabidopsis thaliana*. Isolation was carried out using PureLink Plant RNA Reagent (Ambion). After isolation, the RNA samples were purified by DNAasa I RNAasa-free (BioLabs). The quality and quantity of isolated RNA samples were measured using Nanodrop. Their integrity was visually assessed on ethidium bromide-stained agarose gels.

Gene expression analysis

Isolated total RNA was the first converted to cDNA using Transcription First Strand cDNA Synthesis Kit (Roche). For PCR reactions, diluted cDNA products were used as template. Specific primers were selected from NCIB verified from BLAST and STAIR database. After that, they were delivered from Sigma-Aldrich. A complete list of primer sequences is provided in Table 1. qPCR assay was performed using Kapa Sybr Fast qPCR (KapaBiosystems). The qPCR amplification programme was 95 °C for 3 min; 95 °C for 15 s, 60 °C for 45 s, repeating 40 cycles, 95 °C for 15 s, 60 °C for 45 s, melting curve for 20 min; and 95°C for 15s. The total volume from the qRT-PCR was 20 µL. Actin 2 was used as a housekeeping gene for normalization. Relative quantity ($2^{-\Delta\Delta Ct}$ method) was then used to calculate relative gene expression level (Livak and Schmittgen, 2001).

Table 1 Overview of selected primers for qPCR analysis.

Locus	Genes	Forward primer (5'-3')	Reverse primer (5'-3')
AT3G18780	*ACT2*	CTTGCACCAAGCAGCATGAA	CCGATCCAGACACTGTACTTCCTT
AT1G07890	*APX1*	TGCCACAAGGATAGGTCTGG	CCTTCCTTCTCTCCGCTCAA
AT4G23100	*GSH 1*	CCCTGGTGAACTGCCTTCA	CATCAGCACCTCTCATCTCCA
AT5G27380	*GSH 2*	GGACTCGTCGTTGGTGACAA	TCTGGGAATGCAGTTGGTAGC
At2g37040	*PAL*	GCAGTGCTACCGAAAGAAGTG	CGACCTACATTCCTTGATCCTG
At5g13930	*CHS*	CGCATCACCAACAGTGAACAC	TCCTCCGTCAGATGCATGTG

Legend: ACT 2 – Actin 2, APX 1 - Ascorbate peroxidase 1, GSH 1 – γ-Glutamylcysteine synthetase, GSH 2 – Glutathione synthetase, PAL – Phenylalanine ammonia lyase, CHS – Chalcone synthase

Statistical analysis

The data were statistically analysed by one-way ANOVA variance test with subsequent Tukey's comparison test using software R, version 3.4.0 for windows (www.r project.org).

RESULTS AND DISCUSSION

Effect of toxicant on the growth

Growth was suppressed by an increasing concentration of the toxicant. Concentration of 500 µM SeZn-NPs, 500 µM Zn, 50 µM Se and the combination of 500 µM Zn with 50 µM Se were extremely phytotoxic and absolutely inhibited the growth. Concentration of 250 µM SeZn-NPs, 250 µM Zn, 25 µM Se and the combination of 250 µM Zn with 25 µM also highly reduced the growth (Figure 1). Only the lowest concentrations were used for next analysis.

Figure 1 Influence of ZnSe-NPs treatments on the growth of Arabidopsis thaliana

Antioxidant response to toxicant's treatment

NPs as heavy metals are generally considered to be phytotoxicity because of producing reactive oxygen species. The main defence antioxidant mechanism is formed by secondary metabolites such as glutathione (GSH), phytochelatins, ascorbate, phenols, flavonoids, tocopherols, carotenoids and their associated enzymes (Sharma and Dietz, 2009). The evaluating of antioxidant response

was done by gene expression analysis and spectrophotometric analysis of total polyphenols, flavonoids, and total antioxidant activity.

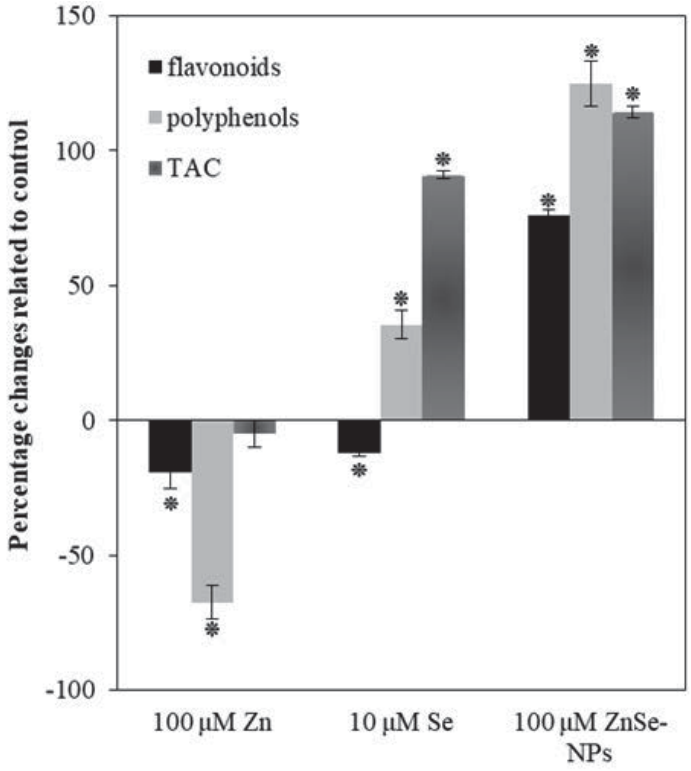
PAL is vital enzymes catalyses reaction of converting L-phenylalanine to ammonia and trans-cinnamic acid that result in thousands of polyphenols. Increased expression of gene coding PAL was manifested in the situation of 100 µM ZnSe-NPs and 10 µM selenium treatments (Figure 2). Simultaneously in these cases, it was established the higher amount of polyphenols compared to control (Figure 3). The total amount of polyphenols in the control samples was 51 mg GAE/g FW. Amount of polyphenols was expressed as the equivalent to milligrams of Gallic acid per 1 g of fresh weight (mmol GAE/1g FW).

Figure 2 Overview of relative expressions of antioxidant related genes

	100 µM ZnSe-NPs	100 µM zinc	10 µM selenium
GSH 1	increased expression ↑↑	increased expression * ↑	increased expression ↑↑ *
GSH 2	increased expression ↑↑	increased expression * ↑	decrease expression
PAL	increased expression ↑↑↑	decrease expression	increased expression ↑
CHS	increased expression ↑↑↑	decrease expression	decrease expression
APX 1	increased expression ↑↑	decrease expression	increased expression ↑

Legend: The relative gene expression level (2−ΔΔCt method for quantitation): ↑ *relative expression 1–3*, ↑ *relative expression 3–5*, ↑ *relative expression 5–>; APX 1 - Ascorbate peroxidase 1, GSH 1 - γ-Glutamylcysteine synthetase, GSH 2 - Glutathione synthetase, PAL - Phenylalanine ammonia lyase, CHS - Chalcone synthase. Stars (*) indicate significant differences compared to the control (p <0.05), (n=3).*

Figure 3 Percentage changes in amount flavonoids, polyphenols and TAC (total antioxidant capacity) related to control. Error bars correspond to standard error of mean. (*) indicate significant differences compared to the control (p <0.05, n=3) according to one-way ANOVA test with subsequent Tukey's comparison test.



Chalcone synthase (CHS) is responsible for synthesis of flavonoids. Except 100 µM zinc treatment, their transcriptomic response to toxicant was higher (Figure 2). This upregulation was followed by increased synthesis of flavonoids (Figure 2). The total amount of polyphenols in the control samples was 4.3 mg RE/g FW. Amount of polyphenols was expressed as the equivalent to milligrams of Gallic acid per 1 g of fresh weight (mmol GAE/1g FW). The exposure to NPs and heavy ions generally induce the biosynthesis of polyphenols and flavonoids involved in the response to abiotic stress (Landa et al. 2012, Moulick et al. 2015, Ma et al. 2013)

Ascorbate peroxidase 1 (APX 1) is part of ascorbate glutathione cycle and belong between the key enzymes to remove H_2O_2 (Panchuk et al. 2005). Higher gene expression for APX 1 was in the samples treated by 100 µM ZnSe-NPs and 10 µM selenium (Figure 1).

Glutathione (GSH) is important molecule that prevents the cell from the oxidative stress. There are two enzymes which are necessary for its biosynthesis (Ma et al. 2013). Except 10 µM selenium in the case of GSH 2, genes were upregulated (Table 1). Total antioxidant activity was higher in every treatment related to control (Figure 2). Total antioxidant activity was expressed as the equivalent to mmol/l of Trolox per 1 g of fresh weight (mmol/l trolox/1g FW). TAC of control samples was 6 mmol/l Trolox/1g FW.

CONCLUSION

Growth was suppressed by an increasing concentration of the toxicant. 250 and 500 µM concentrations were extremely phytotoxic and inhibited the growth. More antioxidant related genes were upregulated than suppressed. Concurrently, there were higher productions of secondary metabolites which are often synthesis during abiotic stress. The strongest antioxidant response and the highest biosynthesis of antioxidant molecules have been found in 100 µM ZnSe-NPs treatment. The weakest antioxidant response was found in 100 µM zinc treatment. The objective of this first experiment was to find out the border of toxicity. The further research will be focus on plant response more deeply and other concentration options will be selected.

ACKNOWLEDGEMENTS

The research was financially supported by the Internal Grand Agency of Mendel University in Brno IP 42/2017 and CEITEC 2020 (LQ1601) with financial support from the Ministry of Education, Youth and Sports of the Czech Republic under the National Sustainability Programme II.

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ROOT PHENOTYPING OF SOYBEAN [*GLYCINE MAX* (L.) *MERRILL*] GENOTYPES BASED ON IMAGE ANALYSIS

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Abstract: Drought most affects the crop growth and production. The first organs of plants that respond to drought in the soil are roots. Classical approaches evaluating root traits in field conditions are still considered standard techniques. In the recent decade, the novel phenotyping methods have been introduced into root research. These methods make use of advances in automation and computer-aided analysis. The experiment focused on the gradual effect of dehydration on the structure of root, where four genotypes of soybean [*Glycine max (L.) Merrill*, SA-027, SA-047, SA-072, SA-217, all originally from China] were used. In growth stage (19 BBCH), gradual dehydration was induced by interruption of substrate watering. The intensity of drought, was quantified the gravimetric method and dehydration took 9 days. After the dehydration was complete, the roots were extracted from soil by washing method and then dried. The root structure was evaluated by automatic RGB imaging analysis using phenotyping PlantScreen™ platform. Using the software Image-J, we evaluated the number of nodules formed on the roots. Our findings lead to the conclusion that gradual dehydration led to a decrease in the absorption area and weight of roots. On the contrary, we have seen a significant increase in the growth of roots in to length as the thickness.

Key Words: soybean, water stress, RGB imaging analysis, root growth and structure

INTRODUCTION

Soybean [*Glycine max* (L.) *Merrill*] is one of the most important legumes. It is a valuable source of protein for human food, is a component of livestock feed, and is also used in industry in the production of biofuels (Hartman et al. 2011). Impact of global climate change on crop production over the last decade emerged as a major research priority (Shanker et al. 2014). Drought is considered a limiting factor of crop productivity (Brestic and Zivcak 2013). Soybean plants are sensitive to drought for the relatively high water requirement (Yang et al. 2015). Roots are the first organs that perceive and respond to drought. The distribution of roots, especially those that can penetrate deeper into the soil, plays an important role in determining the ability of plants to capture important resources such as water, as well as minerals. Based on this, root architecture has a significant impact on growth and yield crops (Fenta et al. 2014). At present, it is difficult to study the growth and architecture of the root system, especially in field conditions. Classical approaches to analysis of characters in field conditions, such as the method of excavation and analysis of soil cores, are time consuming, but still accepted as standard techniques (Hoogenboom et al. 1986, Nielsen et al. 1997, Trachsel et al. 2011). Modern and non-destructive approaches to the evaluation of the root system architecture are at the forefront. These approaches make use of advances in automation and computer-aided analysis, based on the principles of red-green-blue (RGB), computed tomography (CT), magnetic resonance imaging (MRI) or positron-emission tomography (PET) imaging (Lynch 1995, Comas et al. 2013, Postma et al. 2014, Osmont et al. 2007, Makbul et al. 2011). The aim of the work was to evaluate the effect of gradual dehydration on growth and root architecture as well as the number of nodules created using RGB imaging and automatic and semi-automatic image analysis.

MATERIAL AND METHODS

In 2016, four genotypes of soybean [*Glycine max (L.) Merrill*; cvs. SA-029, SA-046, SA-072, SA-217, all originating from China] were grown in a pot vegetation experiment in natural conditions. Inoculated seeds of Nitrazon (Farm Žiro, s.r.o., Nehvizdy, Czech Republic) were sown in 15-liter pots filled with soil substrate (brown soil; pH=7.04; N-P-K contents were 7.4, 30.0 and 150.0 mg per kg, respectively). In the growth stage of 19 BBCH (Munger et al. 1997), was experimentally induced gradual dehydration by interruption of substrate watering.

We have identified the intensity of drought by determining the relative water content (RWC) in adults, a fully developed leaf using the gravimetric method. The duration of gradual dehydration was identical for all genotypes for 9 days.

The roots of the plants were extracted from the pots in the bath filled with water and washed by hand (Trachsel et al. 2011) in terminal phase of vegetation growth (BBCH 49). Soil block with plants was selected from pot and immersed under water. After short time, soil block was subtly comminuted by water flow and hand movement. Root system of plant was cut from shoot in place 20 mm above first lateral roots. Isolated roots were naturally dried. The root system was placed on a blue pad and phenotyping with the PlantScreen™ automatic RGB imaging unit (PSI, Drásov, Czech Republic). The system recorded an RGB projection at a resolution of 2560 × 1920 pixels, and the root system area was automatically analyzed by PlantScreen Data Analyzer (PSI, Drásov, Czech Republic). The length of the root system and the number of nodules formed was evaluated from the obtained RGB image in .png format semiautomatic software ImageJ version 1.46r (http://imagej.nih.gov/ij) after binarization and skeletonization image.

The weight of the roots was weighed by the analytical Scales ALS 220-4N (Kern & Sohn GmbH, Balingen, Germany).

Statistical analysis of the experimental data was performed using Statistics Version 10 software (StatSoft Inc., Tulsa, Oklahoma, USA). ANOVA has been performed between the different treatment (well-watered and water stress) and between genotypes at a significance level of 0.05, and Duncan´s post hoc test was used. The variability between investigated genotypes was tested by the HSD test.

RESULTS AND DISCUSSION

In the experiment, the soybean plants were exposed to water stress (WS). The plant root system is a complex three-dimensional (3D) structure (Lynch 1995). The development of modern methods of root phenotyping allows to identify the dynamics of the formation of the root system structures under various environmental factors (Comas et al. 2013, Postma et al. 2014). Drought affects the growth and root architecture. A typical reaction of plants to the decrease in water content in the soil is the increase in the absorption area of the root system and acceleration of growth of lateral roots (Osmont et al. 2007). These morphological characteristics under conditions of declining soil moisture promote better water and mineral extraction.

The level of water stress was monitored according to the decline of relative water content (RWC) in mature leaves. Drought resulted in significant decline of RWC for genotypes SA-029 and SA-072 (31.5%) (Table 1).

Table 1 Relative water content (RWC; %) in mature leaves of soybean genotypes grown under well watered (WW) and water stressed (WS) conditions. The data are the means ± S.E. (n=4).

Genotype	RWC (%)	
	WW	WS
SA-029	74.0 ± 0.2^{Aa}	31.5 ± 4.6^{Bc}
SA-046	71.5 ± 6.5^{Aa}	43.0 ± 2.4^{Ba}
SA-072	73.4 ± 5.6^{Aa}	31.5 ± 3.1^{Bc}
SA-217	73.8 ± 0.7^{Aa}	37.7 ± 1.7^{Bb}

Legend: S.E. – standard error. Capital letters denotes significant differences at P<0.05 obtained by Duncan´s post-hoc test between WW and WS conditions and the small letters indicate genotypes differences.

In the experiment, we observed significant differences between genotypes in evolution of underground biomass under well-watered conditions. Genotype SA-217 reached the highest level of root surface area and root dry weight. Dehydration resulted in a significant reduction in the absorption area of roots in genotypes SA-029, SA-072 and SA-217. In the genotype SA-046, we observed the nonsignificant reduction in this parameter (Table 2).

Table 2 Root projected area (cm2) and root dry weight (g) in soybean genotypes grown under well watered (WW) and water stressed (WS) conditions. The data are the means ± S.E. (n=5).

Genotype	Root projected area (cm2)		Root dry weight (g)	
	WW	WS	WW	WS
SA-029	75.3 ± 14.0^{Ab}	57.5 ± 11.3^{Ba}	2.7 ± 0.5^{Ab}	2.3 ± 0.6^{Aa}
SA-046	29.8 ± 11.1^{Ac}	26.5 ± 16.4^{Ac}	1.2 ± 0.4^{Ac}	1.4 ± 0.6^{Ab}
SA-072	54.5 ± 17.8^{Ad}	35.6 ± 23.5^{Bbc}	2.2 ± 0.6^{Ab}	1.5 ± 0.9^{Ab}
SA-217	117.9 ± 0.6^{Aa}	62.5 ± 23.1^{Ba}	3.9 ± 0.0^{Aa}	2.7 ± 0.9^{Ba}

Legend: S.E. – standard error. Capital letters denotes significant differences at P<0.05 obtained by Duncan´s post-hoc test between WW and WS conditions and the small letters indicate genotypes differences.

Genotypes responses in root length on water stress shown in Table 3. In well-watered conditions, genotype SA-217 compared to other genotypes achieved significantly the largest length of root. Water stress has led to increased growth of roots, especially in genotype SA-029. In contrast, the SA-217 genotype showed higher sensitivity to drought and root growth was reduced.

Table 3 Root length (mm) in soybean genotypes under well-watered (WW) and water stressed (WS) conditions. The data are the means ± S.E. (n=5).

Genotype	Root length (mm)	
	WW	WS
SA-029	376.8 ± 27.8^{Bb}	554.4 ± 57.3^{Aa}
SA-046	377.3 ± 54.7^{Ab}	433.5 ± 61.4^{Ac}
SA-072	362.1 ± 23.0^{Bb}	484.8 ± 42.5^{Ab}
SA-217	489.5 ± 87.8^{Aa}	455.9 ± 28.2^{Abc}

Legend: S.E. – standard error. Capital letters denotes significant differences at P<0.05 obtained by Duncan´s post-hoc test between WW and WS conditions and the small letters indicate genotypes differences.

Table 4 Comparison of root nodules number (RNN) countered manually and RNN measured automatically from image analysis in soybean genotypes under well-watered (WW) and water stressed (WS) conditions. The data are the means ± S.E. (n=5).

Genotype	RNN manually countered		RNN automatically measured	
	WW	WS	WW	WS
SA-029	29.6 ± 3.9^{Ab}	20.4 ± 2.1^{Ba}	20.0 ± 5.6^{Ab}	13.0 ± 2.2^{Bb}
SA-046	10.0 ± 2.7^{Ac}	11.6 ± 3.0^{Ab}	5.4 ± 1.8^{Ac}	6.8 ± 2.4^{Ac}
SA-072	39.0 ± 7.6^{Aa}	24.6 ± 7.2^{Ba}	32.8 ± 6.7^{Aa}	18.2 ± 6.1^{Ba}
SA-217	0.0 ± 0.0^{nd}	0.0 ± 0.0^{nd}	3.5 ± 2.1^{Ac}	1.8 ± 2.2^{Ac}

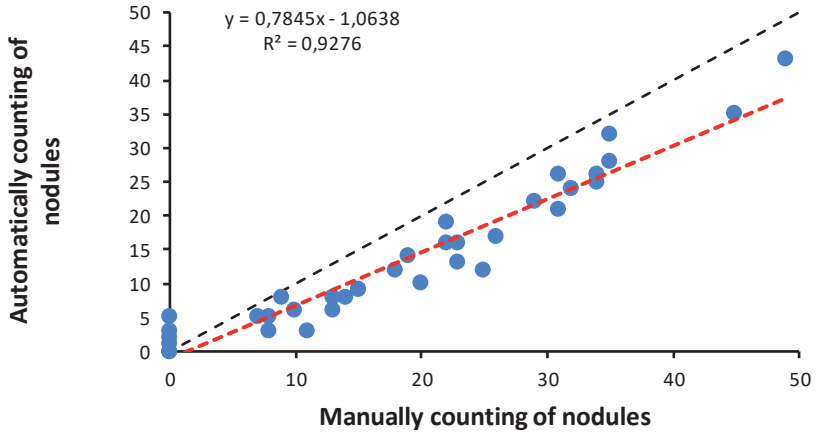
Legend: S.E. – standard error. Capital letters denotes significant differences at P<0.05 obtained by Duncan´s post-hoc test between WW and WS conditions and the small letters indicate genotypes differences.

Dehydration accelerated, except SA-217, growth of roots into length without (Table 3) breaking their geometry and root area (Table 2). Root dry mass per root area (RMA) significantly increased in all genotypes under drought, from mean level 373.9 to 445.4 g per m2. This finding leads to the conclusion that root growth was preferentially oriented on the length of root and root area and not into thickening. Makbul et al. (2011) in its work observed similar changes in the dynamics growth of roots of soybean in conditions of drought.

Creating a 2D image of the root system make also utilization for automatic or semi-automatic evaluation of generated nodules. By using the Image-J program, the system after image

skeletonization counted the quasi-circular objects on the roots (Barbedo et al. 2012), corresponding to the nodules. We have found a close correlation between the automatically and manually calculated number of nodules with rp = 0.95 (Figure 1). Similarly high correlation coefficients of this relationship have been found in the works (Barbedo et al. 2012, Lira and Smith 2000). However, this approach analysis a 2D image from one perspective. We found that nodules that are created on the reverse side of the root of the 2D image, the system is not able to detect and so leads to underestimation of the number of nodules evaluated automatically. Another potential flaw may be the optimum setting of the trace and image skeletonization, which can also count objects that are not real nodules. As noted by genotype SA-217 (Table 4).

Figure 1 The relationship between automatic and manually calculated number of nodules.



Legend: The point represented an individual root analysis. Equation: y = 0.6739x + 2.0193; R^2 = 0.91; P<0.0001, the black dot line represents a 1 : 1 ratio.

CONCLUSION

Modern methods of root phenotyping based on RGB image are useful tool for characterizing root growth under stress conditions.

ACKNOWLEDGEMENTS

This work is supported by APVV-15-0721, VEGA-1-0923-16 and VEGA1-0831-17.

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THE EFFECTS OF RED, BLUE AND WHITE LIGHT ON THE GROWTH AND DEVELOPMENT OF *CANNABIS SATIVA* L.

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Abstract: The aim of this study was to investigate the effect of red (600–700 nm, peak 660), blue (400–500 nm, peak 450) and white light on the morphological and photosynthetic qualities of *Cannabis sativa* L. The two treatments were the white light (WL), and a combination of blue red lights (BR). Plants grown under WL were 23% taller than those grown under the BR light emitting diodes. The leaf area was also greater under WL than BR by 20%. The number of lateral branches and length of dominant lateral branch weren´t significantly different. It was concluded WL that emit a full spectrum of light affects plant growth and development better than BR light. The quantum efficiency ranged from 0.81 to 0.845 indicating the plants were not in stress.

Key Words: Cannabis sativa L., morphology, light quality, light emitting diode.

INTRODUCTION

Many species of medicinal and aromatic plants are cultivated for industrial uses (Lubbe and Verpoorte 2011). Herbs are used in pharmaceutical and cosmetic industry for extracting active ingredients (Roxana-Gabriela 2016). Processing of plant-derived pharmaceuticals must take place under tightly controlled conditions, using production standards (Hefferon 2010). Since 2003 medicinal grade cannabis is provided in the Netherlands on prescription through pharmacies. Domestic production of cannabis has been increasing in most European countries and export flows are dynamic and changing. Denmark appears to be a center of cannabis production, and the Czech Republic and Slovakia have become important cannabis producers and exporters to neighboring countries (Hazekamp 2006). The European market for cannabis is extremely large, and supplying cannabis, whether it is at the importation, production or distribution level, requires organization and logistics, human and other resources, and the need to generate and distribute income and profits (EMCDDA 2012). To increase the production capacity, controlled growing systems using artificial lighting have been taken into consideration (Darko et al. 2014).

A closed system for plant production with artificial light is an innovative method of plant cultivation (Schroeter-Zakrzewska et al. 2017). The majority of plants are grown in sealed rooms; these being fitted with bright lights specifically designed to emit wavelengths that maximize plant growth (EMCDDA 2012). Study conducted by Potter and Duncombe (2012), has shown that, when light intensity is increased, the Δ^9-tetrahydrocannabinol (THC) content of the cannabis is boosted because plants in brighter conditions produce proportionally more female flowers, which contain a greater concentration of THC. As an artificial light source, light-emitting diodes (LEDs) can be used to make the plants grow more quickly in closed-type plant production systems, especially in the environment of the light intensity is insufficient (Xu et al. 2016). LED lights do not consume much power, do not require ballasts and produce a fraction of the heat of High intensity discharge (HID) lamps (Thomas 2012). Their small size, durability, long operating lifetime, wavelength specificity, relatively cool emitting surfaces, and linear photon output with electrical input current make these solid-state light sources ideal for use in plant lighting designs (Massa et al. 2008).

Because light is such an important environmental parameter, plants have evolved numerous biochemical and developmental responses to light that help to optimize photosynthesis and growth

(Müller et al. 2001). Light regulates crop growth, plant development (including flowering), as well as how quickly plants use water. Managing light is obviously critical to the production of crops grown in controlled environments. When considering the different dimensions of light, we usually focus on photoperiod (day length), light quantity (intensity) and light quality (the spectral distribution) (Runkle 2017). The spectral quality of lights is the relative intensity and quantity of different wavelengths emitted by a light source and perceived by photoreceptors such as phytochromes, cryptochromes, and phototropins, and plants generate a wide range of specific physiological responses through these receptors. A specific light quality can be used to improve the nutritional quality of vegetables and yields in commercial production (Kuang-Hung et al. 2012, Takemiya et al. 2005).

The objective of this study was to examine growth and development of *Cannabis sativa* L. plants grown in controlled conditions under different light wavelengths. In this study, we used pure white LEDs (Light emitting diodes) compared to red and blue LEDs (R:B = 1:1) as a light source.

MATERIALS AND METHODS

Plant material

Plants of six female drug type varieties of *C. Sativa* (High Potency breeds acquired from CBD Botanic, Spain) were grown in a controlled indoor growing facility at the Mendel University in Brno, Czech Republic. The growing conditions were maintained at temperature of 25 ± 3 °C and relative humidity (RH) of 50 ± 5%. Indoor light was measured at ~200 ± 30 μmol m^{-2}/s (Quantum sensor SQ-500 series, USA) at plant canopy level for a photoperiod of 18 hours of day. Six cuttings were made from each plant for the study. The cuttings were dipped in an auxin solution to promote rooting and directly planted in rock wool cubes of 36x36x40 mm. The cuttings were kept in dark for 24 hours and then transferred to the Climacell (BMT Technologies, Germany) at 24 °C and 80% RH and allowed to be rooted. After proper rooting out of the 36 cuttings, 24 healthy and randomly selected female clones were used for the experiment.

Experimental set-up

The experiments were carried out in the Climacell Evo. The clones were divided into 12 clones each group and placed in two different LED light treatment groups namely Red-blue (R:B) and White light (Control). The two LED light treatments used were (1) 100% Blue and RED light and (2) 50% White. The temperature and RH in the Climacell were maintained at 24 °C and 60% (Day) and 18 °C and 70% (night) respectively. During the vegetative cycle 18h photoperiod was maintained for 2 weeks and during the flowering cycle 12h photoperiod was maintained for another 7 weeks. The wavelengths of blue and red LEDs used were in the range of 420–490 nm and 630–680 nm.

Plant growth measurements

Main measured quantities in this study were plant height, number of lateral branches, leaf area, and quantum efficiency. The leaf area was measured by the method mentioned by Pandey and Singh (2011). Using Adobe Photoshop CS 5 and Canon EOS 1100D. For quantum yield the plants were kept in dark to adapt for 15 mins. Next, randomly chosen leaves from six plants from each treatment were measured using FlourPen FP 100 (Photon Systems Instruments, Czech Republic). Statistical significance was determined by one-way ANOVA and tukeys HSD test to the treatments with significant difference. Twelve plants were used per treatment.

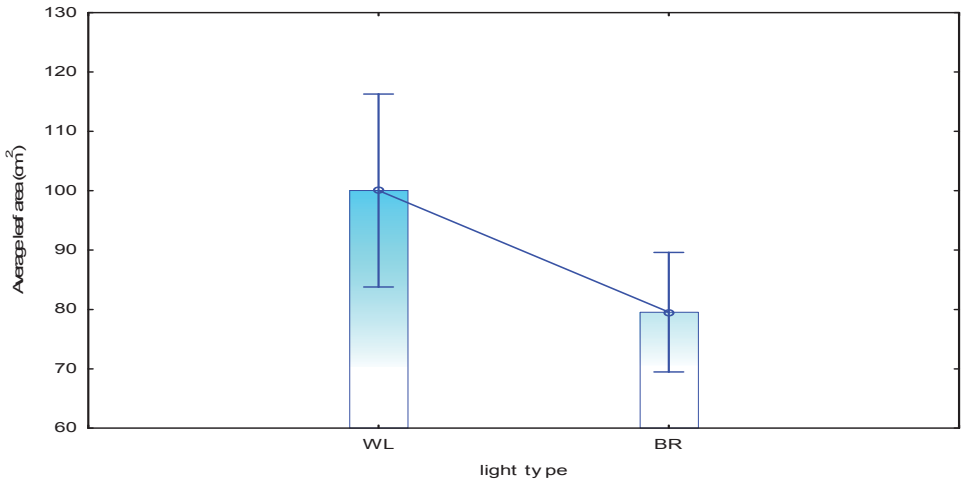
RESULTS AND DISCUSSIONS

Leaf Area

Plants grown under BR light had an average leaf area of 79.54 cm^2 which is smaller compared to plants grown in white light, where average leaf area was 100.03 cm^2 (Figure 1) Many studies show different light quality compared to white light have inhibiting effects on plants and their growth as in the case with pepper, lettuce and tobacco (Brown et al. 1995, Kim 2004, Yang et al., 2016). As Choi et al. (2016) examined effects of LED with different wavelengths on the length of petioles and width of leaflets on strawberry plants they found out that combined illumination with red and blue LED light was the most effective in increasing the length of petioles as well as the width of leaflets.

In comparison with pure red LED and pure blue LED. Arena et al. (2016) found out in an experiment with (*Solanum lycopersicum* L.) and (*Platanus orientalis* L.), growth under Red-Green-Blue (RGB) and BR reduced leaf area compared to growth under WL. Plants that were grown under BR LED lights had smaller leaf area by 20% than those grown under pure WL LED.

Figure 1 Average leaf area of WL and BR treatments.

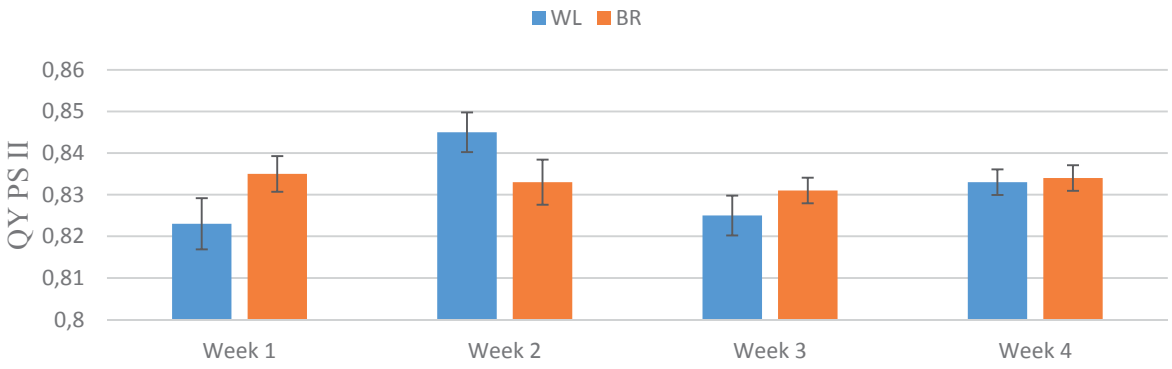


Legend: WL = white light, BR = Blue-Red. Data are mean values ± SE.

Quantum Yield of Photosystem II

The photosynthetic efficiency for both the treatments appeared to be ranging from 0.81 to 0.845. For non-stressed plants, the photosynthetic efficiency fluctuates from 0.75 to 0.85 (Bolhar–Nordenkampf et al. 1989). Which indicated that the plants were in a healthy state (Figure 2).

Figure 2 Quantum yield of photosystem II

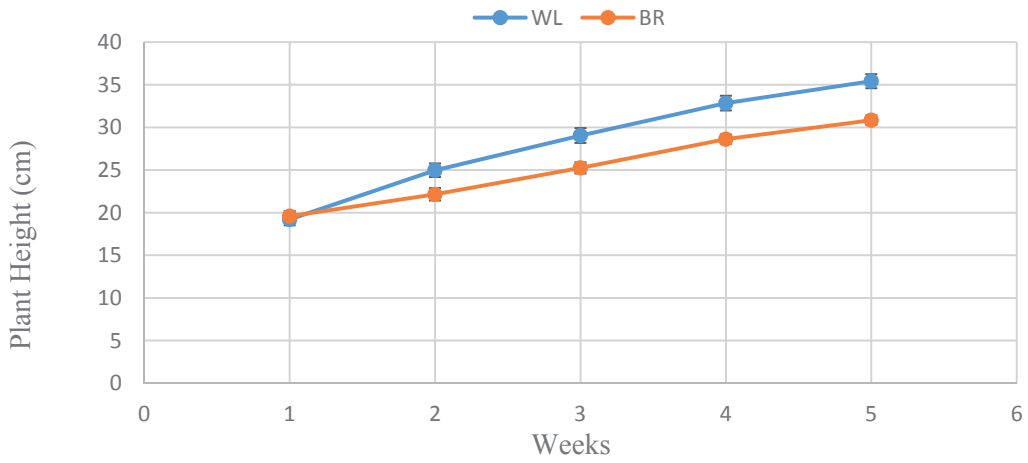


Legends: WL = White light, BR = Blue-Red. Data are mean values ± SE.

Plant Height

There was no significant difference between the two light treated variants until the fourth week of measurement. Since the fifth week, the plants growing in WL treatment started showing a considerable difference in height. The difference in the heights of plants placed under BR light was visible from the first week (Figure 3). Glowacka (2004) found tomato cultivars placed under blue light showed shorter height compared to those kept in day light. In roses and poinsettia blue light was known to reduce stem elongation (Islam et al. 2012, Terfa et al. 2013). In petunia blue light promotes stem elongation on the contrary red light suppresses plant height (Fukuda et al. 2011).

Figure 3 Plant height growth in subsequent experimental weeks.



Legends: WL = White light, BR = Blue-Red. Data are mean values ± SE.

Number of lateral branches

There were fluctuating results for lateral branches but they were not statistically significant (data not shown). Blue light is shown to stimulate bud out growth in *Triticum aestivum* (Barnes and Bugbee, 1992) and *Rosa* (Girault et al. 2008) whereas reduced it in *Solanum tubersum* (Wilson et al. 1993).
In *Lilium* (Vandenbussche et al. 2005) and *Rosa* red light inhibited lateral branching.

CONCLUSIONS

The plants grown under WL had and average leaf area of 100.03 cm^2 compared to plants grown in BR light with an average leaf area of 79.54 cm^2. The WL treatment showed an increased plant height compare to BR treatment. The Quantum yield of photosystem II indicated nonstressed plants. The number of lateral branches were not affected as they did not show any significant difference.

ACKNOWLEDGEMENTS

The research was financially supported by the IGA FA MENDELU No. IP 2017/076.

We would like to express our sincere thanks to CBD Botanic (Spain) for providing us with the plant material and support.

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EFFECTS OF WASTEWATER ON SEED GERMINATION AND PHYTOTOXICITY OF HEMP CULTIVARS (*CANNABIS SATIVA* L.)

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Abstract: To investigate the effects of wastewater on seed germination and phytotoxicity a laboratory experiment was designed on hemp cultivars (*Cannabis sativa* L.). Seeds were germinated in 100% distilled water, 100% wastewater, 50% wastewater. We analyzed the percentage of seed germination and heavy metal accumulation in the germinated seeds. The concentrations used for treatment did not negatively affect seed germination except for 100% wastewater treatment which resulted in seedling mortality. The heavy metal accumulation was greater in some varieties germinated in distilled water compared to 50% wastewater treatment.

Key words: germination, phytoremediation, *Cannabis sativa* L., heavy metals, wastewater.

INTRODUCTION

Mankind rely heavily on natural resources for its survival. Water one of the most important natural resource has been subjected to rigorous exploitation and pollution due to anthropogenic activities. This pollution ranges from emissions, effluent wastes, Insecticides/pesticides, industrial and municipal waste in agriculture (McGrath et al. 2001, Schalscha and Ahumada 1998). Each source of pollution has damaging effects to the ecosystem including human health. Heavy metal contamination of water and land pose a serious threat to plant, animal and humans alike.

As, Cu, Cd, Pb, Cr, Ni, Hg and Zn have been identified in polluted environments. Excessive uptake of these elements by plants may cause toxicity in human nutrition with fatal consequences (Lone et al. 2008). Given the risk of pollution, wastewater from different sources also contain considerable amounts of organic and plant nutrients (N, P, K, Ca, S, Cu, Mn and Zn) and has been reported to increase crop yield (Pathak et al. 1999, Lubello et al. 2004, Nath et al. 2009). With the increasing stress on limited water resources use of wastewater for irrigation purpose has been considered extensively.

There are numerous advances been made to address the problem of heavy metal contamination. Physicochemical methods though effective are costly and have side effects. Use of special types of plants to decontaminate soil or water of heavy metals is called phytoremediation and the plants used for such purposes are called hyper accumulators (Linger et al. 2005).

Hemp (*Cannabis sativa L.*) is suited for various climates in the world and can phytoremediate environments polluted by heavy metals. Contaminated hemp fibers can be used as insulation and as a composite material. The most critical aspect of plant survival and reproduction is seed

germination. The present investigation was conducted to evaluate the impact of different concentrations of wastewater on seed germination of chosen varieties of hemp.

MATERIAL AND METHODS

Domestic wastewater sample

The wastewater was obtained from Kuchyňky landfill (49.2490778N, 17.3121181E). Sample of wastewater was collected from landfill leachate in pre-sterile bottles and brought to the laboratory directly after sampling. The samples were preserved at a temperature of 4 °C ± 1 until analysis. For the present study wastewater was diluted to with distilled water to give a concentration of 50-50%. The wastewater was analyzed for the content of heavy metals (Cd, Cr, Ni, Pb, Zn and Hg) in the Analytical laboratory at the Department of Chemistry and Biochemistry, Faculty of AgriSciences, Mendel University in Brno and is presented in Table 1.

Table1 Heavy metal content of wastewater from Kuchyňky.

Cd		Pb		As		Cr		Ni		Zn		Hg	
c	RSD	c	RSD	c	RSD	c	RSD	c	RSD	c	RSD	c	RSD
0.518	33.1	15.262	5.4	4.50	28.2	108.19	20.6	155.0	5.7	0.4723	0.7	0.00393	0.005

Legend: c – concentration (µg/L), RSD – relative standard deviation in percentage.

Experimental setup

The hemp cultivation experiments were carried out in the laboratory conditions using hemp cultivars registered in the variety catalogue of the European Union (Table 2). The seeds were sterilized with 0.1% w/v aqueous solution of mercuric chloride for 5 minutes to remove the microbes followed by repeated washing in sterile de-ionized water. Wastewater sample was diluted with distilled water to give a final concentration of 50%. Distilled water with the following chemical ingredients (mg/L): (Ca (NO3)2 0.8, KH2PO4 0.2, KNO3 0.2, MgSO4.7H2O 0.2, KCl 0.2, FeSO4 0.01, pH = 5.2).

Table 2 Different varieties of seeds used.

No.	Varieties	Origin	Sex	Year of Registration.
1	Bialobrzeskie	Poland	monoecious	1968
2	Carmagnola	Italy	dioecious	1960
3	Epsilon 68	France	monoecious	1996
4	Ferimon	France	monoecious	1981
5	Futura 75	France	monoecious	1998
6	Kompolti	Hungary	dioecious	1955
7	Santhica 27	France	monoecious	2003
8	Tygra	Poland	monoecious	2009
9	USO 31	Holland	monoecious	1997
10	Wojko	Poland	monoecious	2012

A laboratory experiment was designed with 15 healthy treated seeds per Petri dish. The seeds were germinated in sterilized Petri dish with a filter paper at the bottom. Each Petri dish was irrigated with 2 ml of solution 100% wastewater, 50% wastewater and 100% distilled water respectively and then incubated at 22 ± 2 °C in complete darkness. The experiments were repeated twice and germination in each experimental set was recorded every 48hrs and total germination was calculated and expressed in percentage.

Chemical analysis of germinated seeds

X-ray fluorescence (XRF) was used to measure the heavy metal content on the instrument XRF Delta Professional (Olympus). The dried material (roots and hypocotyls) was crushed in a ceramic bowl. 1 gram of sample was pelleted on the apparatus on an industrial press (pressure -10 tons). The material thus was used to measure the composition by the XRF method.

RESULTS AND DISCUSSION

Germination assessment of different hemp varieties revealed unexpected results (Table 3). The germination percentage of seeds in 100% wastewater was significantly high in all variants. However, in these variants the roots turned brown immediately after germination resulting in mortality because heavy metal accumulation is known to be toxic for plant species affecting amylase, protease and ribonuclease enzyme activity thus retarding seed germination and growth (Ahmad and Ashraf 2011) (see Figure 1). Also seed germination is the most resistant to heavy metals due to the presence of seed coat which serves as a barrier between embryo and the environment (Seregin and Kozhevnikova 2005, Araùjo and Monteiro, 2005).

Table 3 Percentage germination of hemp seeds.

	Variants	Bialobrze-skie	Carmagn-ola	Epsilon - 68	Ferimon	Futura 75	Kompolti	Santhica 27	Tygra	USO 31	Wojko
Germinated seeds Day 2 (%)	a	93.3	66.6	50.0	73.3	63.3	53.3	66.6	76.6	66.6	16.6
	b	63.3	76.6	10.0	50.0	23.0	30.0	33.3	26.0	33.3	3.3
	c	50.0	16.6	23.3	26.6	23.0	13.0	43.3	16.0	36.6	6.6
Germinated seeds Day 4 (%)	a	93.3	76.6	66.6	76.6	70.0	66.6	73.3	83.3	70.0	16.6
	b	76.6	16.6	26.6	63.3	43.0	56.0	40.0	50.0	73.3	6.6
	c	73.3	30.0	30.0	36.6	50.0	36.0	53.3	36.0	50.0	10.0

Legend: a = 100% wastewater, b= 50% wastewater, c = distilled water.

All variants except for Epsilon 68 and Santica 27 showed high rate of germination in 50% wastewater treatment compared to distilled water on the second day. The ability of seeds to germinate in high concentration of salts differs with variety as well as species (Unger 1987). Bialobrzeskie, Ferimon, Kompolti, Tygra, USO 31 all showed significantly higher rate of germination in wastewater compared to distilled water. Ottobang et al. (1997) had suggested that sewage sludge is a common manure and can be used for crop and other plant growth due to the presence of important organic matter. Also the content of organic nutrients (N, P, K, etc.) in wastewater is reported to increase crop yield and can also enhance the germination rates (Pathak et al. 1999, Lubello et al. 2004).

The chemical analysis of the germinated seeds is shown below (Table 4). As, Cr, Cd, and Pb were below the limit of detection. Seeds with 100% wastewater treatment were not examined for heavy metal content since they did not survive past the germination stage. A binding methodology for the preparation of plant material for laboratory measurements using the XRF method is not established (Kalnický 2001), but the most widely used method is the method adopted by Richardson (1995). According to this method, the plant material must first be rinsed to remove the surface dust and soil particles that adhere to it.

Table 4 Heavy metal uptake by hemp seeds expressed in percentage.

Variety	Ni (%)		Zn (%)		Hg (%)	
	a	b	a	b	a	b
Bialobrzeskie	0.110 ± 0.013	0.052 ± 0.010	0.145 ± 0.009	0.125 ± 0.008	0.233 ± 0.011	0.155 ± 0.008
Carmagnola	0.061 ± 0.008	0.051 ± 0.008	0.100 ± 0.006	0.101 ± 0.006	0.244 ± 0.009	0.235 ± 0.009
Epsilon 68	0.071 ± 0.011	0.052 ± 0.008	0.084 ± 0.007	0.083 ± 0.005	0.175 ± 0.009	0.130 ± 0.006
Ferimon	0.068 ± 0.009	0.086 ± 0.013	0.116 ± 0.007	0.096 ± 0.008	0.258 ± 0.010	0.157 ± 0.010
Futura	0.047 ± 0.008	0.069 ± 0.008	0.092 ± 0.006	0.106 ± 0.006	0.199 ± 0.008	0.213 ± 0.008
Kompolti	0.041 ± 0.007	0.060 ± 0.009	0.078 ± 0.005	0.072 ± 0.005	0.160 ± 0.007	0.122 ± 0.007
Santhica 27	0.051 ± 0.008	0.060 ± 0.008	0.121 ± 0.007	0.111 ± 0.006	0.251 ± 0.010	0.256 ± 0.009
Tygra	0.046 ± 0.008	0.058 ± 0.009	0.089 ± 0.006	0.116 ± 0.007	0.184 ± 0.008	0.100 ± 0.006
USO 31	0.052 ± 0.008	0.051 ± 0.008	0.102 ± 0.006	0.099 ± 0.006	0.356 ± 0.011	0.271 ± 0.010
Wojko	0.042 ± 0.009	0.062 ± 0.008	0.116 ± 0.007	0.096 ± 0.006	0.298 ± 0.011	0.221 ± 0.008

Legend: a = distilled water, b = 50% wasterwate

Figure 1 Germination in different wastewater treatments for hemp variety Bialobrzeskie.

Legend: A= 100% wastewater, B= 50% wastewater, C= distilled water.

Interestingly the heavy metal content of seeds treated with distilled water was reported more than those treated with 50% wastewater in some varieties of seeds. Nickle (Ni) is known to inhibit seed germination by affecting the mobilization of carbohydrates and proteins in germinating seeds (Ahmad and Ashraf 2011, Ashraf et al. 2011). Bialobrzeskie recorded almost twice the concentration of Ni in seeds germinated in distilled water compared to 50% wastewater even though the percentage of germination in this variety was almost same.

Mercury (Hg) was known to increase the seed germination *Triticum aestivum* L. varieties (Shagufta et al. 2006). But there was no significant effect of Hg on different varieties. Li et al. (2005) noted that seedling growth for *Arabidopsis thaliana* is more sensitive to heavy metals in comparison to seed germination. The physiology and biochemistry of the toxic effects of zinc in plants are likely to be similar to those reported for other heavy metals. However, zinc is not considered to be highly phytotoxic (Manivasagaperumal et al. 2011).

CONCLUSION

The seeds germinated in 50% wastewater showed higher percentage of germination is certain varieties compared to distilled water. This could be in part due to the readily available nutrients from various sources already present in the wastewater. Hence the treatment used did not negatively affect seed germination. 100% wastewater showed promising result for germination but did not survive past the germination stage. Heavy metal analysis of germinated seeds showed moderate accumulation of heavy metals.

ACKNOWLEDGEMENTS

The research was financially supported by the IGA FA MENDELU No. TP 5/2017.

We would also like to express our appreciation to the management of the landfill DEPOZ, Ltd. Namely, we are very grateful to Ing. Ivan Mohler and his colleagues for their assistance and their willingness to provide their time so generously.

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STUDY OF INDUSTRIAL HEMP PHYTOTOXICITY IN AN EXPERIMENTAL HYDROPONIC CULTURE

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Abstract: Heavy metal contamination of soil is a persistent environmental problem. One of the solution is soil phytoremediation – the ability of several plant species to clean the soil from contaminants. Hemp (*Cannabis sativa* L.) for industrial use is a good candidate for this purpose due to multiple-use and tolerance to heavy metals. This experiment is focused on examining the phytotoxic effect of landfill leachate on growth and development of two cultivars of industrial hemp in the hydroponic culture. Length of roots and shoots, number of nodes, photosynthetic efficiency and overall viability of the plants were measured. Significant differences were found between the experimental groups. Results suggest that *Cannabis sativa* L. is indeed a heavy metal-tolerant plant species. Variety-related sensitivity to heavy metals is to be considered.

Key Words: hemp, *Cannabis sativa* L., hydroponic culture, landfill leachate, heavy metals, phytotoxicity

INTRODUCTION

Pollution of agriculturally used areas, especially by heavy metals is a persisting issue and still one of the major challenges for food production, environmental institutions and public health (Linger et al. 2002, Jarup 2003). In recent years, phytoremediation has become one of a worldwide accepted solutions for cleaning up the soil in contaminated sites, thanks to the potential of several plant species to bioaccumulate contaminants (Prasad 2003).

Hemp (*Cannabis sativa* L.) is a multiple-use plant providing raw material for the production of natural fiber, insulating board, rope, oil, varnish and paper. It can be widely employed in many types of non-food industries, as it is suitable for growing in polluted regions thanks to high tolerance of contamination. Being a tall plant, growing fast and easily in dense stands and producing a high above ground biomass makes it also a good candidate for accumulation of heavy metals, phytostabilization and soil phytoremediation. Unlike another plants used for phytoremediation, it provides additional end uses (Angelova et al. 2003, Citterio et al. 2003, Girdhar et al. 2014).

Hydroponic culture can be used extensively to determine the phytotoxic effects of trace metals, while plants should be grown in a dilute solution which mimics the soil solution to gather some reliable data on the relationship between growth depression and the concentration of the toxic metal in solution (Kopittke et al. 2010).

This paper investigates the effect of landfill wastewater (leachate) on growth and development of two cultivars of industrial hemp in an experimental hydroponic culture. Plant viability, shoot length, number of stem nodes, photosynthetic efficiency and length of the roots were evaluated.

MATERIALS AND METHODS

Setup of the experiment

Two hemp cultivars (*Cannabis sativa* L.) were chosen for this experiment – Bialobrzeskie, a Polish variety registered in 1968 and Monoica, a Hungarian variety registered in 2006 (Bjelková 2011). Seeds provided by institute Agritec Plant Research, Ltd., Šumperk, (Czech Republic) were sown into plastic trays with perlite (AGRO CS Inc., Czech Republic) and left to germinate with regular watering in the greenhouse. The photoperiod was set at 16 hours of light per day. After several days, about 2–3 cm big seedlings were transferred to an experimental hydroponic culture. The plantlets were put into small, conically shaped plastic tubes with gaps for roots on the bottom. The roots were put through the gaps to be submerged into a solution. Tubes with plantlets were set within a tray on top of several opaque plastic boxes with a volume of 2 litres. Each box contained approximately eight plants. There were totally four experimental groups in thirty-two hydroponic boxes, while every group contained eight boxes – Monoica and Bialobrzeskie cultivars irrigated with standard nutrient solution in first two groups as a control, then the same two cultivars irrigated with leachate admixture in the second two contaminated groups. Plants grew in a conditioned greenhouse during a prolonged photoperiod with 18 hours of light per day and with the temperature regime set not to fall below 25 °C. Total duration of the experiment was 7 weeks.

Nutrient solution composition and measured parameters

Standard water soluble fertlizer Peters Professional M-77 (Everris International B.V.) in a solution of 0.4 g/l was used to provide nutrition for hemp plants in control groups. For the contaminated groups, 15% of landfill leachate containing heavy metals from the location Zdounky-Kuchyňky (49.2490778N, 17.3121181E) was mixed with the original solution. In both groups, the solution was replaced with a fresh one every two weeks.

On the first day of every week, the stem length and number of nodes in plants were measured with a common millimetre scale. Overall root length was measured every two weeks as well as photosynthetic efficiency – quantum yield of photosystem II was measured by FlourPen FP 100 (Photon Systems Instruments Ltd.).

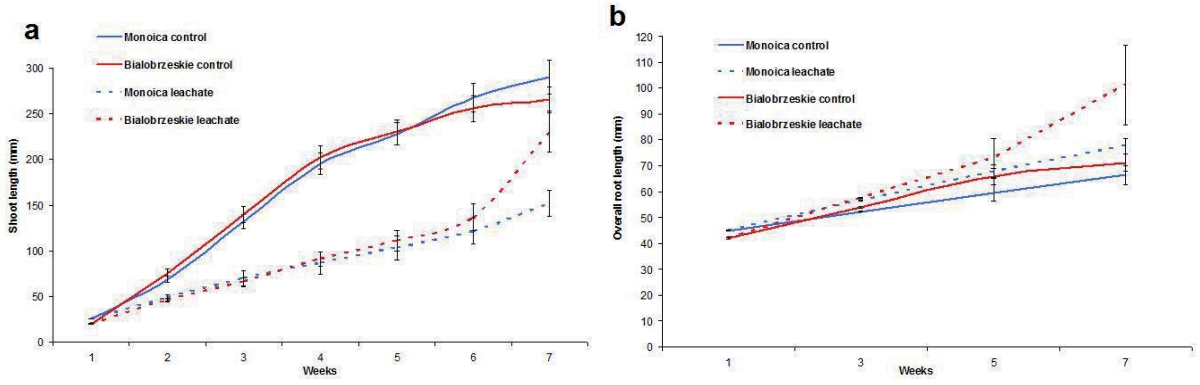
Data of all the parameters were evaluated in Microsoft Excel software, statistical differences were tested by one-way ANOVA „F“ test at the level of significance $\alpha = 0.05$.

Results and outputs of this research were processed in the facilities and by instrumentation financed by project OP VaVpI CZ 1.05/4.1.00/04.0135.

RESULTS AND DISCUSSION

According to expectations, the plant growth in groups treated with wastewater was severely inhibited, at least during the first five weeks of the experiment. Lesser average root and shoot length in both hemp cultivars can be observed (Figure 1). In some previous bioassays in soil, heavy metals were reported to adversely affect the plants growth, both for roots and shoots (Amin et al. 2013).

Figure 1 Growth curves of morphological parameters for both cultivars of hemp



Legend: a) average stem length for every week, b) overall length of the roots

Interestingly, Bialobrzeskie cultivar started to exhibit different behaviour in terms of growth response to stress in the sixth week of the experiment. It seems that the shoot growth was not anymore inhibited by heavy metals contained in the leachate. During the seventh week, average stem length in this group got close to the levels of healthy, non-contaminated Bialobrzeskie group and was even significantly higher compared to Monoica cultivar in the leachate ($P = 0.0407$). During the fifth week of measurement, average shoot length was significantly lower in the groups with leachate for Monoica ($P = 9.82 \times 10^{-11}$) as well as for Bialobrzeskie ($P = 3.76 \times 10^{-10}$).

Regarding the length of roots, no statistically significant differences were found between the experimental groups. Similar behaviour of hemp roots subjected to cadmium, nickel and chromium was observed in a study by Citterio et al. (2003), although the experiment took place in a soil, not in a solution culture and heavy metal concentrations were few orders higher.

Of course, it is important to note that the data for contaminated groups do not represent all the plants from the original setup, because in both cultivars there was a high mortality rate, most likely due to other, potentially toxic chemicals contained in the leachate. Aside from heavy metals, considerable amounts of ammonia, chlorides and phenolic compounds are commonly reported in leachates from landfill sites and municipal waste (Osada et al. 2011, Silva et al. 2015). Ubiquitous contaminants, for example bisphenol A is known to interfere with nitrogen nutrition in the roots of legumes (Sun et al. 2013). For the control groups with nutrient solution, none or negligible mortality was observed, more likely to be attributed to the competition for nutrients. Again, the results are more interesting in contaminated groups, where in case of Bialobrzeskie variety 56% of the plants from the original setup survived in the end of the experiment, while there were only 39% surviving within Monoica variety (Table 1).

Table 1 Comparison of viability between hemp cultivars in leachate

Weeks	Cultivar			
	Bialobrzeskie		Monoica	
	Healthy plants (%)	Plants withered (%)	Healthy plants (%)	Plants withered (%)
1	100	0	100	0
2	94	6	98	2
3	74	26	83	17
4	71	29	61	39
5	69	31	52	48
6	59	41	41	59
7	56	44	39	61

This together with the data for shoot length (Figure 1) can be considered another parameter suggesting the variety-given sensitivity to heavy metal contamination. In ecotoxicological bioassays including the effect of heavy metals on root length, differential sensitivity scale often occurs among not just species but cultivars (Crini et al. 2017).

High percentage of dead plants in this experiment may be related to the high concentration of heavy metals in the chosen solution and conditions, or other unknown compound. However, also the oxygen availability, salinity and concentration of ions in the mixture of fertilizer solution with 15% of landfill leachate, which was used for the contaminated groups in this experiment is more likely to be considered. Previous analysis of undiluted leachate from Zdounky–Kuchyňky landfill used in this experiment reported the content of several heavy metals, mostly zinc (472.3 ± 3.3 µg/l), nickel (155 ± 8.84 µg/l), chromium (108.19 ± 22.3 µg/l), lead (15.26 ± 0.82 µg/l), trace amounts of arsenic (4.5 ± 1.27 µg/l), mercury ($3.93 \pm 2 \times 10^{-4}$ µg/l) and cadmium (0.518 ± 0.17 µg/l) in the samples (Vaverková et al. 2017).

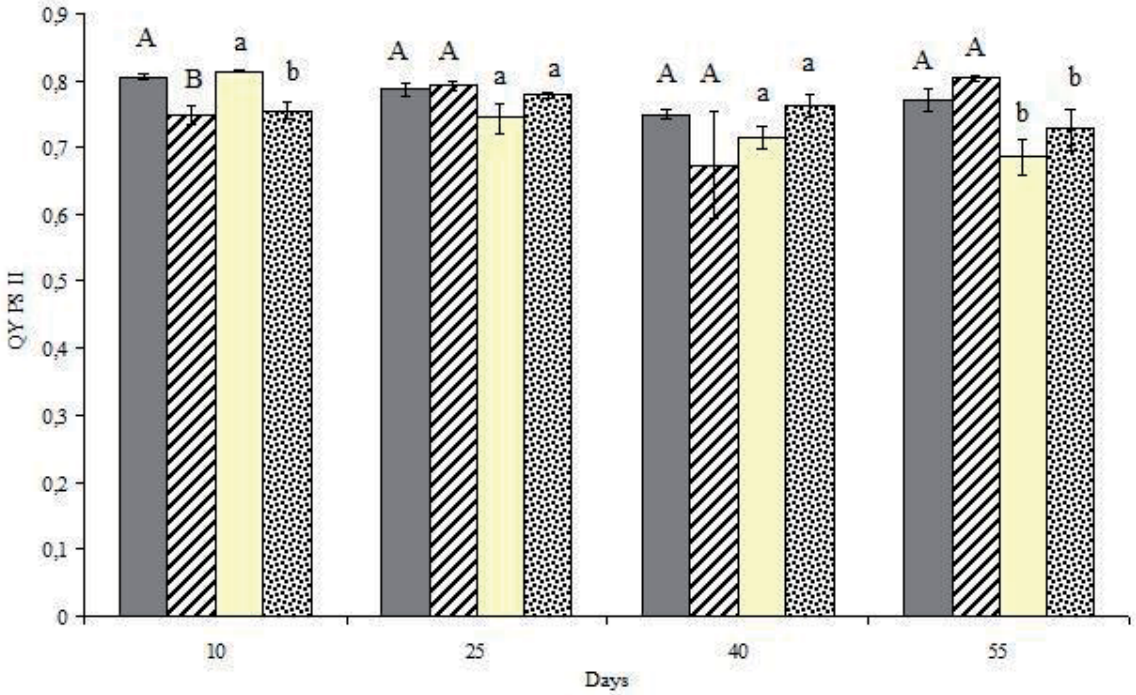
This would imply much lower resulting heavy metal concentration in the chosen 15% leachate solution. A study by Girdhar et al. (2014) has proven the high capability of *Cannabis sativa* L. for hyperaccumulation (up to 44 µg/g for zinc, 11 µg/g for nickel and 15 µg/g for chromium in shoots).

But it is still necessary to bear in mind that phytotoxic effects on the same plant species may be vastly different depending on the contaminated environment, pH, interaction with other compounds and whether the roots are in a soil or an aquatic solution (Baker and Walker 1989).

For the nodal proliferation (number of stem nodes created), the results were mostly correlating with the data for shoot length. In the leachate groups, during the last week of measurement, significantly higher number of stem nodes was recorded in case of Bialobrzeskie cultivar when compared to Monoica (data not shown). Polish variety seemed to have a tendency to create more nodes generally also within control groups. Again, control groups overall exhibited significantly higher number of nodes compared to groups in landfill leachate. The inhibitory effect on stem nodes in the presence of lead in concentration of 50 mg/l was observed in one study on seagrass (Ambo-Rappe et al. 2011). Interestingly, completely contradictory results were found in a greenhouse experiment on potato conducted by Marofi et al. (2013), where the plants were in the soil and concentration of lead in wastewater used for irrigation was much lower (60 µg/l), but still higher than compared to current experiment with hemp. More likely, other factors than heavy metals play the role in this case.

The obtained results for photosynthetic efficiency appeared to be fluctuating during the weeks, but statistically significant differences in quantum yield of photosystem II (QY PS II) between the cultivars were found only during the very last week (Figure 2).

Figure 2 Quantum yield of photosystem II in different experimental groups of hemp



Legend: grey columns – Monoica control group, striped columns – Monoica leachate, white columns – Bialobrzeskie control, dotted columns – Bialobrzeskie leachate; Capital letters represent the statistical differences within the Monoica cultivar, while lower case letters represent the differences within Bialobrzeskie

On the other hand, only in the very first measurement week both varieties within the control group exhibited significantly higher QY PS II when compared to contaminated groups. Statistical significance was evaluated always only within given measurement week, between the cultivars and also between the solutions. It would seem that hemp plants in contaminated groups adjusted to the conditions without sacrificing the photosynthetic ability. Higher photosynthetic efficiency of Monoica cultivar during the last measurement may not have a necessary correlation with heavy metals, as it was also observed in control groups. It could be a result of other factors, such as genotype-specific traits related to senescence or nutrient requirements. On several occasions, lower concentration of heavy metals, especially cadmium was observed to actually increase the chlorophyll content in hemp, thus having a possible influence on photosynthesis, as observed in a study by Linger et al. (2005).

CONCLUSION

Phytotoxic effect of landfill leachate containing heavy metals on growth and development of two cultivars of industrial hemp was observed in an experimental hydroponic culture. Morphological parameters, viability of plants and influence on photosynthesis were studied. Significant differences were found between the solutions and also between cultivars. Obtained results suggest, that cultivar Bialobrzeskie had a higher tolerance to the leachate, thus may be better suited for possible field application, but more comparable experiments are needed for validation. Furthermore, it seems that not only plant species, but also cultivars could be considered before the phytoremediation practices. In the future, more detailed and extensive experiments in both hydroponic culture and soil, supported with the chemical analysis of heavy metals absorbed in plant tissue (roots and shoots) should be conducted to determine the real potential of hemp cultivars for hyperaccumulation of heavy metals.

ACKNOWLEDGEMENTS

The research was financially supported by the IGA FA MENDELU No. TP 5/2017. We would like to express our great appreciation to the management of the landfill DEPOZ, Ltd. Namely, we are very grateful to Ing. Ivan Mohler and his colleagues for their assistance and their willingness to provide their time so generously.

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USE OF RT-qPCR METHOD FOR ANALYSIS OF CYTOKININ-ACTIVATED REPORTER GENE *lacZ* IN *E. coli*

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Abstract: Escherichia coli strain KMI001 (*ΔrcsC*, *cps*::*lacZ*) expressing the CRE1/AHK4 receptor that activates β-galactosidase expression has been previously employed to evaluate cytokinin and/or anti-cytokinin activity of synthetic cytokinin-like compounds. Here, for the first time, we evaluate its response on the transcript level. We tested and optimized mRNA extraction protocol and analyzed expression stability of eight reference genes. We show that the presence of cytokinin may alter the expression pattern of these so-called house-keeping genes of bacteria.

Key Words: E. coli, reference genes, RT-qPCR, cytokinin, heterologous reporter system

INTRODUCTION

Cytokinins are plant growth regulators that affect various processes in plant development and stress responses. Three Arabidopsis thaliana sensor histidine kinases, AHK2, AHK3, and CRE1/AHK4, are known to be cytokinin receptors. Compounds that specifically interact with cytokinin receptors have high utility in research and potential agronomic applications (Koprna et al. 2016). A heterologous system expressing CRE1/AHK4 in a manner that functionally complements a two-component signalling pathway in a *E. coli* (Suzuki et al. 2001, Yamada et al. 2001) has been used as a tool to search compounds with agonist or antagonist properties toward the CRE1/AHK4 cytokinin receptor (Spíchal et al. 2004, Romanov et al. 2005, Klimeš et al. 2017). In this *E. coli* strain, CRE1/AHK4 signaling triggers expression of a β-galactosidase reporter gene *lacZ*. Subsequently, β-galactosidase activity is detected by highly sensitive fluorescence measurements. However, the *lacZ* expression at the transcript levels has been not assayed and possible benefits of RT-qPCR to determine the time-course and extent of *lacZ* activation were neglected.

Common features of bacterial cells as rigid cell wall and relative short half-life of mRNA, and, shortcomings in methodology complicate reliable transcriptome analysis. In this study, we validated a method for immediate cell lysis and RNA stabilization. Further, we first analyzed expression stability of several candidate reference genes for reliable RT-qPCR analysis under cytokinin treatment in the *E. coli* strain harbouring cytokinin-activated reporter gene *lacZ*.

MATERIAL AND METHODS

E. coli strain, cultivation, sample preparation and live-cell cytokinin-binding assay

E. coli strain KMI001 (*ΔrcsC*, *cps::lacZ*) harboring the plasmid pIN-III-AHK4 was used (Suzuki et al. 2001, Yamada et al. 2001). *E. coli* cultures expressing CRE1/AHK4 cytokinin receptor were grown in LB liquid medium supplemented with ampicillin (100 μg/ml). The starting culture was divided into five aliquots which were supplemented with 0.02% (v/v) DMSO (mock) or *trans*-zeatin (*tZ*) in DMSO (final concentration, as for the mock) to get final *tZ* concentration of 10 nM, 100 nM, 1 μM, or 10 μM, respectively. Lysozyme (0.8 mg per ml of bacterial culture) was added 10 min before *tZ* binding assay performance and harvest of bacteria for expression analysis. For RT-qPCR analysis, cells in liquid cultures were concentrated by centrifugation, supernatant was discarded, pellets were frozen in liquid nitrogen and kept at -80 °C.

The strength of the ligand-receptor interaction was described as fluorescence intensity of the β-galactosidase-catalyzed reaction product. A Nanodrop II liquid handling system (BioNex Solutions, USA) was used for all pipetting steps. Optical densities (OD_{600}) and fluorescence intensities of the β-galactosidase-catalyzed reaction product (ex. and em.: 365 and 448 nm) were measured using an Infinite M1000Pro plate reader (Tecan, CH).

RNA extraction, cDNA synthesis and quantitative PCR

Frozen pellet from centrifuged lysozyme-pre-treated bacterial culture was resupended in 0.5 ml of TRI reagent (Invitrogen) to quickly lyse bacterial cells, protect and isolate total RNA. Initial RNA isolation steps were performed according to manufacturer's instruction to separate an aqueous phase containing RNA. Subsequently, 2 volumes of ethanol were added to the aqueous phase and total RNA was isolated using the RNAeasy Mini Kit (Qiagen) following the manufacturer's protocol. Extracted RNA was treated with TURBO DNase (Ambion) for 5 min at 37 °C and DNase activity was then abolished with EDTA (final EDTA concentration in the RNA sample was 2.5 mM). RNA integrity was assessed by non-denaturing agarose electrophoresis and RNA concentration was determined using NanoDrop (Thermo Fisher Scientific). First-strand cDNA was synthesized from 1 μg of total RNA using the RevertAid Reverse Transcriptase (Thermo Fisher Scientific) and random hexamer primers according to the manufacturer's instructions including initial incubation at 65 °C for 5 min (DNase denaturation, secondary RNA structure relaxation). Quantitative PCR (qPCR) was performed using specific UPL probes and primers designed by ProbeFinder Software (Roche) and LightCycler 480 Probes Master in a LightCycler 480 Instrument (Roche). For each gene and sample, the Cp values were identified using the second derivative maximum method and they were converted to a linear scale using relative standard curve method. The gene expression data were normalized by geometric averaging using the GeNorm VBA applet for Microsoft Excel (Vandensompele et al. 2002) with the candidate reference genes and are presented as relative to control treatment. Primer sequences and UPL probes are listed in Table 2 in the section Results and Discussion. A Student's *t*-test ($p < 0.05$) was utilized to identify statistically significant differences between mock- and *tZ*-treated variants.

RESULTS AND DISCUSSION

β-galactosidase activity in cytokinin-activated *E. coli* strain KMI001

In the *E. coli* strain KMI001 expressing the CRE1/AHK4 cytokinin receptor, an activating ligand in the growth medium initiates a signal transduction pathway that triggers an engineered operon and results in an expression of the reporter enzyme β-galactosidase (Suzuki et al. 2001). It is believed that this expression is proportional to the ligand's concentration and activating properties (Yamada et al. 2001, Spíchal et al. 2004). However, this conclusion is based only on the reporter enzyme activity and the transcript level of *lacZ* gene and the time-course of its induction in the presence of CRE1/AHK4 activating ligand(s) have not yet been reported.

To evaluate the cytokinin-activated reporter system, the overnight culture of *E. coli* (strain KMI001) was exposed to *trans*-zeatin (*tZ*) (final concentrations of 10 nM, 100 nM, 1 μM, or 10 μM) for 17 h. We observed a concentration-dependent increase in galactosidase activity but we also found that at the micromolar *tZ* concentrations the optical density was significantly lower (Table 1). This indicates that the overexpression of β-galactosidase inhibits bacterial growth or that the *tZ* treatment may have a toxic effect on the culture.

Table 1 Optical density and β-galactosidase activity in the presence of cytokinin tZ *in* E. coli *strain KMI001*

tZ [nM]	0	10	100	1000	10000
OD_{600} (200 μl)	0.18 ± 0.02	0.18 ± 0.01	0.21 ± 0.01	0.11 ± 0.00*	0.10 ± 0.00*
Activity [RFU]	2646 ± 57	3780 ± 84*	9515 ± 190*	16162 ± 364*	16066 ± 492*

Data are means ± SD of three biological repeats. Asterics indicate significant differences between mock- and tZ-treated variants at $p < 0.05$ (t-test).

Expression analysis in cytokinin-activated *E. coli* strain KMI001

The mRNA levels change on a minute scale due to the degradation and *de novo* transcription and significant changes may occur in the time required for the sample harvest and RNA isolation. In case of bacteria, this procedure includes incubation with lysozyme to ensure the complete lysis of the cells. Thus, we aimed to ensure quick lysis of the cells and RNA protection to minimize unwanted changes of mRNA levels. The lysozyme treatment was implemented before the sample harvest and the total mRNA was extracted by acid guanidinium thiocyanate-phenol-chloroform extraction (TRI reagent) and a column-based RNA purification using RNAeasy Mini Kit (Qiagen). This procedure provided a sufficient RNA yield from 1 ml of overnight bacterial culture.

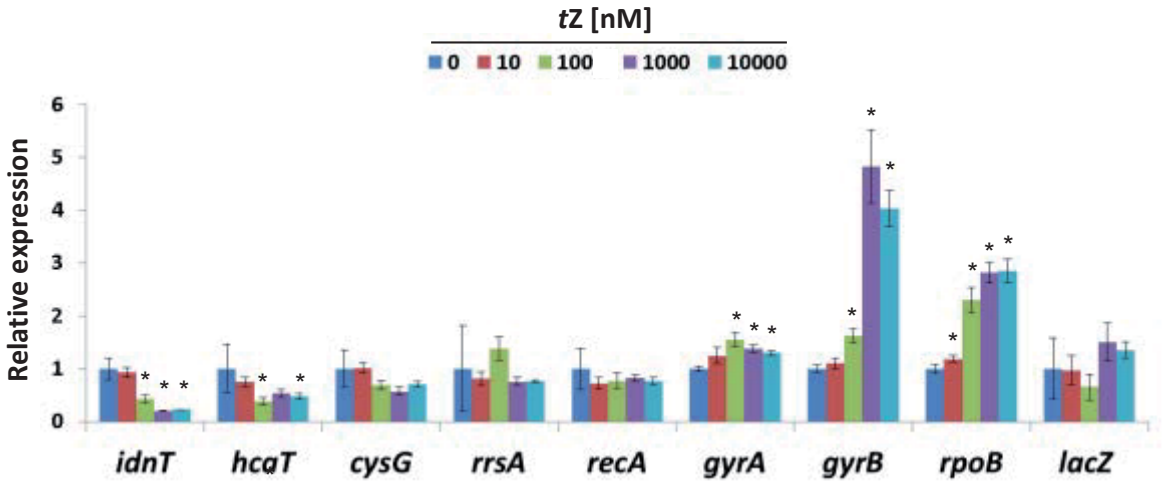
Isolated RNA was processed for a reverse transcription-quantitative real-time PCR (RT-qPCR) analysis of the cytokinin-activated *lacZ* over-expression. Accurate interpretation of RT-qPCR data requires normalization using constitutively expressed reference genes. However, the utility of reference genes must be experimentally validated for particular organisms, tissues, cell types and specific experimental designs. The use of multiple stable reference or housekeeping genes is generally accepted as the method of choice for RT-qPCR data normalization (Vandesompele et al. 2002, Bustin et al. 2009, Souček et al. 2017). As the choice of reliable reference genes in the presence of cytokinins has not been systematically validated in bacteria, an objective of this study was to identify reference genes for the accurate normalization of gene expression in cytokinin-activated *E. coli* strain KMI001.

Table 2 Genes, primers, UPL probes and stability M-value. Candidate reference genes were selected based on current studies (e.g. Zhou et al. 2011, Peng et al. 2014, Rocha et al. 2015). The geNorm was used to calculate the expression stability value (M-value)

Gene symbol	Gene description	Left primer Right primer	Probe number	M-value
gyrA	gyrase A	ggcgaagacgaagtaatgct atcgcacggacagaagactc	161	0.702
gyrB	gyrase B	gcgtaaccgcaagaacca ctttctcgacgttgaggatttt	161	1.187
recA	recombinase A	acgtaaactgggcgtcgata gggcgtcacagatttcca	99	0.735
rpoB	DNA-directed RNA polymerase, beta subunit	atcaacggtactgagcgtgtt aaagaagacgcccggactac	33	0.928
rrsA	16S ribosomal RNA	tccctagctggtctgagagg cgtaggagtctggaccgtgt	5	0.875
cysG	uroporphyrin III C-methyltransferase	cgatcgcgactgtctgatt cctgcgtctaacagcaacct	9	0.771
ldnT	L-idonate/5-ketogluconate/ gluconate transporter	gatcaccggtctggttgtg tcctgatgatgctacgatgg	18	1.241
hcaT	HcaT MFS transporter	catgctgctcggctttct ctctcctgctggcgactt	5	0.889
lacZ	β-galactosidase	gacccgcattgaccctaac tgcttcggcctggtaatg	11	-

In our conditions, we found that most of the reference genes demonstrated a significant change in the expression pattern (Figure 1, Table 2). geNorm´s algorithms address the problem of multiple reference genes to evaluate the expression stability of the candidates even if the stability and reliability of the available candidate reference genes is poor. For each reference gene, reference gene stability value (M-value) is calculated as the average pairwise variation of a particular reference gene with all other tested candidate reference genes. The M-values was lowest for *gyrA*, *recA* and *cysG* suggesting these genes as the most stable and convenient for an accurate normalization procedure (Table 2). However, the normalization using this three reference genes provided very similar *lacZ* expression values as the use of all candidate reference genes for RT-qPCR data normalization. The insignificant *lacZ* activation even under high *tZ* concentrations suggests a mis-interpretation of *lacZ* transcript levels. In this line, we suggest that the cytokinin-upregulated group of genes related to DNA metabolism (*gyrB*, *rpoB* and also *gyrA*) obscures the actual *lacZ* transcript levels and that cytokinin-downregulated genes related to the transport may represent the actually stable genes in the presence of high *tZ* concentration. However, the generally unstable expression of candidate reference genes in our experimental design points to necessity to test other reference genes specifically suited for our experiment.

Figure 1 Candidate reference genes and lacZ in response to cytokinin (tZ) in E. coli strain KMI001



Data are means ± SD of three biological repeats. Asterics indicate significant differences between mock- and tZ-treated variants at $p < 0.05$ (t-test).

CONCLUSION

This study showed an optimized mRNA extraction protocol and revealed variant expression of reference genes under the cytokinin treatment in *E. coli*. The poor stability of the candidate reference genes obscured reliable normalization method for accurate quantification of *lacZ* overexpression at the transcript level in *E. coli* strain KMI001. Thus, selection and evaluation of other candidate reference genes specifically suited for our experimental design is necessary. Consequently, the advanced RT-qPCR method including validated reference genes will provide a useful tool for characterizing extent and time-course of *lacZ* activation in response to specific ligands and conditions in the strain KMI001.

ACKNOWLEDGEMENTS

This research has been financially supported by the Czech Science Foundation under project GA15-19266S, the Ministry of Education, Youth and Sports of the Czech Republic under project CEITEC 2020 (LQ1601).

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EFFECT OF ZINC-SELENIUM NANOPARTICLES ON MICROALGAE *SCENEDESMUS QUADRICAUDA*

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Abstract: The increasing industrial use of nanomaterials in recent years poses a potential risk to the environment. The first organisms that come into contact with these substances include aquatic organisms, and therefore this study focuses on microalgae that are at the beginning of the food chain. In this study, the toxicity of ZnSe nanoparticles in the freshwater green microalga *Scenedesmus quadricauda* was investigated. The effect of zinc in the form of zinc sulphate heptahydrate ($ZnSO_4.7H_2O$) and the combination of zinc and nanoparticles were also monitored. The microalgae were exposed to these nanoparticles at concentrations of 10, 50, 100 and 250 µM for 7 days. The microalgae responses were analysed at the level of chlorophyll-*a*, chlorophyll-*b*, carotenoids, flavonoids and total antioxidant capacity. For chlorophyll-*a*, -*b* and carotenoids, similar results were obtained. The most significant effect was found in the sample with a combination of zinc and ZnSe nanoparticles. This sample also affected the most the flavonoid content, especially at concentrations of 50, 100 and 250 µM, where increased synthesis of these compounds was observed. Similar results were obtained in the total antioxidant capacity assay, where a sample with combination of zinc and ZnSe nanoparticles showing an increasing trend, particularly at a concentration of 250 µM.

Key Words: microalgae, nanoparticles, zinc nanoparticles, secondary metabolism, flavonoids

INTRODUCTION

With the rapid development of nanotechnology and the widespread use of nanomaterials, there is an increasing risk of environmental contamination by these particles. Nanoparticles are defined as particles with at least two dimensions between 1–100 nm (Bhatt and Tripathi 2011). They are a natural part of the environment. However they are also artificially synthesized for the needs of the industry. Their increased production and use can lead to release into the environment where they can interact with biotic and abiotic components. In spite of their great advantages, the presence of nanoparticles in nature can have a dangerous biological effect (Bhatt and Tripathi 2011). In particular, heavy metal nanoparticles may have a negative impact on the environment. The potential consequences of such contamination are currently difficult to assess as the toxicity of nanoparticles is not well known (da Costa et al. 2016). Heavy metal nanoparticles are one of the most commonly used nanoparticles in the industry (Nagajyoti et al. 2010). Due to their unique physical and chemical properties, nanoparticles of metals are increasingly being used in various commercial products, leading to concerns about their potential toxicity. In the industry, it has a wide range of applications including catalysis, sensors and environmental remediation, personal care products (e.g. sunscreen creams), coatings or paints (Franklin et al. 2007). ZnSe can be used as a material for n-type semiconductors. Due to the ability to emit fluorescent light can be used as quantum labels for biological use (Iwahori et al. 2005). It can also be used as a semiconductor material, which is potentially used in the diodes of blue-green light, laser diodes and solar cells (Shakir et al. 2009).

Zinc is an essential micronutrient, which is important for normal growth of algae. Its deficiency leads to poor growth and low dry weight (Li et al. 2006). Zinc plays an important role in maintaining the stability of cell membranes in the activation of more than 300 enzymes in protein and nucleic acid metabolism (Soto et al. 2011). However, it can also be toxic when applied in higher amounts. It was found that zinc affects chlorophyll content due to the peroxidation of chloroplast membranes (Li et al. 2006). It has also been found that zinc in the form of zinc oxide (ZnO) and zinc oxide nanoparticles (ZnO NPs) show algal toxicity in the form of growth inhibition (Aruoja et al. 2009). Nanoparticles of zinc (ZnO NPS) substantially reduces the viability of the cells, increases the activity of antioxidant enzyme superoxide dismutase (SOD), increases the level of lipid peroxidation and causes substantial morphological changes and damage to the cell wall of microalgae (Suman et al. 2015) In the study Chen et al. 2012 was also found distortions of the morphology, viability and integrity of the microsurface membrane resulting from the dissolution of zinc ions.

It is generally assumed that nanoparticles will persist in aquatic systems and that their bioavailability may be significantly higher than for larger particles. There are scientific concerns that these nanoparticles may pose an increased health and environment risks. A small size of nanoparticles results in both greater mobility and potentially increased permeability through biological membranes. This can be reflected by cell-level responses (Franklin et al. 2007).

There is little information about the environmental fate of nanomaterials and their possible toxicity to aquatic biota. There are many studies on algal zinc toxicity, but in the form of NPs their toxicity is scarcely explored.

MATERIAL AND METHODS

Biological material

Green freshwater 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 vitro on Petri dishes in a cultured room with controlled conditions (23 ± 1 °C, 70 μmol m^2/s of light intensity, light/dark cycle 12:12 h) for several weeks until a significant percentage increase in biomass. Microlgae were then transferred to liquid TAP (Tris Acetate Phosphate) media with varying concentrations of ZnSe nanoparticles, zinc sulphate heptahydrate ($ZnSO_4.7H_2O$) and combinations thereof. The concentrations were chosen in the range of 10–250 μM (10, 50, 100 and 250 μM). Samples were taken after 7 days of culture, then lyophilized and analysed.

Chlorophylls and carotenoids determination

The chlorophyll content was determined according to the method of Lichtenthaler and Wellburn (Lichtenthaler and Wellburn 1983). The ethanolic extract was centrifuged and measured at wavelengths of 470, 649 and 665 nm. The calculation was carried out according to the above method.

Spectrophotometric determination of flavonoids

The sample of lyophilized algae was extracted with 80% methanol in an automatic homogenizer. The extract thus prepared was analysed using a 5% $NaNO_2$, 10% $AlCl_3.6H_2O$ and 1 ml of NaOH. The reaction mixture was then measured at 415 nm. The rutin was used as the reference standard.

Spectrophotometric determination of TAC

Total antioxidant capacity (TAC) was determined in a lyophilized sample extracted with 80% methanol according to phosphomolybdate assay system with some modifications (Shabbir et al. 2013). The prepared extract was placed in an ultrasonic bath at room temperature in the dark for 45 minutes. The samples were then centrifuged. The analysis is based on reaction with special reagent (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium

molybdate). The reaction mixture was incubated at 95 °C for 60 minutes and then measured at 695 nm against water as a blank. The trolox was used as the reference standard.

Statistical analysis

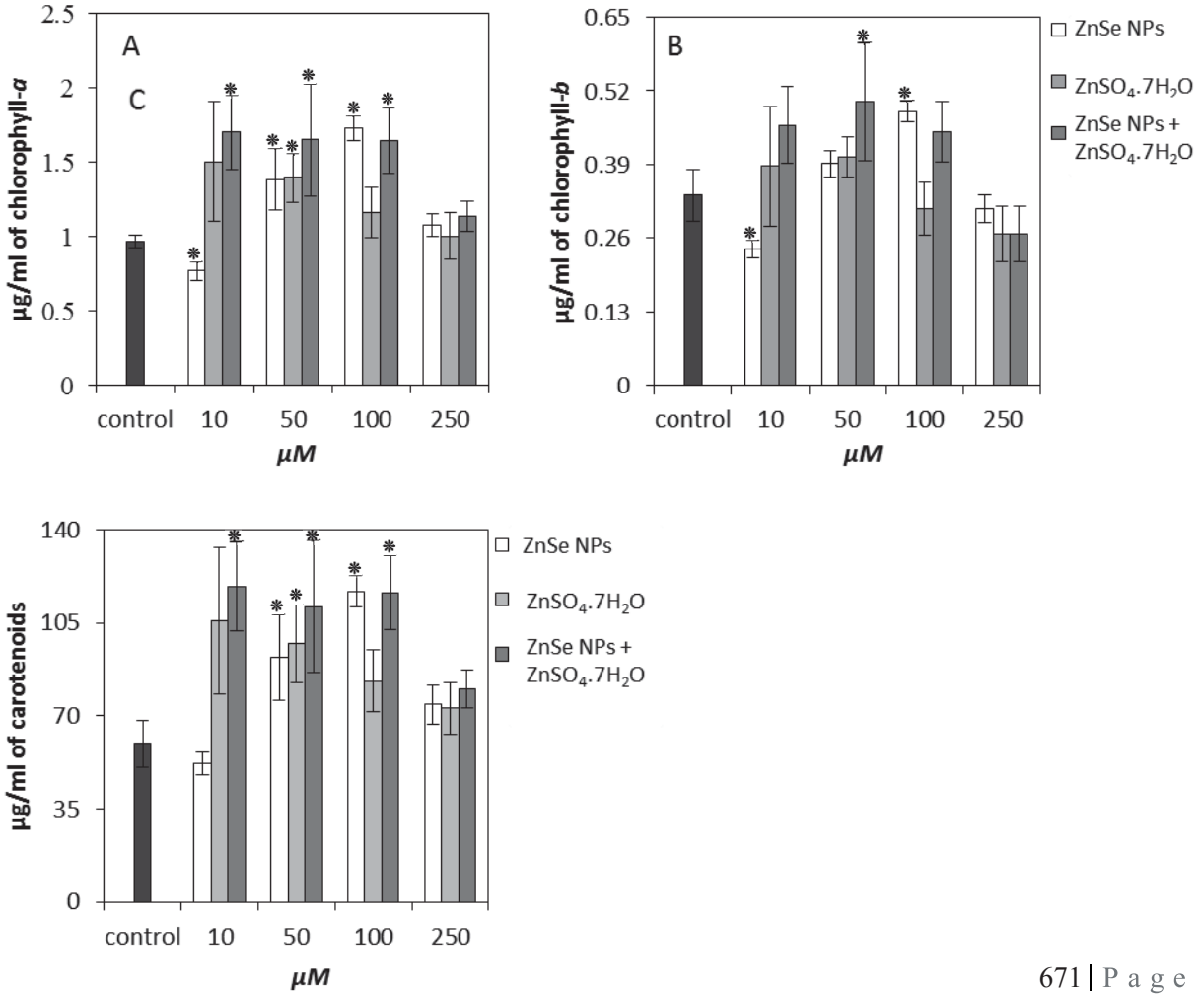
The data were statistically analysed by using software R, version 3.4.0 for windows (www.r project.org). Significant differences compared to the control samples are shown as the asterisk and represent statistically significant differences compared to the control samples ($p < 0.05$, $n = 3$). Statistical analysis was carried using student's *t*-test.

RESULTS AND DISCUSSION

Chlorophylls and carotenoids

For ZnSe nanoparticles, a slight decrease of chlorophyll-*a* (Figure 1A) was first observed compared to control. Then there was a gradual increase up to a concentration of 250 µM, in which we observed a decrease. On the other hand, the samples with zinc ($ZnSO_4.7H_2O$) and a combination of zinc and nanoparticles have increased since the concentration of 10 µM. We observed a stagnation or a slight decrease at the other concentrations, which is most pronounced at a concentration of 250 µM (Figure 1A), when all the samples return to the control value. Similar results were found for chlorophyll-*b* (Figure 1B) and for carotenoids (Figure 1C). This is in contradiction with the outcome of the study Soto et al. 2011, where concentration of chlorophyll-*a* decreased significantly at zinc ($ZnSO_4.7H_2O$) concentration of 0.075 mg/l. Differences could have been caused by a different type of tested algae (*Pseudokirchneriella subcapitata*) that could otherwise react to the presence of zinc (Soto et al. 2011).

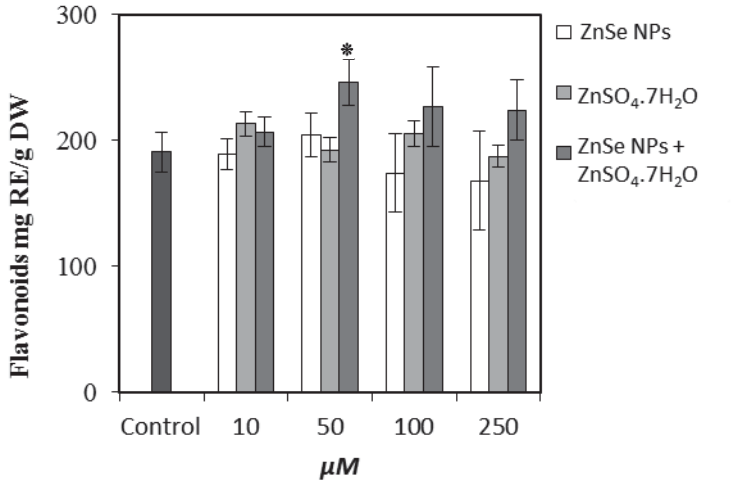
Figure 1 The concentration of chlorophyll-a (A), chlorophyll-b (B) and carotenoids (C) in Scenedesmus quadricauda depending on different concentrations of zinc sulphate heptahydrate ($ZnSO_4.7H_2O$), ZnSe nanoparticles and combination thereof. Error bars correspond to standard error of mean (n=3).



Flavonoids

Flavonoids belong to a large heterogeneous group of substances that are important secondary metabolites of plants (Goiris et al. 2014). They are an important part of the plant's antioxidant mechanism that prevents oxidative stress. Plants under strong stress conditions accumulate flavonoids that are effective reactive oxygen species (ROS) separators. It was assumed that changes in cellular redox homeostasis due to stress activate the flavonoid biosynthesis (Agati et al. 2012). In the sample with ZnSe nanoparticles, we did not notice significant differences in flavonoids content compared to control at concentrations of 10 and 50 µM. At a concentration of 100 and 250 µM, there was a slight downward trend. On the other hand, the zinc ($ZnSO_4.7H_2O$) sample did not show significant differences compare to control at any of these concentrations. The most significant effect on flavonoid content was found in a sample of both nanoparticles and zinc, in which we recorded a slight increase in concentrations of 50, 100 and 250 µM. At these concentrations, the microalgae attempted to prevent oxidative stress by increasing the synthesis of these substances.

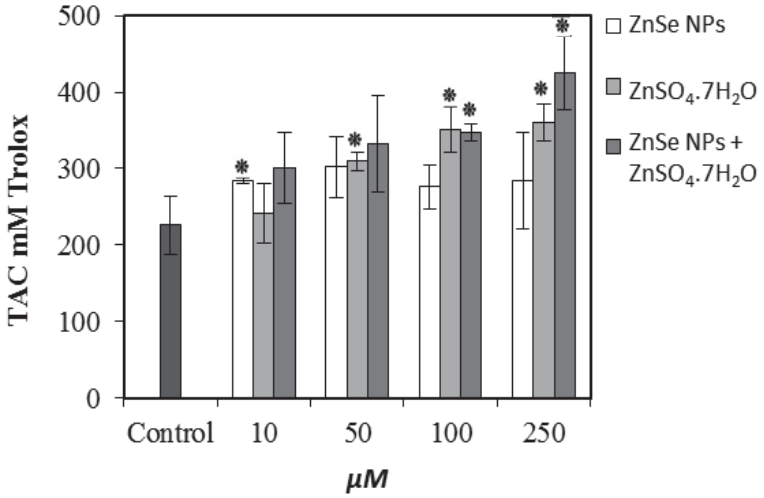
Figure 2 The concentration of flavonoids in Scenedesmus quadricauda depending on different concentrations of zinc sulphate heptahydrate ($ZnSO_4.7H_2O$), ZnSe nanoparticles and combination thereof. Error bars correspond to standard error of mean (n=3).



Total Antioxidant Capacity – TAC

The ZnSe nanoparticle sample did not have a significant effect on total antioxidant capacity. On the other hand, a significant increase in concentration was observed for the zinc sample and the zinc-nanoparticle combination. The most significant increase was observed at concentrations of 50, 100 and 250 µM, consistent with results in flavonoid assays. This confirms the hypothesis that the algae at these higher concentrations increased the activity of the antioxidant system to inhibit oxidative stress (Miazek et al. 2015), especially in the sample with a combination of zinc nanoparticles and zinc sulphate heptahydrate.

Figure 3 Total antioxidant capacity in Scenedesmus quadricauda depending on different concentrations of zinc sulphate heptahydrate ($ZnSO_4.7H_2O$, ZnSe nanoparticles and combination thereof. Error bars correspond to standard error of mean (n=3).



One explanation for this result is the possibility of releasing zinc ions from ZnSe nanoparticles, which could increase the concentration of Zn^{2+} ions themselves together with $ZnSO_4$. Therefore, the combined effect of zinc and ZnSe nanoparticles could have these significant effects.

CONCLUSION

Toxicity of ZnSe nanoparticles on green freshwater microalgae *Scenedesmus quadricauda* was investigated. Zinc can be toxic in both bulk and nano forms. The results of this study compare the effect of zinc in the form of bulk, nano and combination of both. It has been shown that zinc in bulk and nanoparticles form can affect chlorophyll-*a*, chlorophyll-*b* and carotenoid levels, resulting in an increase in the content of these pigments, especially at concentrations of 10, 50 and 100 μM. The most significant effect on flavonoid content and total antioxidant capacity was shown by a combination of zinc and nanoparticles. In this sample, increased flavonoid synthesis and increasing concentration of antioxidant capacity were observed. This combination could have the greatest effect due to the release of Zn^{2+} ions from ZnSe nanoparticles, which together with zinc produced a significant effect.

ACKNOWLEDGEMENTS

The work was supported by Internal Grant Agency of Mendel University in Brno (Project No. Tym003) and CEITEC 2020 (LQ1601) with financial support from the Ministry of Education, Youth and Sports of the Czech Republic under the National Sustainability Programme II.

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CYTOKININS IN REGULATION OF COTYLEDONARY BUD OUTGROWTH IN PEA (*PISUM SATIVUM* L.)

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Abstract: This work was aimed on the role of cytokinins in regulation of cotyledonary bud outgrowth in intact pea (*Pisum sativum* L.) plants. It is well known that application of cytokinins to the cotyledonary buds of intact plants activates their outgrowth. Here we show by immunolocalization rapid polarization of PIN1 proteins in the cytokinin treated buds, on contrary to untreated buds. It is therefore obvious that cytokinins directly influence polarization of PIN1 proteins and the subsequent canalization of the polar auxin transport from the buds enabling their outgrowth.

Key Words: Cytokinins, apical dominance, polarization of PIN1 proteins

INTRODUCTION

The polarized auxin flux in the main stem of the plant can inhibit the growth of the lateral buds by being able to regulate the ability of the lateral bud to establish its own auxin export into the main stem (Li and Bangerth 1999). Sachs (1981) observed in his attempts to regenerate vascular bundles after mechanical damage to the stem. The theory assumes that auxin has a polarizing effect on the tissue, and it is based on the auxin feedback induced by polarization of its transport at the level of one cell. The regulation and direction of the auxin flow is realized at the cellular level by auxin-induced changes in the polarity of PIN proteins (Sauer et al. 2006b).

If we have two auxin sources nearby, there is competition between them and one source can block the auxin canalisation from the underlying one (Sachs 1968). If the shoot apex is decapitated, the axillary buds are released from inhibition and begin to grow. This growth is initially uniform for both buds, but after several days of growth it can be observed that one of the shoots remain completely inhibited, and the second growing bud will take over the function of the new shoot apex.

This means that there is competition for dominance, based on the blockage of canalisation of auxin from one bud by another one (Balla et al. 2016). Export of the auxin from the lateral buds is only possible if the primary auxin source is removed or weakened (e.g. by decapitation). As a result of decapitation, auxin export from the lateral buds due to the polarization of the PIN proteins will occur to create the auxin channel. In addition, vascular bundles necessary for the growth of lateral buds are formed along these channels (Balla et al. 2011). The main aim of the described experiments was to elucidate the action of exogenously applied cytokinin, 6-benzylaminopurine (BAP), on the cotyledonary buds of intact pea plants (*Pisum sativum* L.). We studied the release of cotyledonary pea buds from apical dominance and the role of cytokinin on polar auxin transport in the buds.

MATERIAL AND METHODS

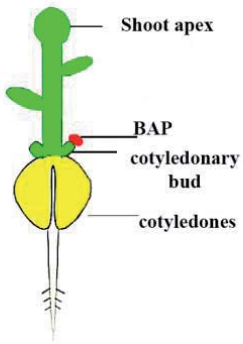
Plant material

For the experiments seeds of pea (*Pisum sativum* L.), variety Vladan (Semo a.s., Smržice, Czech Republic) were used.

Experimental setup

The exogenous application of 1% BAP (Sigma-Aldrich, USA) to the cotyledonary buds of intact pea plants was performed on day 7, calculated from the time when imbibed seeds were sawn onto Perlite, and treated plants were sampled 0, 6, 12, 24, 48, 72, 96 hours after BAP application (Figure 1). Parallel samples of cotyledonary buds from control, untreated plants were collected at 0 and 120 hours.

Figure 1 The application of 1% BAP on the cotyledonary bud in intact pea plant (Pisum sativum L.)



Imunolocalization of PIN1

The segments of pea buds were left in fixation mixture until next day at -20 °C. Fixation mixture consists of methanol together with acetic acid in ratio 3 : 1. After the segments were pulled out from fixation mixture, they were 3 times washed for 15 minutes in PBS buffer at pH 7.4. The dehydration of segments was performed by using the ethanol series to achieve complete water removal of the segments of pea buds. The concentrations of ethanol in ethanol series were 10/30/50/70/90/96/100%. The pea buds were after that immersed into solution xylene : ethanol in different ratio (Sauer et al. 2006a). Individual segments were immersed in xylene solution overnight. The next day paraffin Paraplast plus (Kendall, USA) was gradually added to the flasks with a melting point of 56 °C. First, a mixture of paraffin and xylene added to the segments and was incubated at room temperature for 12 hours. After that, paraffin was added again for 12 hours at 42 °C and 4 hours at 58 °C. This was followed by the gradual removal of the xylene solution with paraffin and its gradual replacement with pure and dissolved paraffin (Sauer et al. 2006a). Cutting was performed to 12 µm thin slices on a Leica RM2255 rotary microtome (Leica biosystems, Germany). The glasses with segments that were selected for further processing were subsequently rehydrated by descending ethanol series of 90/70/50/30/10% concentration. Finally, the sample glasses were immersed for 10 minutes in PBS buffer (Sauer et al. 2006a). Blocking solution was pipetted onto microscope slides (3% bovine serum albumin dissolved in PBS buffer at pH 7.4) and left in the dark for 60 minutes. After this time, the blocking solution was removed from the microscope slides and then 100 µl of the solution with the primary anti-PIN1 antibody (anti-PIN1 antibody was provided by the Laboratory of developmental and cell biology of plants-IST Austria) was pipetted onto microscope slides. Antibody dilution was performed with blocking solution in a ratio of 1 : 1000. To prevent it from drying out, the glass was covered by parafilm. The following day was removed from the microscope slides with parafilm, a blocking solution with the first antibody, and the microscope slides were washed three times in PBS buffer containing 0.2% Tween-20 (Sigma-Aldrich, USA) (Sauer et al. 2006a). Blocking solution was pipetted onto the glasses and was then incubated in a humid environment and in the dark for 30 minutes. The blocking solution was then removed and solution of the secondary antibody conjugated to the fluorescent dye-polyclonal Cy3-anti rabbit (Sigma-Aldrich, USA) was pipetted onto the glass. The antibody was pre-diluted with blocking solution in a ratio of 1: 500. Then, to prevent the sample from drying out was again used parafilm. After 4 hours of incubating the samples at 37 °C in the dark, the secondary antibody was removed from the glass and the glass was washed three times in PBS buffer containing 0.2% Tween-20 at room temperature for 10 minutes (Sauer et al. 2006a).

Microscopy

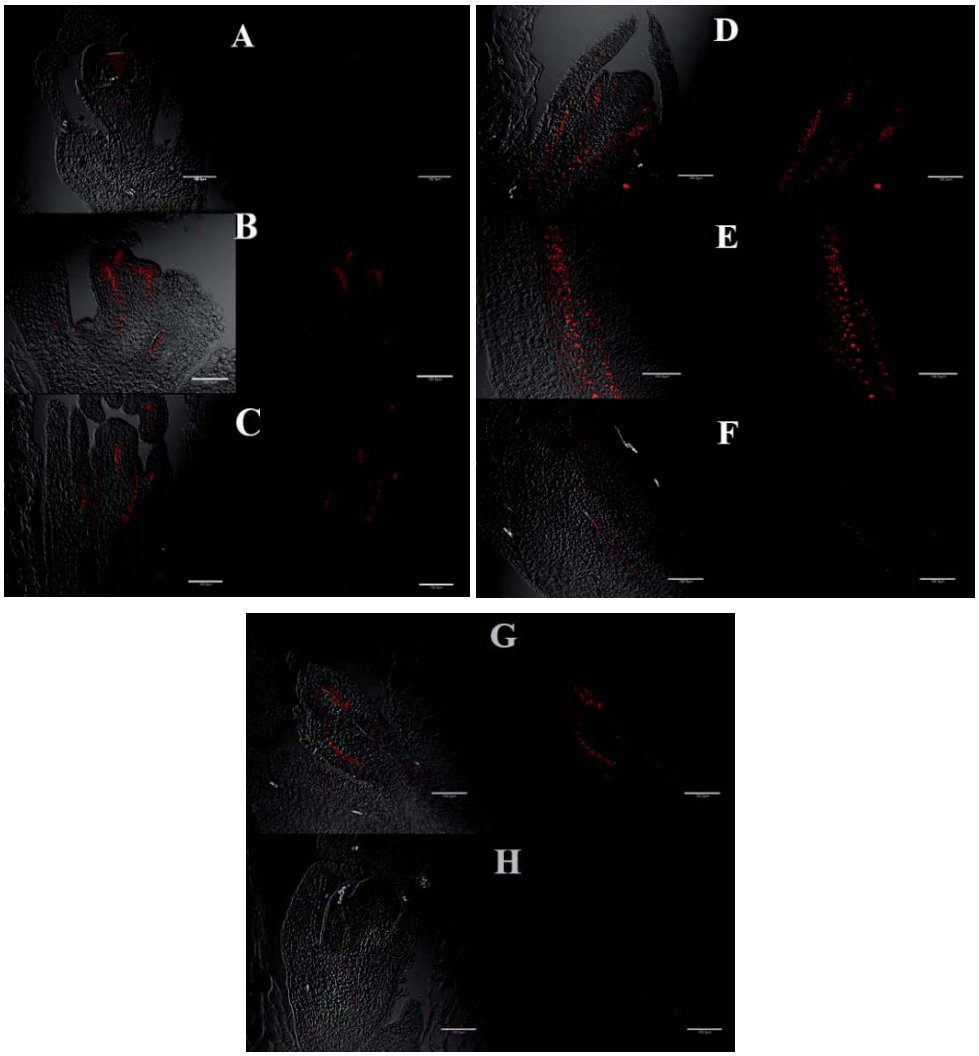
To prevent protein degradation, they were mounted in mounting medium after removal (Sauer et al. 2006). Object microscopy was performed on a BX 60 LSM Fluoview 300 confocal laser scanning microscope (Olympus, Japan) using a 568 nm krypton-argon laser and a BA 585IF barrier filter.

RESULTS AND DISCUSSION

The effect of cytokinin application to cotyledonary buds of intact pea plants was observed by confocal microscopy of immunolocalized PIN1 proteins.

Files of cells with polarly localized PIN1 protein clearly define channels with established polar auxin transport. In our experiment we observed, that while in the buds of intact plants the PIN1 proteins are not polarized (Figure 2A, 0 hours; Figure 2H, 120 hours nontreated plants), already after the first 6 hours after application of BAP, polarized PIN1 proteins can be observed that define the transport channels of auxin (Figure 2B). During 12–96 hours after the BAP application (Figure 2C–2G) to the cotyledonary buds this auxin channels are gradually increasing indicating the location of the future vasculature.

Figure 2 Imunolocalization of PIN1 proteins observed by confocal microscope showing polarization of PIN1 proteins in procambial cells in cotyledonary buds of intact pea plants in the time (A) 0 hours, (B) 6 hours, (C) 12 hours, (D) 24 hours, (E) 48 hours, (F) 72 hours, (G) 96 hours after application 1% BAP. Figure 2H; 120 hours, represents untreated bud from control plant. Objective 20x, scale is 100 μm.



Immunoassay of cotyledonary buds of intact plants showed that no PIN1 proteins were polarized in the apical region of the bud. However, after BAP application to these cotyledonary buds, it was possible to observe polar localization of PIN1 proteins already 6 hours after application. This indicates that the buds had been released from inhibition and that auxin transport proceeded in a similar way as is known in the axillary buds (Kalousek et al. 2010). The amount of polarized auxin transmembrane transporters PIN1 in the bud continued to increase during the time, and auxin transport channels were formed, the buds were released from apical dominance, and were growing out. It is known that polar auxin transport enabled by PIN1 protein polarization is essential for the whole process of axillary bud release from dormancy and outgrowth (Balla et al. 2011). Since polarization of PIN1 proteins occurred in the cotyledonary buds only a few hours after the BAP treatment, the results of the experiment indicate that cytokinin stimulates the polarization of PIN1 locally and has a direct effect on the initiation of polar auxin transport.

CONCLUSION

The results are in accordance with the theory of auxin canalization (Prusinkiewicz et al. 2009, Balla et al. 2011), and cytokinins have been observed to accelerate the polarization of PIN1 proteins and thereby initiate export of auxin from the axillary bud (Kalousek et al. 2010). This also provides explanations, that cytokinins initiate the growth of cotyledonary buds on intact plants by initiating polarization of PIN1 proteins, and consequently export of auxin from this bud.

ACKNOWLEDGEMENTS

This research was carried out under the project CEITEC 2020 (LQ1601) from the Ministry of Education, Youth and Sports of the Czech Republic under the National Sustainability Programme II, and by the project "CEITEC – Central European Institute of Technology" (CZ.1.05/1.1.00/02.0068).

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ANIMAL BIOLOGY

THE EFFECT OF CURCUMIN ON *IN VITRO* INDUCED BACTERIAL CONTAMINATION OF RABBIT EJACULATES BY *ENTEROCOCCUS FAECALIS*

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Abstract: The aim of this study was to compare the effect of traditionally used antibiotics (penicillin, gentamicin, kanamycin) with a selected bioactive substance (curcumin; CUR) on rabbit spermatozoa in the presence of *Enterococcus faecalis*. Rabbit sperm motility was analysed using the CASA (Computer-assisted sperm analysis) system. Production of reactive oxygen species (ROS) was determined by the chemiluminescence assay. Mitochondrial activity expressed through the mitochondrial membrane potential was analysed using the fluorimetric dye JC-1. Chromatin-dispersion (SCD) test was used to determine the DNA damage. At times of 0 and 6 hours, we observed changes in the structural integrity and functional activity of male reproductive cells. The CASA analysis showed that gentamicin was the most effective supplement, which significantly increased ($P < 0.001$) the motility after 6 hours. CUR increased the sperm motility with a significance level of $P < 0.01$. The results of the ROS analysis showed that CUR was the most capable in effectively neutralize the bacterium-induced oxidative stress ($P < 0.001$). At the same time, CUR was able to maintain the mitochondrial membrane potential with a significance level of $P < 0.01$. The results of the DNA fragmentation analysis indicate that the bacteria in combination with antibiotics significantly ($P < 0.01$ in case of gentamicin and kanamycin; $P < 0.05$ with respect to penicillin) damaged the DNA of male gametes. On the other hand, CUR significantly reduced ($P < 0.05$) DNA fragmentation. Among the selected experimental compounds CUR was able to preserve spermatozoa most effectively. We may conclude that CUR did not only equalize, but it also exceeded the effects achieved using antibiotics in three of the four assessments. Therefore, we may propose the use of curcumin as a supplement for semen extenders.

Key Words: Enterococcus faecalis, curcumin, bacterial infection, antibiotics, spermatozoa

INTRODUCTION

Bacterial contamination of ejaculates and its subsequent consequences on the sperm fertilizing potential is an intensively discussed topic. Clinical studies are not fully in compliance with the effect of bacteriospermia on the function of male gametes (Moretti et al. 2009, Barraud-Lange et al. 2011). The majority of urogenital infections remain asymptomatic (Vilvanathan et al. 2016). Collecting semen samples on animal farms is not a sterille process. Semen samples may be contaminated by bacteria, particularly during the storage, with a subsequent decrease of semen quality. Moreover, the health condition of recipient can be compromised. Prevention of the destructive effects of bacteria during semen preservation may be facilitated by the addition of antibiotics to semen extenders (Salamon and Maxwell 2000). In general, antibiotic therapies used in treatment of many diseases have baleful effects on fertility (Khaki 2015).

Due to the increasing resistance of bacteria to antibiotics, it is necessary to seek for alternatives. Except that curcumin abounds the antimicrobial effect, provides also antioxidant effect and improves spermatozoa vitality (Tvrdá et al. 2015). The aim of the study was to analyse the efficiency of a selected alternative biomolecule (CUR) and antibiotics, which are traditionally used in animal biotechnologies (penicillin, gentamicin, kanamycin), during the co-cultivation of rabbit spermatozoa with uropathogenic bacteria (*Enterococcus faecalis*) isolated from rabbit ejaculates.

MATERIAL AND METHODS

Rabbits possess numerous advantages in comparison to mice, rats or larger animals, such as their peaceful and compliant nature. Therefore, they are used as standard experimental subjects (Wang et al. 1998). Ejaculates from 12 rabbits (New Zealand white brolier line) were observed on a regular collection schedule using an artificial vagina in Animal Production Research Center Nitra (Slovak Republic). Immediately after collection, the sperm motility and concentration were assessed. A sample above 70% motility (> 5 μm/s) was considered to an acceptable sample. In laminar box were samples aliquoted in 100 μL into Petri dishes with growth medium and keep them in incubator (35 ± 2 °C) during 48–72 hours. By MALDI-TOF MS (Bruker Daltonics, USA) were identified *Enterococcus faecalis* in 10 from 19 isolated bacteria. We prepared liquid cultivation medium consisting of casein hydrolysate, beef infusion solids, starch and water (pH 7.4 ± 0.2; 25 °C). Bacteria were cultivated during 24–48 hours at the temperature 36 °C. The grown culture was diluted to 0.3 McF (DEN-1 McFarland Densitometer, Grant-bio, UK). The culture was divided to 4 experimental groups treated by antibiotics: penicillin (P) – 300 μg/mL, gentamicin (G) – 1000 μg/mL, kanamycin (K) – 80 μg/mL and curcumin (CUR)– 1 μmol/mL. As a control, we established 2 groups: positive control (PC) –with bacteria and negative control (NC) – without bacteria. In a ratio 1:30, we mix in ejaculates into each group and cultivated during 6 hours at 37 °C. Each methodical steps are focused on complex assessment of essential components of structural integrity and functional activity of male reproductive cells. Motility was assessed by Computer-asissted semen analysis (CASA, Version 14.0 TOX IVOS II.; Hamilton-Thorne Biosciences, Beverly, MA, USA) (Tvrdá et al. 2016a).

ROS generation was assessed by the chemiluminescent assay using luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma-Aldrich) as the reagent. We used 96-well plates, while blank, negative and positive control were measured in triplicates. Blank consisted of 100 μL of PBS (Dulbecco's Phosphate Buffer Saline, Sigma-Aldrich, USA), negative control contained (except for 100 μL of PBS) 2.5 μL of luminol. Positive control contained 100 μL PBS, 2.5 μL luminol and 12.5 μL of 30% H_2O_2 (8.8 M; Sigma-Aldrich). Chemiluminescence was measured 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/10^6 sperm (Tvrdá et al. 2016b).

Mitochondrial membrane potential is an important marker of mitochondrial function and was measured by Mitochondrial Membrane Potential Assay Kit JC-1 (Abnova, Taiwan). Approximately 1 million cells were colored by JC-1 dye. After 30 min. of incubation (37 °C) the samples were centrifuged (Hettich Rotina 420, Deutschland) during 5 min. at 2100 rpm. Subsequently, supernatant was removed and pellet was gently washed with buffer solution (200 μL). These steps are repeated until the third centrifugation. Then supernatant is removed and pellet was resuspended with 100 μL and quantitatively displaced into a black 96-well plate. The samples were analysed by Glomax Multi$^+$ (Promega, USA) using a blue filter, subsequently via a green filter. The results are expressed as a ratio of JC-1 complexes to JC-1 monomers (Zorova et al. 2017).

DNA fragmentation (SCD test) were assessed through the Halomax Kit (Halotech, Spain). The SCD test is based on controlled DNA denaturation process in agarose matrix to facilitate the subsequent removal of the proteins contained in each spermatozoon. The result after DNA dyeing is the presence/absence of the halo effect around fragmented/compact DNA (Choucair et al. 2016).

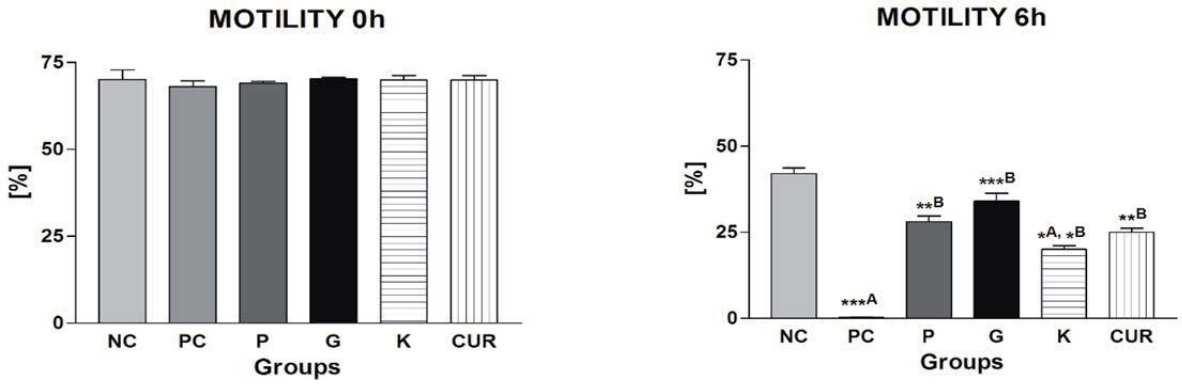
All data were subjected to statistical analysis using the GraphPad Prism program (version 6.0 for Windows, Graphpad Software incorporated, San Diego, California, USA, www.graphpad.com). Results are quoted as arithmetic mean ± standard error of mean (SEM). Differences between control and experimental groups were statistically evaluated by One-Way ANOVA with the Dunnet's comparison test. The level of sifnificance for the comparative analysis was set at * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

RESULTS AND DISCUSSION

After 6 hours spermatozoa in the PC were nearly immotile. In contrast, NC showed over 40% motility. Fraczek et al. (2014) simulated in vitro bacterial infection and tested fertilization potential of male reproductive cells. The effect of bacterial species significantly decreased the sperm motility. In

the group treated with gentamicin we observed the highest motility (34,00 ± 4,26%) among the experimental groups. On the other hand, Kováčová (2015) noticed that gentamicin (1000 µg/mL) can decrease sperm motility already after 1 hour. In the group treated with CUR after 6 hours we observed a significant increase ($P < 0.01$) of motility against PC.

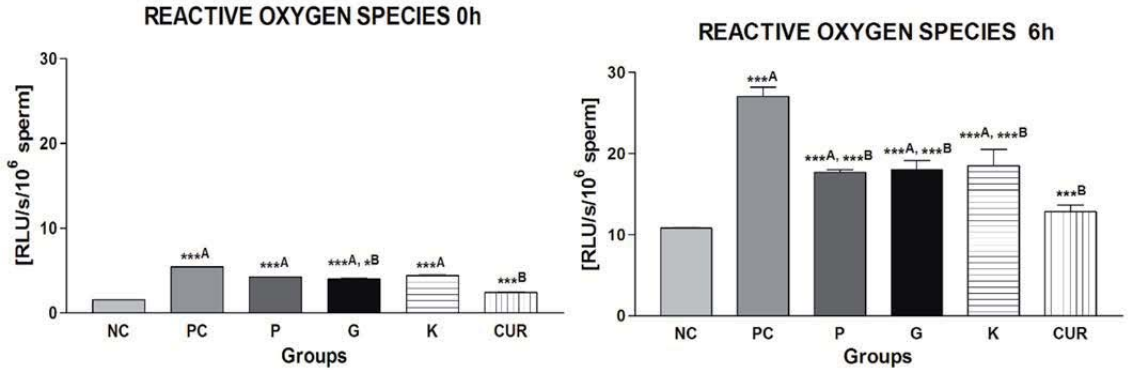
Figure 1 Comparation of motility results of Control and Experimental groups assessed at initial time and after 6 hours



*Legend: NC – negative control; PC – positive control; P – group treated with penicillin; G – group treated with gentamicin; K – group treated with kanamycin; CUR – group treated with curcumin; * P < 0.05; ** P < 0.01; *** P < 0.001*

Already at initial time, a significantly increased ($P < 0.001$) ROS generation was recorded in all experimental groups compared to NC, except for the group treated with curcumin. Tvrdá et al. (2016b) discussed about the antioxidant and motion-maintaining effect of curcumin, which correlates with our results. The selected antibiotics did not reach as good antioxidant effect as curcumin. That may be caused by inducing complex redox alterations that contribute to cellular damage and death (Dwyer et al. 2014).

Figure 2 Comparation of ROS generation results of Control and Experimental groups assessed at initial time and after 6 hours

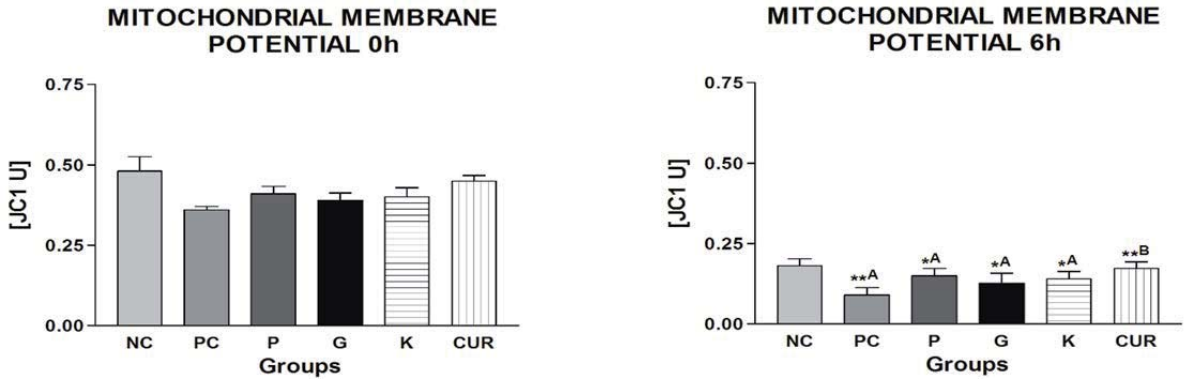


*Legend: NC – negative control; PC – positive control; P – group treated with penicillin; G – group treated with gentamicin; K – group treated with kanamycin; CUR – group treated with curcumin; * P < 0.05; ** P < 0.01; *** P < 0.001*

The assessment of the mitochondrial membrane potential showed a significant increase ($P < 0.01$) of JC-1 units in the group treated with curcumin compared with PC. The selected antibiotics were not effective enough in the maintance of mitochondrial membrane potential.

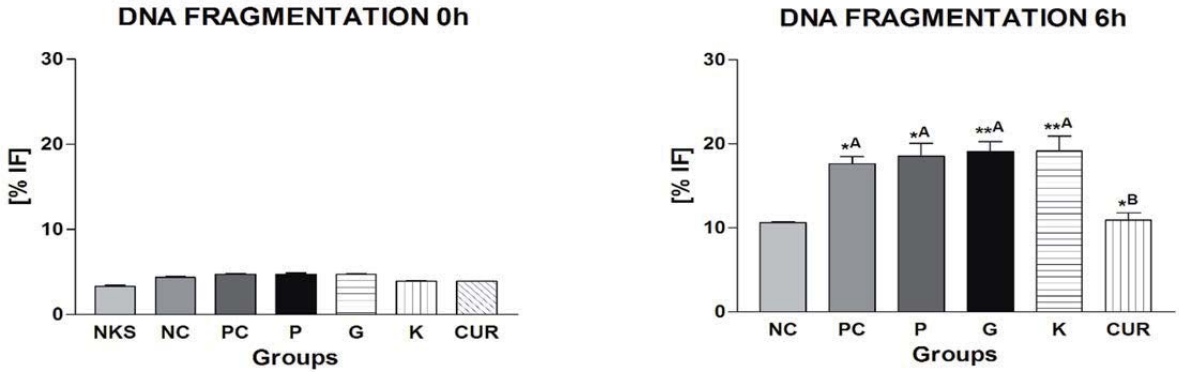
Analysis of DNA fragmentation showed a destructive effect of bacteriospermia. Surprisingly, the selected antibiotics did not decrease the fragmentation index, conversely, they increased the percentage of fragmentation index. CUR remarkably decreased ($P < 0.05$) the fragmentation index and therefore was able to protect the structure of the DNA molecule against deleterious effects of oxidative stress. Kalghatgi et al. (2013) pointed out detrimental side effects of antibiotic treatment, including oxidative stress leading to damage of membrane lipids, proteins and DNA.

Figure 3 Comparation of Mitochondrial membrane potential results of Control and Experimental groups assessed at initial time and after 6 hours



*Legend: NC – negative control; PC – positive control; P – group treated with penicillin; G – group treated with gentamicin; K – group treated with kanamycin; CUR – group treated with curcumin; * P < 0.05; ** P < 0.01; *** P < 0.001*

Figure 4 Comparation of DNA fragmentation results of Control and Experimental groups assessed at initial time and after 6 hours



*Legend: NC – negative control; PC – positive control; P – group treated with penicillin; G – group treated with gentamicin; K – group treated with kanamycin; CUR – group treated with curcumin; * P < 0.05; ** P < 0.01; *** P < 0.001*

CONCLUSION

Our results showed that *E. faecalis* considerably destructs spermatoza in each observed markers of the cell damage. By adding antibiotics or curcumin, we were able to affect the viability of spermatozoa. On the basis of the obtained results, we may conclude that the protective effects of curcumin in a global aspect outweighed the protective effects of selected antibiotics in three of the four observed parameters. Due to the constantly increasing bacterial resistance against traditional antibiotics commonly used in semen extenders, it is necessary to re-evaluate their usage and search for new supplements that would effectively supress bacterial colonization or to provide a particular advantage, thereby prolonging the viability of male reproductive cells. Of course, further studies are needed.

ACKNOWLEDGEMENTS

The research was financially supported by the Slovak Research and Development Agency Grant no. APVV-15-0544 and by the Research Centre AgroBioTech built in accordance with the project Building Research Centre „AgroBioTech“ ITMS 26220220180.

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MICROELEMENTS AND MACROELEMENTS IN SEMINAL PLASMA AFFECT OXIDATIVE BALANCE OF STALLION SEMEN

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Abstract: The concentrations of seminal elements can indicate the physiological balance in the body. Imbalance between the levels of reactive oxygen species (ROS) and the levels of antioxidant systems is called oxidative stress (OS). The objective of this study was to evaluate correlations between macroelements and microelements, which have impact to oxidative balance in seminal plasma of stallions. Fresh semen was obtained from 12 breeding stallions at National Stud Farm in Topoľčianky in age of 5–26 years composed of following breeds: Arab thoroughbred, Holsteiner, Hucul, Lipican, Selle francaise, Shagya-arab. Horses were stabled in boxes with bedding straw and feed with oat and hay. Semen was collected by the trained veterinarian equipped with lubricated pre-warmed artificial vagina at the start of the breeding season in February. Monitored results show a significant correlation between production of reactive oxygen species (ROS), malondialdehyde production (MDA) and magnesium (each $p < 0.01$). Furthermore, Fe correlated very significantly with total antioxidant status (TAS) ($p < 0.001$). Also Fe correlated significantly with protein carbonyls (PC) ($p < 0.01$). Correlation analysis indicated positive correlation between copper and reactive oxygen species (ROS) ($p < 0.05$). Finally, these results indicate that seminal chemical elements influence protection against oxidative stress. At the same time, oxidative stress and spermatozoa abnormalities are caused by mineral imbalance in the seminal plasma.

Key Words: macroelements, microelements, oxidative stress, seminal plasma, stallions

INTRODUCTION

Seminal plasma is a complex mixture secreted from the testes, epididymis and accessory sex glands. It can influence spermatozoa morphology, motility, acrosome reaction and fertility (Asadpour 2012).

Seminal plasma includes many enzymes, proteins, lipids, microelements [copper (Cu), iron (Fe), zinc (Zn)] and macroelements [sodium (Na), potassium (K), calcium (Ca), magnesium (Mg)] (Zambelli et al. 2010). Some others are demanded in narrow limits [manganese (Mn), selenium (Se, cobalt (Co)] (Tvrda et al. 2013b). The osmotic balance is established by sodium (Na) and potassium (K) cations in the seminal plasma, while the calcium (Ca) is needed for stimulation of steroidogenesis in Leydig cells of the testis (Asadpour 2012). Imbalance between the levels of reactive oxygen species (ROS) and the levels of antioxidant systems is called oxidative stress (OS) (Patricio et al. 2016, Kawai et al. 2017). Lower concentrations of zinc in the organism for long-term induce oxidative stress, which

was confirmed on animal studies (Valko et al. 2005). According to study of Hsu and Guo (2002), the higher levels of oxidative stress as well as low levels of lead increased production of reactive oxygen species (ROS). Crucial element in cell physiology is magnesium which plays a role in spermatogenesis (Wong et al. 2001).

ROS represents a main factor in several physiological processes and in spermatozoa acts as trigger for the regulation of hyperactivation, sperm-oocyte binding as well as acrosome reaction. Nowadays, there are not enough studies about macroelements and microelements, which have impact on oxidative balance in seminal plasma (Agarwal et al. 2006, Rivlin et al. 2004).

Thus, the objective of this study was to evaluate correlations between macroelements (Ca, Na, K, Mg) and microelements (Zn, Fe, Cu) to oxidative balance in seminal plasma of stallions.

MATERIAL AND METHODS

Animals

Fresh semen was obtained from 12 breeding stallions at National Stud Farm in Topoľčianky in age of 5–26 years composed of following breeds: Arab thoroughbred, Holsteiner, Hucul, Lipican, Selle francaise, Shagya-arab. Horses were stabled in boxes with bedding straw and feed with oat and hay. Movement of horses was secured by carousel, where they walked one hour a day as well as by individual fields, where horses stay four hours a day.

Semen collection and processing

Stallions were sexually stimulated by the presence of mare situated close to the breeding phantom. Consequently, semen was collected by the trained veterinarian equipped with lubricated pre-warmed artificial vagina at the start of breeding season in February.

Samples were immediately after collection centrifuged (15 minutes at 10000 x g) and the supernatant was separated. Samples of seminal plasma were then stored at 7 °C to prevent further production of oxygen reactive species.

Detection of microelements and macroelements

The concentrations of the metals, Ca, Cu, Fe and Zn, were measured in samples following the oven drying (60C, SUP – 100W dryer, WAMED) and mineralization with hot nitric acid (65%, Baker Analyzed, JT Baker, Phillipsburg, NJ, USA) in the open mineralization system (Velp Scientifica DK20). Mineralized solutions were diluted with ultrapure water (18.2 MV cm at 25 °C, Direct-Q 3, Merck-Millipore, Germany) up to 10 mL and analyzed with a flame atomic absorption spectrometer (AAnalyst 200, Perkin Elmer, Waltham, MA, USA). The final results, after comparison with the limits of quantification and recalculations, were expressed as parts per million (ppm). Hg measurements were realized using automated Hg analyzer (NIC MA – 2) without the external mineralization (conducted in two repetitions). The final results, after comparison with the limits of quantification and recalculations, were expressed as parts per million (ppm) (Binkowski et al. 2016).

Assessment of oxidative balance

ROS production in each group was assessed by the chemiluminescence assay using luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione; Sigma-Aldrich) as the probe (Kashou et al. 2013). The test samples consisted of luminol (10 µL, 5 mM) and 400 µL of sample. Negative controls were prepared by replacing the spermatozoa suspension with 400 µL of each culture medium. Positive controls included 400 µL of each medium, 10 µL luminol and 50 µL hydrogen peroxide (30%; 8.8 M; Sigma-Aldrich). Chemiluminescence was measured on 48-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/10^6 spermatozoa cells (Tvrda et al. 2016).

Total antioxidant status (TAS)

An improved enhanced chemiluminescence antioxidant assay was applied for the determination of the total antioxidant capacity of the sample. The TAS assay is based on enhanced horse radish peroxidase (HRP) – catalyzed luminol chemiluminescence. To analysis 5–100 µmol/L Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; Sigma-Aldrich) was used as the standard,

while a signal reagent consisting of 0.1 mol/L Tris-HCl (Sigma-Aldrich), 12 mol/L H_2O_2 (Sigma-Aldrich), 41.8 mmol/L 4-iodophenol (Sigma-Aldrich) and 282.2 mmol/L luminol (Sigma-Aldrich) was used to induce the chemiluminiscent reaction. Chemiluminescence was measured on 96-well plates in 10 one minute lasting cycles using the Glomax Multi + Combined Spectro-Fluoro Luminometer (Promega Corporation). The results are expressed as relative light units (RLU's) (Duracka et al. 2016a, Tvrda et al. 2016).

Malondialdehyde (MDA)

The MDA content was assessed with the help of the TBARS assay, modified for a 96-well plates and ELISA reader. Each sample was treated with 5% sodium dodecyl sulfate, 0.53% tiobarbituric acid (TBA; Sigma Aldrich, St. Louis, USA) in 20% acetic acid adjusted with NaOH (Centralchem, Bratislava, Slovak Republic) to pH 3.5 and boiled at 90–100 ºC for 1 h. Subsequently, the samples were placed on ice for 10 min and centrifuged at 1750 × g for 10 min. Supernatant was used to measure the product formed by the reaction of MDA and TBA under high temperature and acidic conditions at 530–540 nm (Tvrda et al. 2013a).

Protein carbonyls (PC)

Carbonyl group quantification was done using the traditional 2,4-dinitrophenylhydrazine (DNPH) method. 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 11828 × g for 15 minutes. 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 re-suspended in 1 mL of 6 M guanidine-HCl (Sigma-Aldrich) before absorbance measurement at 360 nm. The molar absorption coefficient of 22000 1/M.cm was used to quantify the concentration of protein carbonyls groups. Protein carbonyls were expressed as nmol/L (Duracka et al. 2016b).

Statistical analysis

All data were analysed using the Statistical Analyses System (SAS 9.2. using of application Enterprise guide 5.1). Pearson's correlations between different parameters of macroelements and microelements to oxidative balance were used.

RESULTS AND DISCUSSION

The concentrations of seminal elements can indicate the physiological balance in the body (Massanyi et al. 2003). In our study selected elements in seminal plasma of stallions were analysed (see Table 1). Average level of microelements such as Zn and Fe were lower, but level of Cu was similar to results in the study of Massanyi et al. (2003). Concentration of Cu was higher in study of Usuga et al. (2017) than in our study. In the study of Pesch et al. (2006), the level of Na, K and Mg was higher, but level of Ca was lower compared to our results. In results of Tvrda et al. (2013b), who analysed bovine seminal plasma, level of Na, K and Mg was lower than in our study. According to Usuga et al. (2017), concentration of magnesium was approximately equal to our concentration. The concentrations of Pb in our study were similar to results, which reported Massanyi et al. (2003).

Table 2 displays the results of oxidative balance in stallion seminal plasma. Concentration of MDA in our results was higher than in the study of Tvrda et al. (2013b), where bull samples were analyzed (2.94 µmol/L). In contrary to results of El Sisy et al. (2016) level of MDA was higher compared to present study. Furthermore, Patricio et al. (2016) determined higher values of TAS 1530 [eq. µmol Trolox/L] in the human semen compared to our study. The level of ROS and PC was 97.8 [RLU/s/L] and 141.3 [nmol/L].

Correlation analyses

In our results a significant correlation between reactive oxygen species (ROS), malondialdehyde (MDA) and magnesium (each $p < 0.01$) was found. The analyses of Tvrda et al. (2013b) show

negative significant correlations between MDA and Mg. In study of Abdul-Rasheed (2010) positive associations between Mg and antioxidant markers were found. Monitored Mg correlations reflect the regulatory effect of the activity of Mg-dependent ATPase on the motility of the spermatozoa (Stegmayr et al. 1982).

Table 1 Concentration of selected elements in stallion seminal plasma (n = 12)

Elements	Mean	SD
Pb [mg/kg]	0.4	0.4
Ca [mg/kg]	236.9	136.5
Mg [mg/kg]	99.8	125.3
Zn [mg/kg]	64.0	107.9
K [mg/kg]	731.3	46.5
Na [mg/kg]	3601.3	115.0
Fe [mg/kg]	1.6	0.5
Cu [mg/kg]	1.2	0.5
Hg [mg/kg]	0.00	0.00

Legend: SD – standard deviation, Pb – lead, Ca – calcium, Mg – magnesium, Zn – zinc, K – potassium, Na – sodium, Fe – iron, Cu – copper, Hg – mercury.

Table 2 Level of oxidative parameters in stallion seminal plasma (n = 12)

Parameters	Mean	SD
MDA [µmol/L]	4.7	3.3
ROS [RLU/s/L]	97.8	47.6
PC [nmol/L]	141.3	101.4
TAS [eq. µmol Trolox/L]	381.0	407.6

Legend: SD – standard deviation, MDA – malondialdehyde, ROS – reactive oxygen species, PC – protein carbonyls, TAS – total antioxidant status.

Furthermore, Fe correlated very significantly with total antioxidant status (TAS) ($p < 0.001$). Also Fe correlated significantly with protein carbonyls (PC) ($p < 0.01$). Catalase, the enzyme catalyzing the hydrogen peroxide decomposition, contains ferric (Fe^{3+}) heme which is eminently engaged in catalase action what supports the importance of iron prevalence (Josephy and Mannervik, 2006).

The copper is an important element for various metalloproteins and metalloenzymes which are affected in energy or antioxidant metabolism (Usuga et al. 2017). In the study of Hamad et al. (2014) ionic form of copper and at high levels, this element could rapidly become toxic. Toxic effect of elevated cooper concentration on the seminiferous epithelium as well as on the immune system was observed in study of Vrzgulova et al. (1993).

Physiological concentrations of Fe and Cu suggested that they have stimulating impact on motility as well as antioxidant status of bovine semen (Tvrda et al. 2013b). Microelements such as iron and copper are part of the antioxidative system present in stallion seminal plasma. They are able to neutralize or remove certain ROS (Waheed et al. 2013). Correlation analysis indicated positive correlation between copper and reactive oxygen species (ROS) ($p < 0.05$). Variations of the Cu and Fe concentrations imply that physiological levels of both trace elements may stimulatively affect motility as well as antioxidant status of the semen (Tvrdá et al. 2013b). In other parameters there were positive and negative correlations, but without statistical signification (see Table 3). Based on these results, relation between selected elements and oxidative balance in the seminal plasma of stallion were stated.

Table 3 Correlations between selected elements and oxidative balance in stallion seminal plasma (n = 12).

	Pb [ppm]	Ca [ppm]	Mg [ppm]	Zn [ppm]	K [ppm]	Na [ppm]	Fe [ppm]	Cu [ppm]	Hg [ppm]
MDA [µmol/L]	0.331	-0.083	0.814**	-0.019	0.029	-0.151	-0.278	0.609	-0.082
ROS [RLU/s/L]	0.220	-0.028	0.844**	0.015	0.076	-0.114	-0.095	0.744*	-0.077
PC [nmol/L]	-0.188	-0.635	0.140	-0.203	-0.297	-0.320	0.804**	0.571	-0.202
TAS [eq. µmol Trolox/L]	-0.412	-0.510	0.027	-0.352	-0.261	-0.142	0.910***	0.541	-0.107

*Legend: The level of significance was set at *P < 0.05;**P < 0.01;***P < 0.001, MDA – malondialdehyde, ROS – reactive oxygen species, PC – protein carbonyls, TAS – total antioxidant status, Pb – lead, Ca – calcium, Mg – magnesium, Zn – zinc, K – potassium, Na – sodium, Fe – iron, Cu – copper, Hg – mercury.*

CONCLUSION

In conclusion, our results indicate that seminal chemical elements influence protection against oxidative stress. At the same time, oxidative stress and seminal abnormalities are caused by mineral imbalance in the seminal plasma. Therefore, based on the literature sources and found results it is highly believable that synergism of Mg, Fe and Cu act as the factor responsible for decreased formation of ROS, but higher amounts of copper stimulate certain forms of ROS.

ACKNOWLEDGEMENTS

The research was financially supported by the VEGA 1/0760/15, VEGA 1/0857/14, APVV-16-0289, APVV-15-0544, KEGA 006/SPU-4/2015 and AgroBioTech Research Centre built in accordance with the project Building „AgroBioTech" Research Centre ITMS 26220220180.

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INFLUENCE OF OESTRADIOL AND PROGESTERONE LEVELS ON THE NUMBER OF MAST CELLS IN THE FELINE MYOMETRIUM

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Abstract: The aim of the study was to describe the influence of the female sex hormone levels on the numbers of mast cells in the myometrium of queens. Samples were fixed in 4% formaldehyde solution. Mast cells were detected by toluidine blue staining. The highest number of mast cells was found in the follicular phase, when the oestradiol level was at its highest. The lowest number of mast cells was described in the luteal phase, when the progesterone level was at its highest. However, the differences between the stages were not significant. Weak positive correlation was described between the oestradiol level and the number of mast cells. To conclude, the numbers of mast cells were influenced by the sex hormone levels.

Key Words: cat, oestrous cycle, uterus

INTRODUCTION

A considerable attention was paid to the role of mast cells (MCs) in the female reproductive organs. The membranes of MCs contain receptors for oestradiol and progesterone. Their stimulation induces degranulation of MCs. Metalloproteinases and proteases of MCs degrade extracellular matrix, VEGF modulates neovascularization (Jensen et al. 2010). Histamine influences the implantation of conceptus, ovulation and regulation of blood flow in the placenta and contractility of the myometrium (Noor et al. 2010). MCs can participate in the induction of pre-term deliveries (Woidacki et al. 2013).

Distribution of MCs in the uterus in accordance to the stage of the oestrous cycle was described in women (Mori et al. 1997, Sivridis et al. 2001), bitches (Goericke-Pesch et al. 2010), mares (Walter et al. 2012), rats (Karaca et al. 2007), mice (Schmerse et al. 2014), buffalos (Shahrooz et al. 2005) and goats (Karaca et al. 2008, 2009).

The number of MCs in the uterus of buffalo increased in the luteal phase compared to the follicular phase (Shahrooz et al. 2005). The highest number of MCs in a goat was described in dioestrus and prooestrus, the lowest number in metoestrus (Karaca et al. 2008, 2009).

A positive correlation between the oestradiol concentration and the number of MCs in uterine cervix was described in a mare (Walter et al. 2012), and between progesterone level and number of MCs in the uterus of bitch (Goericke-Pesch et al. 2010). No differences in the number of MCs were found between the uterus of gravid and non-gravid mares 15 days after artificial insemination (Klein et al. 2016).

There was no significant difference in the number of MCs in the uterus of women between proliferative, secretory and menstrual phase according to Mori et al. (1997). Nevertheless, Sivridis et al. (2001) mentioned a significantly increased number of MCs at the end of the menstrual cycle.

The highest number of MCs in the endometrium of rats was found in oestrus, myometrium revealed the highest number of MCs in metoestrus (Karaca et al. 2007). The number of MCs was the highest during oestrus in mice (Schmerse et al. 2014).

A lower number of MCs in the uterus was proved in rats after bilateral ovariectomy compared to the control group. The following oestradiol administration increased the MC number (Jensen et al. 2010).

Significantly fewer MCs in the uterine horn on the site, on which there was a unilateral ovariectomy performed (ipsilaterally) were described in rats (Razi et al. 2010).

As to the queens, the effect of the estradiol and progesterone levels on the number of MCs has been described in ovaries. The lowest number was detected in anoestrus when the levels of hormones were basal (Hamouzova et al. 2017).

The aim of this study was to determine the association between the number of MCs in the feline myometrium and the levels of the female sex hormones.

MATERIAL AND METHODS

All animals, whose uteruses were used were kept as pets and were castrated because of preventive and therapeutic reasons. Blood collection was performed for routine preoperative examination. Eleven samples of uterine horns in anoestrus and in follicular phase were used. Nine samples of uterine horns in luteal phase were used. Queens at a developed stage of gravidity were excluded from the study.

The samples were immediately fixed in 4% formaldehyde solution, subsequently dehydrated in a graded alcohol series, acetone and xylene, then infiltrated with hot paraffin and embedded in paraffin wax. Three to four μm thin sections were cut in a routine manner.

The sections were stained with acidified toluidine blue (0.5% solution in 0.25 M HCl, pH 1.2) for 15 minutes. The stage of the oestrous cycle was determined by measuring oestradiol and progesterone levels in the blood serum. Uterine horns from cats with the oestradiol level lower than 20 pg/ml (= 73.42 pmol/l) were classified as anoestrous, uterine horns from cats with oestradiol level higher than 20 pg/ml (= 73.42 pmol/l) were considered to be in the follicular phase and uterine horns from cats with progesterone level higher than 1.5 ng/ml (= 4.77 nmol/l) were considered to be in the luteal phase. Mast cells were counted in the *stratum vasculare* of the at 400x magnification per 1 mm^2.

Results were statistically evaluated by the ANOVA method of analysis of variance. The differences between mean values were evaluated (after a previous error analysis and evaluation of the influential values) by the Tukey HSD test in the SAS computer program (SAS Institute, Carry, USA), version 9.4 at the level of significance $p = 0.05$. Correlation analysis was performed by Pearson's method.

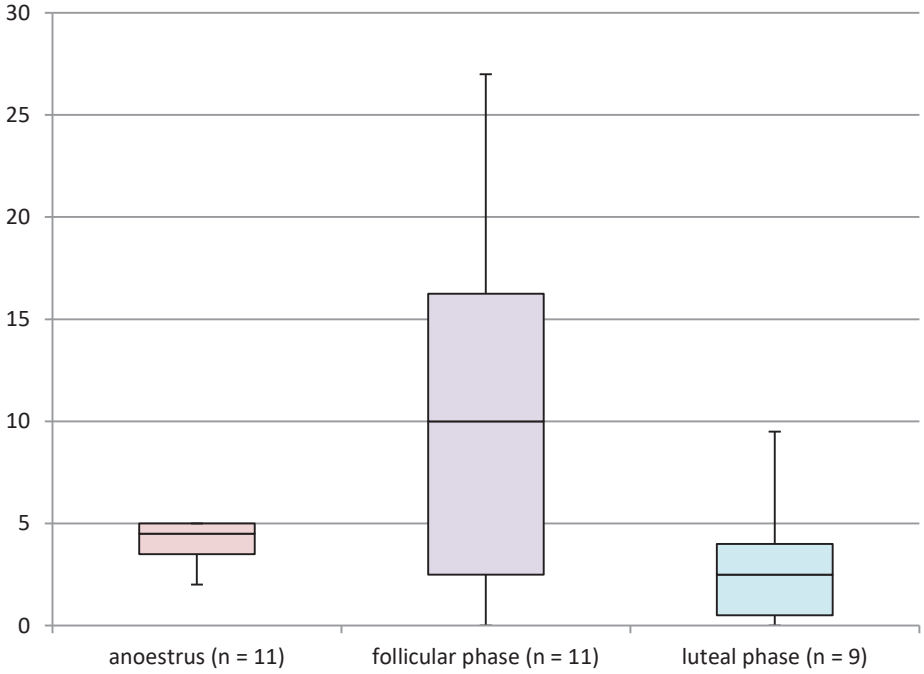
RESULTS AND DISCUSSION

The number of MCs in the *stratum vasculare* of the myometrium was 4.0 ± 1.2 in anoestrus, 11.8 ± 6.3 in the follicular phase and 2.7 ± 3.0 in the luteal phase (Table 1, Figure 1). Nevertheless, no significant difference between various phases of the oestrous cycle, determined by oestradiol and progesterone levels, was found ($p > 0.05$). There was a weak positive correlation (Pearson correlation coefficient = 0.39308, $p < 0.05$) between the oestradiol level and the number of MCs in the myometrium (Figure 2). No correlation was found between the progesterone level and the number of MCs in the myometrium (Figure 3).

Table 1 The number of MCs in various phases of the oestrous cycle

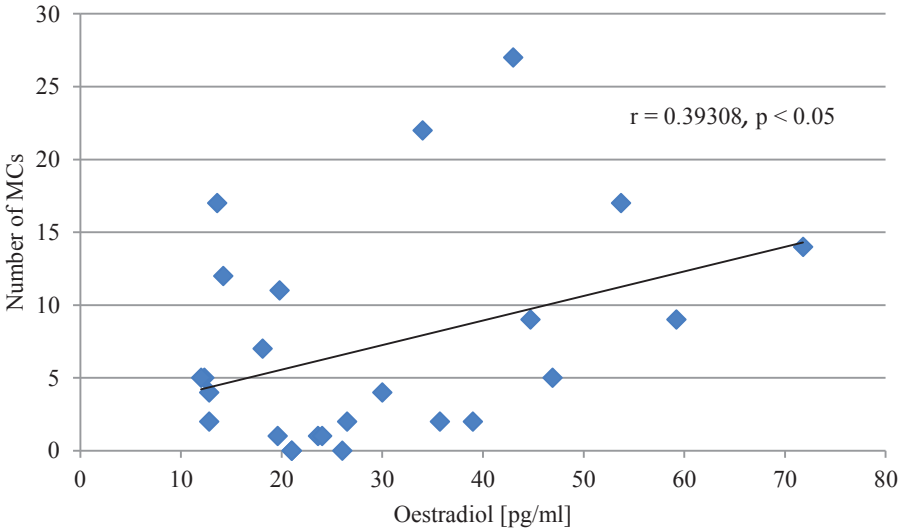
Stage of the oestrous cycle	Average number of MCs	Standard deviation
Anoestrus (n = 11)	4.0	1.2
Follicular phase (n = 11)	11.8	6.3
Luteal phase (n = 9)	2.7	3.0

Figure 1 The number of MCs in various phases of the oestrous cycle



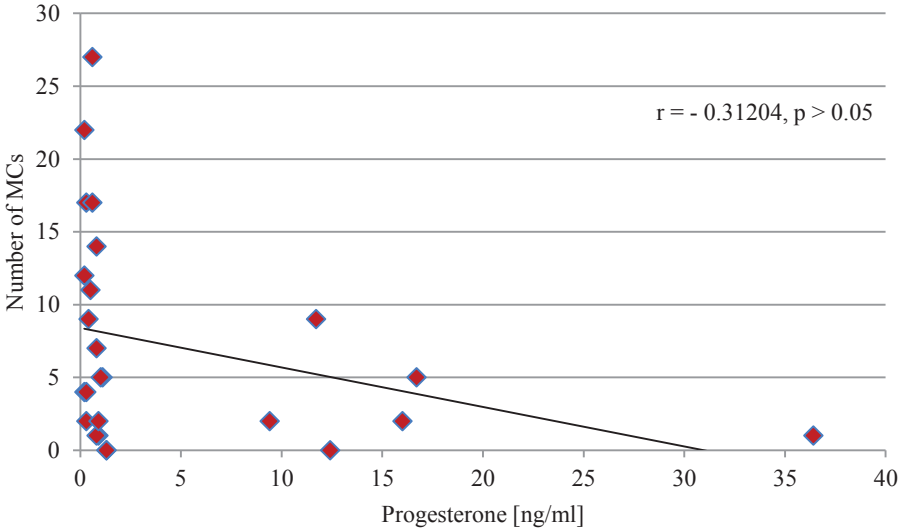
The figure shows the number of MCs in the myometrium in different stages of the oestrous cycle. In the anoestrus, the number of MCs was low in all cases, while in the follicular and luteal phase there were considerable differences between individual cats. In most cats in the luteal phase, the number of MCs was even lower than in anoestrus. In the follicular phase, the number of mast cells in most cats was high.

Figure 2 Dependence of the number of MCs in the myometrium on the oestradiol level



The figure shows a weak positive correlation between the number of MCs and the oestradiol level, as was confirmed by the statistical correlation analysis (Pearson correlation coefficient = 0.39308, p < 0.05).

Figure 3 Dependence of the number of MCs in the myometrium on the progesterone level



The figure shows that the number of mast cells is not correlated with the progesterone level (Pearson correlation coefficient = -0.31204, p > 0.05).

According to the results of the previous studies, a decrease of female sex hormone levels in the blood serum lead to a decrease of the number of MCs in the uterus of rats (Razi et al. 2010, Jensen et al. 2010) and mares (Walter et al. 2012). The same results were described in feline ovaries (Hamouzova et al. 2017).

The positive effect of the oestradiol on the number of MCs presented in our study was reported in rats (Jensen et al. 2010, Karaca et al. 2007), mice (Schmerse et al. 2014) and also in the feline ovaries (Hamouzova et al. 2017). We described a weak positive correlation between the oestradiol level and the number of MCs. The same results were found in a uterine cervix in a mare (Walter et al. 2012). However, in our study, the differences between the stages of the oestrous cycle were not significant. The reason could be that the oestrous cycle varies significantly in individual cats (i.e. spontaneous ovulation, pseudogravidity after ovulation without parturition, daylight length effect). Moreover, the queen is the only animal with induced ovulation in which the influence of female sex hormones on the number of MCs in uterus was described. It is difficult to compare the results and make conclusions due to this reason. Morever, the queen is not the only mammal without significant difference between the phases of the oestrous cycle. Mori et al. (1997) did not find any in women either.

The positive effect of the progesterone on the number of MCs reported in bitches (Goericke-Pesch et al. 2010) and buffalos (Shahrooz et al. 2005) was not found in queens. Nevertheless, the positive correlation between the progesterone level and the number of MCs bitches (Goericke-Pesch et al. 2010) was proved in uterine cervix but not in the uterine horns. Luteal phase in queens is very variable, from the first days after ovulation (induced or spontaneous) to different stages of the gravidity. Moreover, the pseudogravidity also can occur.

CONCLUSION

The number of MCs in *stratum vasculare* of the feline myometrium was influenced by the oestradiol level in the blood serum. Weak positive correlation was described between the oestradiol level in the blood serum and the number of MCs in the myometrium. No correlation was described between the progesterone level and the number of MCs. The highest number of MCs was found in the follicular phase, the lowest one in the luteal phase. Nevertheless, the differences between the numbers of MCs in the individual phases of the oestrous cycle were not significant.

ACKNOWLEDGEMENTS

Research reported in this publication was supported by IGA VFU (grant 101/2016).

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ASSOCIATION OF SELECTED GENES WITH MILK FAT IN TWO BREEDS OF CATTLE

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Abstract: The aim of the work was to evaluate genotype and allelic frequencies of selected genes affecting milk fat synthesis and to investigate possible association between gene polymorphisms and milk production traits. For this purpose, the analysis of *DGAT1* (diacylglycerol acyltransferase 1), *FASN* (fatty acid synthase) and *LEP* (leptin) was carried out on 754 dairy cows distributed in five farms located in the Czech Republic. The tested animals were divided into three groups: Czech Fleckvieh (CF; n=266), Holstein (H; n=329) and crossbreds (CB; n=159, with 87% of CF crossbreeds). The dominant genotypes for all observed groups were: *AA* genotype for *DGAT1* gene (0.962, 0.973 and 0.912; $p<0.01$), *GG* genotype for *FASN* gene (0.770, 0.662 and 0.774; $p<0.01$), and *MM* genotype for *LEP* gene (0.714, 0.783 and 0.826; $p>0.05$). The evaluation of milk production traits in the first lactation has shown, that the highest fat yield was found out in dairy cows with genotypes *AA* (327 kg), *AG* (330 kg) and *MM* (328 kg). The differences were statistically significant only for *DGAT1* gene. These results suggested, that milk from dairy cows with *DGAT1* gene *A* allelic variant would be more appropriate for the production of dairy products (butter, cheeses).

Key Words: DGAT1, *FASN*, *LEP*, milk fat, genetic polymorphism

INTRODUCTION

The content, composition, as well as physical-chemical properties of milk fat are important aspects in the production of dairy products. In comparison with other milk constituents, milk fat is the most variable compound, mainly influenced by feed factors (Kalac and Samkova 2010). Nevertheless, animal factors including genetic variability between -and within- breed have been also extensively studied over the last decades (Soyeurt et al. 2006, Samkova et al. 2012). The knowledge of genetic relationships plays a key role here (Goddard 2001, Dekkers 2012).

Milk fat belongs to quantitative traits whose composition and content are controlled by numerous genes. Among genes involved of milk fat biosynthesis belongs for example *DGAT1* (diacylglycerol acyltransferase 1), *GH* (growth-hormone), *GHR* (growth-hormone receptor), *SCD1* (stearoyl-CoA desaturase 1) – Buitenhuis et al. (2014) and Fontanesi et al. (2014), *FASN* (fatty acid synthase) – Alim et al. (2014), *LEP* (leptin) – Tomka et al. (2016).

DGAT1 gene encodes diacylglycerol O-acyltransferase 1, enzyme that catalyzes final step in biosynthesis process of triacylglycerols (TAGs) – Ibeagha-Awemu et al. (2008). TAGs, the main component of milk fat, are composed of glycerol and fatty acid (FA) esters. *DGAT1* gene is particularly associated with content of milk fat. Furthermore, according to Nafikov et al. (2014), mutations in acyltransferase genes might change their specificity for particular FA. It leads to changes in composition of milk fat, e.g. dinucleotide alleles of *DGAT1*: g.10433–10434 AA>GC mutation. This mutation results in substitution of lysine to alanine (K232A) in final protein (Grisart et al. 2002). Schennink et al. (2008) showed, that *A* allele was associated with a higher proportion of C18:1*c*9 (oleic acid), which is important from technological point of view (Hillbrick and Augustin 2002).

FASN gene encodes fatty acid synthase, a multifunctional enzyme that catalyses *de novo* synthesis of FA in mammals (Lock and Garnsworthy 2003). *FASN* gene, which is considered as

a probable candidate gene for milk production traits, is located within a linkage region harbouring quantitative trait loci (QTL) for milk fat (Alim et al. 2014). The studies on the bovine *FASN* gene structure have revealed occurrence of several single nucleotide polymorphisms (SNPs) linked to the fat content and FA composition in milk (Roy et al. 2006).

Also *LEP* gene is considered as a potential QTL, influencing different production traits in cattle, including fat content (Buchanan et al. 2003). SNP in *LEP* gene is associated with content and yield of fat and protein, milk yield or some reproductive traits (Tomka et al. 2016). Madeja et al. (2004) showed that the *LEP Hph*I gene polymorphism, C>T substitution of bases resulting in a change from alanine to valine (Haegeman et al. 2000) had an effect on the breeding value of milk production.

The aim of this work was to evaluate genotype and allelic frequencies of selected genes affecting milk fat synthesis in Czech Fleckvieh, Holstein and crossbreds and to investigate possible association between gene polymorphisms and milk production traits among cows in the first lactation.

MATERIAL AND METHODS

Experimental design

The analysis of selected genes (*DGAT1*, *FASN*, *LEP*) was carried out on 754 dairy cows distributed in five farms located in the Czech Republic. The tested animals were divided into three groups: 266 of Czech Fleckvieh (CF), 329 of Holstein (H) and 159 of crossbreeds with prevailing of CF crossbreds (87%).

Data for 305-day milk production from the first lactation including overall milk yield (kg), milk fat yield (kg), milk protein yield (kg) as well as contents of these components were collected from the official farm records based on monthly milking tests.

DNA extraction, amplification and sequencing

Genomic DNA from milk samples was isolated by Automated Nucleic Acid Extractor MagCore®HF16 Plus (RBC Bioscience Corp., Taiwan) with the aid of commercial blood kits. The genotyping of *A* and *L* alleles in *DGAT1*, *A* and *G* alleles in *FASN* and *M* and *W* alleles in *LEP* locus was done by the PCR-RFLP methods. PCR was done on a thermocycler Biometra T-1 Thermoblock (Biometra, Germany). For RFLP analysis, the amplified DNA was digested with restriction enzymes specific for tested gene (Table 1).

Table 1 Characteristics of tested genes and parameters of PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism) analysis

Gene	Number of sampling	Product size (bp)	Primer sequences	RE[1]
DGAT1	754	411	F 5′- CAA GGC CAA GGC TGG TGA G - 3′ R 5′- GGG GGC GAA GAG GAA GTA GTA G - 3′	*Cfr*I
FASN	752	382	F 5′- AGA GCT GAC GGA CTC CAC AC - 3′ R 5′- GCC GAT GCA CTC GAT GTA G - 3′	*Msc*I
LEP	643	331	F 5′- GGG AAG GGC AGA AAG ATA G - 3′ R 5′- TGG CAG ACT GTT GAG GAT C - 3′	*Hph*I

Legend: [1]RE – restriction endonuclease; DGAT1 – diacylglycerol acyltransferase 1, FASN – fatty acid synthase, LEP – leptin;

The individual fragments were separated by electrophoresis on an agarose gel with ethidium bromide and genotyped on a UV transiluminator in 302 nm wavelength.

Statistical analysis

The allele frequencies were calculated by simple allele counting. The differences between observed and expected frequencies of genotypes were tested using a χ^2 test in order to verify Hardy-Weinberg equilibrium.

Statistical analysis was done using the Statistica CZ 12 (Statsoft CR). Chi-squared test was used to compare the differences in genotype frequencies among the breeds. ANOVA and Student *t*-test were used to evaluate differences in milk production traits among the different genotypes.

RESULTS AND DISCUSSION

Gene frequency

Genetic variability is useful tool for breeding programmes. The degree of genetic variability is expressed by genotype and allelic frequencies. These frequencies were determined in the groups of purebred (CF and H) and in the group of their crossbreds. For the *DGAT1* gene and *FASN* gene, statistically significant differences in genotype frequencies among breeds were found. The differences in *LEP* gene were not significant for these breeds (Table 2). The dominant genotypes for all observed groups were: *AA* genotype for *DGAT1* gene (0.962, 0.973 and 0.912; $p<0.01$), *GG* genotype for *FASN* gene (0.770, 0.662 and 0.774; $p<0.01$), and *MM* genotype for *LEP* gene (0.714, 0.783 and 0.826; $p>0.05$).

Between the Holstein and Czech Fleckvieh purebred animals, the highest difference in relative frequencies of the dominant alleles was found in *FASN* gene (0.883 and 0.831). No differences were found in *DGAT1* gene (0.981 and 0.986). The crossbreds differed particularly in *LEP* gene. The relative frequency of dominant allele *M* was in this group 0.902, whereas 0.835 and 0.870 for CF and H. The breeds were in Hardy-Weinberg equilibrium for all the polymorphisms studied.

The genotype and allelic frequencies of *DGAT1*, *FASN* and *LEP* gene among various breeds (Holstein, Brown Swiss, Simmental, Swedish Red) were studied by several authors (Dokso et al. 2015, Tomka et al. 2016, Näslund et al. 2008 etc.). These authors also found out, that the relative frequencies among breeds differed. Milanesi et al. (2008) suggested, that observed and expected allelic frequencies could be associated with different selective purpose of breeds.

Polymorphism and milk production traits

Economically, fat and protein yield are important traits for production of dairy products. These traits were influenced mainly by milk yield, which values widely varied among genotypes of genes analysed in our group (Table 3). For the *DGAT1* gene, statistically significant differences were found out. The differences in *FASN* and *LEP* genes were not significant. The highest values of milk yield were found for *DGAT1* gene in genotype *AA* (7955 kg; $p<0.05$), for *FASN* gene in genotype *AG* (8030 kg) and for *LEP* gene in genotype *MM* (7989 kg). The values of fat and protein yield were also statistically significant for *DGAT1* gene in genotype *AA* (326.8 kg and 272.7 kg, respectively; $p<0.05$).

Association between genotypes and milk production traits was confirmed by several authors (Buchanan et al. 2003, Roy et al. 2006, Selvaggi et al. 2017). Dokso et al. (2015) found out, that dairy cows with *LA* genotype in *DGAT1* gene produced more milk compared to *AA* genotype. However, the observed differences were not significant. On the other hand, many authors (e.g. Thaller et al. 2003, Oikonomou et al. 2008) observed a positive effect of allele *A* in *DGAT1* gene on milk yield. Ciecierska et al. (2013) showed, that milk obtained from cows with the *FASN* gene with genotype *AA* was characterized by higher fat content. This was confirmed also by Schennink et al. (2009). Authors described, that milk collected from dairy cows with genotype *AA* was characterized by higher fat content than milk from dairy cows with *GG* genotype. Our results do not correlated with these authors. In our group statistically significant differences were not found for *FASN* gene. For *LEP* gene, the differences were also not statistically significant.

CONCLUSION

The genotype and allelic frequencies statistically differ among the groups of Czech Fleckvieh, Holstein and their crossbreds. For milk production traits (milk, fat and protein yield), statistically significant differences were found in *DGAT1* gene. The results showed, that there happened the selection in favour of *AA* genotype and *A* allele respectively, as they are linked with higher milk and protein yield in the dairy cattle.

Table 2 Frequencies of genes DGAT1 (diacylglycerol acyltransferase 1), FASN (fatty acid synthase) and LEP (leptin) according to group of dairy cows

	Breed															
	Czech Fleckvieh					Holstein					Crossbreeds[1]					*P*
DGAT1 (n = 754)	Genotype			Allele		Genotype			Allele		Genotype			Allele		
	AA	*LA*	*LL*	*A*	*L*	*AA*	*LA*	*LL*	*A*	*L*	*AA*	*LA*	*LL*	*A*	*L*	
Observed	256	10	0	522	10	320	9	0	649	9	145	14	0	304	14	
Relative	0.962	0.038	-	0.981	0.019	0.973	0.027	-	0.986	0.014	0.912	0.088	-	0.956	0.044	0.0074**
Expected	0.962	0.037	0	-	-	0.972	0.028	0	-	-	0.914	0.084	0.002	-	-	
χ^2	0.003ns					0.004ns					0.219ns					
FASN (n = 752)																
	GG	*AG*	*AA*	*G*	*A*	*GG*	*AG*	*AA*	*G*	*A*	*GG*	*AG*	*AA*	*G*	*A*	
Observed	204	60	1	468	62	217	111	0	545	111	123	35	1	281	37	
Relative	0.770	0.226	0.004	0.883	0.117	0.662	0.338	0	0.831	0.169	0.774	0.220	0.006	0.884	0.116	0.0083**
Expected	0.780	0.207	0.014	-	-	0.691	0.281	0.029	-	-	0.781	0.205	0.013	-	-	
χ^2	0.889ns					4.057ns					0.487ns					
LEP (n = 643)																
	MM	*MW*	*WW*	*M*	*W*	*MM*	*MW*	*WW*	*M*	*W*	*MM*	*MW*	*WW*	*M*	*W*	
Observed	160	54	10	374	74	220	49	12	489	73	114	21	3	249	27	
Relative	0.714	0.241	0.045	0.835	0.165	0.783	0.174	0.043	0.870	0.130	0.826	0.152	0.022	0.902	0.098	0.1232
Expected	0.697	0.276	0.027	-	-	0.757	0.226	0.017	-	-	0.814	0.177	0.010	-	-	
χ^2	1.644ns					5.174ns					1.793ns					

*Legend: [1]crossbreeds with prevailing of CF crossbreds (87%); P significancy of differences among breeds; **P<0.01; χ^2 – chi-square test among relative and expected genotype frequencies; nsnon-significant;*

Table 3 Milk production traits ($\bar{x} \pm s_x$) of different genotypes of DGAT1 (diacylglycerol acyltransferase 1), FASN (fatty acid synthase) and LEP (leptin) genes in dairy cows[1]

	DGAT1			*FASN*			*LEP*		
n	704	33	-	537	196	2	482	119	25
Genotype	*AA*	*LA*	*LL*	*GG*	*AG*	*AA*	*MM*	*MW*	*WW*
DIM[2]	294 ± 30	291 ± 28		293 ± 30	294 ± 30	291 ± 20	295 ± 29	292 ± 24	284 ± 59
Milk yield (kg)	7955[a] ± 2163	7153[b] ± 2075	-	7886 ± 2155	8030 ± 2195	5632 ± 1641	7989 ± 2153	7725 ± 2011	7482 ± 2654
Fat yield (kg)	326.8[a] ± 87.1	288.2[b] ± 84.6	-	323.6 ± 87.4	329.6 ± 87.4	252.5 ± 58.7	327.9 ± 85.5	317.7 ± 85.0	311.2 ± 113.0
Fat (%)	4.12 ± 0.33	4.02 ± 0.30	-	4.11 ± 0.33	4.12 ± 0.33	4.53 ± 0.28	4.12 ± 0.33	4.12 ± 0.34	4.11 ± 0.40
Protein yield (kg)	272.7[a] ± 65.2	244.6[b] ± 60.0	-	270.9 ± 65.2	273.3 ± 65.6	202.5 ± 50.2	272.7 ± 64.9	267.6 ± 61.3	257.2 ± 84.4
Protein (%)	3.46 ± 0.23	3.47 ± 0.26	-	3.47 ± 0.23	3.44 ± 0.22	3.62 ± 0.17	3.45[b] ± 0.23	3.50[a] ± 0.21	3.48 ± 0.24

Legend: [1]Czech Fleckvieh, Holstein and crossbreeds; [2]DIM – days in milk; [a,b]means with different superscripts in row differ within genotypes of each gene at $p<0.05$;

ACKNOWLEDGEMENTS

The research was supported by the Ministry of Agriculture of the Czech Republic (No. QJ1510336) and the Grant Agency of University of South Bohemia (GAJU-002/2016/Z).

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NEW MODIFICATION OF CULTIVATION MEDIUM FOR ISOLATION AND GROWTH OF INTESTINAL SULFATE-REDUCING BACTERIA

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Abstract: Different genera of sulfate-reducing bacteria (SRB) are always detected in the large intestine of humans and animals with diseases like an ulcerative colitis and Crohn's disease or even cancer. The final metabolism product of these anaerobic microorganisms is hydrogen sulfide which is known as a toxic substance and can lead to damage of epithelial cells of the bowel in high concentration. Some genera of the intestinal SRB included to the *Desulfovibrionaceae* family are hard to cultivate or even uncultivable. Isolation of these genera is also complicated because there are others satellite microorganisms. Up to now, Postgate's medium and other media do still not solve the cultivation problem and are created generally for *Desulfovibrio* species from nature environment but not for SRB species from the intestine. The object of our research was to modify the principle of isolation of intestinal SRB and cultivation medium based on their physiological and biochemical properties. Thus, there is no selective medium for intestine SRB which would improve cultivation and isolation of these important microorganisms. New created medium can be useful for more opportunities of intestinal SRB cultivation and understanding their involvement in inflammatory bowel diseases.

Key Words: sulfate-reducing bacteria, *Desulfovibrio*, cultivation media, modification of conditions, bacterial growth.

INTRODUCTION

Inflammatory bowel disease (IBD) including ulcerative colitis (UC) or Crohn's disease is characterized by chronic inflammation of the gut in genetically susceptible individuals of unknown etiology (Podolsky 2002, Schirbel and Fiocchi 2010). One of the hypothesis is, that UC is caused by the toxic molecule of hydrogen sulfide (H_2S). This compound in high concentration can lead to damaging of epithelial cells of human and animal large intestine (Kushkevych 2014a).

In persons, with rheumatic diseases, and with ankylosing spondylitis, etc. are often found sulfate-reducing bacteria (SRB) (Barton and Hamilton 2010, Sekirov et al. 2010), which in the increased numbers of them and intense process of dissimilatory sulfate reduction in the gut can cause these inflammatory bowel diseases (Loubinoux et al. 2002). Moreover, the increased number of SRB was found in feces from people with ulcerative colitis compared with healthy individuals (Gibson et al. 1991, 1993a, Levitt et al. 1999, Macfarlane et al. 2000). There is also an assumption that intestinal SRB can cause some forms of cancer of the rectum through the production of hydrogen sulfide which can affect the intestinal epithelial cells (Levitt et al. 1999). Because of this, SRB is important to study more in detail.

Sulfate-reducing bacteria are anaerobic microorganisms, which use sulfate as an electron acceptor in the process of dissimilatory sulfate reduction. This process is also called "dissimilatory sulfate respiration". To obtain energy and sulfate reduction, the electron donor is also necessary. Such electron donors in large intestinal can be lactate, ethanol, butyrate, succinate, acetate, propionate, pyruvate and some amino acids or even molecular hydrogen (Gibson et al. 1993b). All of these electron donors are the products of fermentations of following microorganisms, including genera *Clostridium*, *Escherichia*, *Saccharomyces*, *Bacteroides*, *Fusobacterium*, *Butyrivibrio*, and other. Described microbial genera can produce not only electron donors for SRB but also other important

biologically active substances, including vitamins or amino acids. On the other hand, the final product of SRB metabolism and their sulfate dissimilation is hydrogen sulfide.

SRB, especially *Desulfovibrio* genus, have been studied for over a century because of their ubiquity and their important roles in chemical processes and elemental cycles (Voordouw 1995). Also, *Desulfovibrio* genus is the most common species of SRB and its species are most often isolated from the large intestine of human and animals (Gibson et al. 1988, Moore et al. 1991).

Isolation of SRB from the mixture of human and animals' microbiota and their cultivation is also difficult. Some species of SRB such as *Bilophila wadsworthia* and *Lawsoni intracellularis* are uncultured. Other genera of the *Desulfovibrionaceae* family also grow poorly in cultivation medium or are uncultured. However, in our previous research, two genera of SRB isolated from human intestine and grew up well in cultivation medium were identified and described (Kushkevych 2013, Kushkevych et al. 2014d). It is known that in the intestine can be other genera of SRB and their species but isolation of which is complicated because not all intestinal SRB species can grow in classic Postgate medium or other media for cultivation of natural SRB isolated from the environment.

The aim of our research was to modify isolation conditions and create the optimal medium for cultivation of intestinal SRB based on their biochemical and physiological properties and conditions present in the large intestine of human and animals.

MATERIAL AND METHODS

Object of the study

Strains of SRB were isolated and identified from the large intestine of rats and have been kept in the collections of microorganisms in the Laboratory Anaerobic Microorganisms of Department of Experimental Biology at Masaryk University (Brno, Czech Republic). Other strains of SRB, *Desulfovibrio piger* were isolated from the healthy human large intestine as described previously (Kushkevych, 2013, Kushkevych et al. 2014b) and have also been kept in the same collection.

New modification of the medium for intestinal SRB growth, based on composition of well-known media described below and necessary conditions for SRB in bowel was created.

Media

Postgate's medium B which is a general-purpose medium for detecting and cultivating *Desulfovibrio* and *Desulfotomaculum* (pH was between 7.0 and 7.5).

Postgate's medium C which is a clear medium for biomass culture of *Desulfovibrio* (pH 7.5).

Postgate's medium E which is for isolation of pure cultures (pH 7.6).

The medium of Baars which gives a considerable precipitate after sterilization what is no problem in crude culture (pH 7.0–7.5).

Isolation of intestinal SRB

Samples were cultivated in sterile Eppendorf tubes full of liquid medium (pH 8.8 and flushed with N_2 to attain anaerobic condition) in the thermostat at +37 °C. Each positive SRB suspension was diluted in the same modified agar medium at temperature +43–45 °C and poured into plastic bags with a volume of 20 mL.

After cultivation, the black colonies grown in deep of the agar medium were selected and suspended in sterile saline (0.9% solution of NaCl). Suspensions with isolated colonies were pipetted (100 μL) in the standard media: one with sulfate (concentration 3.5 mM), one with elemental sulfur (without sulfate ions), and another medium without sulfate to be sure that the selected microorganisms belong to the SRB. Selected SRB was transferred to tempered Eppendorf tubes with the liquid medium. These procedures were 3–5 times repeated to selective and obtain pure cultures of intestinal SRB.

The contribution of new modified medium was verified by comparison of intestinal SRB growth rate in this medium and in media described above as well as the diversity of these microorganisms.

RESULTS AND DISCUSSION

As a result of our research is a comparison of a composition of different media for cultivation of SRB of various genera. It is known that mesophilic strains of SRB can grow up at temperature optimum around +30 °C but they can tolerate and grow up to +42 °C (Postgate 1984). On the other hand, the thermophilic strains are able to grow between temperatures from +50 to +70 °C (Kluyver and Baars 1932).

These microorganisms can tolerate pH from 5 to 9.5 and a wide range of osmotic conditions which all depend on the environment where they live (Postgate 1984). However, the pH range in the large intestine of humans or animals is limited and depend on many factors, including composition and enzymatic activity of intestinal microorganisms, substrates which they use, and the process of digestion and quality of consumed food. Basically, the pH in the human colon can be from neutral to alkaline (pH 7.6–8). Despite the wide range of temperatures of environmental SRB, their intestinal species always grow at temperature +37 °C what is a consistent temperature of the warm-blooded animal species and humans.

The composition of different cultivation media and composition of modified medium for isolation intestinal SRB is presented in Table 1.

Table 1 Composition of different cultivation media.

Salts (gram per liter)	Baars	Postgate B	Postgate C	Postgate E	Modified
Na_2SO_4	1	–	4.5	1	3
KH_2PO_4	–	0.5	0.5	0.5	0.3
K_2HPO_4	0.5	–	–	–	0.5
NH_4Cl	1	1	1	1	1
$CaCl_2 \times 6H_2O$	0.1	–	0.06	1	0.06
Yeast extract	–	1	1	1	1
Sodium Citrate $\times 2H_2O$	–	–	0.3	–	0.3
Sodium lactate	5	3.5	6	–	6
$MgSO_4 \times 7H_2O$	2	2	0.06	2	0.1
$CaSO_4$	–	1	–	–	–
Ascorbic acid	–	0.1	–	0.1	0.1
Thioglycolic acid	–	0.1	–	–	–
$(NH_4)_2SO_4$	–	–	–	–	0.2

The concentration of sulfate in the intestine depends on its introduction with food. Oxidized forms of sulfur including sulfate and sulfite are present in such food as commercial bread, dried fruits and vegetables, nuts, fermented beverages and brassica vegetable is sulfate mainly in the free anionic form. About 2–15 mM of sulfate is introduced with foods in human gastrointestinal tract every day. However, the concentration of sulfate ions in the feces is much lower and it is about 0.26 mM/day. It was also observed that 95% of sulfate is absorbed in the gastrointestinal tract and only 5% is in the remaining and detected in the feces (Florin et al. 1993). Other researchers have reported that absorption of sulfate by the human gastrointestinal tract is believed to be badly (Goodman and Gilman 1975, Wilson 1962). Apparently, such a concentration of sulfate (0.26 mM = 0.025 g/L) is sufficient for growth of SRB. This concentration was increased for initiation of SRB growth rate. Thus, the final concentration of the main acceptor in the modified medium is 3 g/L which corresponds 22.69 mM.

Another important factor for the SRB growth is present organic compounds which are electron donors in the process of sulfate reduction, carbon sources, and energy (Kushkevych et al. 2015a). The main electron donor in the large intestine is lactate which can be produced by lactic acid bacteria such genera as *Lactobacillus*, *Streptococcus*, *Bifidobacterium*, and other. Their final product of metabolism, lactic acid, is used by intestinal SRB. As was mentioned by Younes et al. (1996), the concentration of

lactic acid in the large intestine is approximately 80 mM. However, the concentration of this electron donor in the modified medium is 6 g/L which corresponds 53.54 mM.

A varied microflora can be observed during cultivation anaerobically, but comparatively few characteristic SRB can be seen (Butlin et al. 1948). Unfortunately, the contaminations are very persistent and cannot be eliminated by repeated transfer of inoculum to fresh medium. Both Baars (1930) and Starkey (1938) were unlucky with their methods for isolations of pure SRB culture, even by addition 4 ml of 10% of H_2S to 60 mL of medium. The addition of 3% $Na_2SO_3 \times 7H_2O$ to the medium has been shown even in the Butlin's research with halophilic SRB as a positive inhibition for others persistent neighboring contaminating colonies. Thus, at the beginning of the isolation of intestinal SRB, 15 g of Na_2SO_3 (concentration 118.98 mM) in the medium was added for inhibition other representatives of intestinal microbiota. SRB, except for sulfate, can use also another electron acceptor (sulfite) which involved enzymes of sulfate reduction (Kushkevych 2014c, 2015b,c, Kushkevych et al. 2014d, Kushkevych and Fafula 2014e).

One of the main chemical properties of ascorbic acid is that it is a reducing agent. Also, Borsook et al. (1937) have observed in electrometric measurements of the oxidation-reduction potential rapid negative potential drifts of ascorbic acid in pH higher than 6.0. Another important compound which can be used to lower the redox potential is $Na_2S_2O_4$ (30 mg/L what is corresponds 0.17 mM) (Langendijk et al. 2001).

Escherichia coli is an only part of microbiota which is involved in the process of digestion and is able to a synthesis of biologically active substances, its absorption, and synthesis of some vitamins, including vitamin K. It can be also important for the cultivation and growth of intestinal SRB. The addition of 1 mg of vitamin K_1 to the liquid cultivation medium compensate the presence of vitamin K as it is in the large intestine. Others vitamins are compensated by the addition of yeast extract

The compounds used for the modified medium are mainly used from Postgate's medium C which is used for mass cultivation. K_2HPO_4 which is used by Baars in cultivation media for thermophilic strains of SRB was used in our cultivation with +37 °C (Baars 1930). Ammonium ions occurred in a colon is compensated in the medium by the $(NH_4)_2SO_4$ without using more chloride ions. Also, the concentration of hydrogen sulfide is lowered to balance a growth. As reducing agents to lower redox potential was used only ascorbic acid instead of ascorbic acid and thioglycolic acid. However, the medium for isolation was not used Postgate's medium E but modified Postgate's medium C only with the addition of agar.

For the presence of the intestinal SRB during isolation, 10 ml of 10% solution (w/L) of Mohr's salt $[(NH_4)_2Fe(SO_4)_2 \times 6H_2O]$ should be used and added after sterilization to media. This salt is easily dissociated and free Fe^{2+} interact with H_2S produced in the process of dissimilatory sulfate reduction. As a result, black FeS complex is formed and positive bacterial colonies are black colored. However, it is known that H_2S can produce not only SRB but this property have also sulfur-reducing bacteria, including species of *Desulfurella* and *Desulfuromonas* genera, which use elemental sulfur or in some case sulfate as an electron acceptor (Kushkevych, 2013). Other intestinal bacteria which can produce H_2S are species of *Clostridium*, *Salmonella, Enterococcus, Enterobacter, Klebsiella* genera, and numerous of *Bacteroides* species via the expression of the iron flavoprotein sulfite reductase (Linden 2014). Given this fact it is important to confirm that isolated colonies belong to SRB. The confirmation of this type is a cultivation of the isolates in both medium without and with sulfate, and also only with the elemental sulfur but without sulfate. The cultivation of intestinal SRB was carried at +37 °C and pH 7.6 which consistent with pH of human colon lumen (Charalambides and Segal 1992).

As a result, new strains of intestinal SRB from the animals' large intestine were isolated. Based on their growth in different media and modified medium as well as their physiological and biochemical properties, it was confirmed and identified that all isolates belong to the SRB group. The perspective of our research is studies of physiological and biochemical properties of these isolates in detail. Moreover, the testing activity of different new synthesized compounds against intestinal SRB with their subsequent application as promising drugs for the treatment of bowel diseases is also perspective. Because in our previous research, newly synthesized salicylamide derivatives showed inhibition effect against intestinal SRB and process of dissimilatory sulfate reduction (Kushkevych et al. 2015d, 2016).

CONCLUSION

Based on the studied biochemical and physiology properties of intestinal SRB and their condition in the large intestine and comparing different media for cultivation of SRB, the modified optimal medium was created. The modified medium contains a bigger concentration of sulfate for higher growth rate. For inhibition of persistent contaminating colonies in crude culture was used sulfite, which helped with an isolation of SRB colonies without intestinal bacterial satellites, which can be species of *Bacteroides*, *Pseudomonas*, *Clostridium* genera or other microorganisms.

ACKNOWLEDGMENTS

This study was supported by Grant Agency of the Masaryk University (MUNI/A/0906/2016).

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INFLAMMATORY CYTOKINES PRODUCED BY LEUKOCYTES OF BOVINE MAMMARY GLAND

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Abstract: The aim of this work was to study inflammatory cytokines – the tumor necrosis factor alpha (TNF-α) and the interleukin 10 (IL-10), which are produced by bovine mammary gland leukocytes. Eight clinically healthy heifers were selected for this study. Obtained leukocytes were incubated for 1, 2 and 18 hours with lipopolysaccharide (LPS) or muramyldipeptide (MDP) under *in vitro* conditions. Concentration of TNF-α and IL-10 was measured by ELISA method. Production of TNF-α by leukocytes of bovine mammary gland was higher (LPS:552.57 pg/ml [±27.35]; MDP:680.20 pg/ml [±31.32]) than production of TNF-α by blood leukocytes (LPS:344.87 pg/ml [±23.12]; MDP:285.56 pg/ml [±19.87]). Concentration of TNF-α was increased after 18 hours of incubation. IL-10 production by bovine mammary gland leukocytes was higher (LPS:26.02 u/ml [±3.44] and 36.98 u/ml [±7.78]; MDP:22.33 u/ml [±3.02] and 33.45 u/ml [±8.28]) than production of IL-10 by blood leukocytes (LPS:12.67 u/ml [±2.47]; MDP:9.45 u/ml [±3.61]). IL-10 concentration of bovine mammary gland leukocytes was increased after 2 hours of incubation. Differential blood leukocyte count was determined by method of light microscopy.

Key Words: mammary gland, blood, immune system, cytokines, mastitis

INTRODUCTION

Mastitis (also known as mammary gland inflammation) is an important and economically serious production cow disease, especially those with market milk production (Kumar et al. 2017). Therefore, it is called multifactorial disease. Mostly, mastitis symptoms are triggered by these bacteria: *Staphylococcus aureus*, *Streptococcus agalastiae*, *Streptococcus dysgalactiae*, and *Streptococcus uberis* (Kubekova 2007).

The mammary gland inflammation is usually connected with high somatic cell count in milk yield. Approximately 99% of the somatic cells are leukocytes (Kubekova 2007). Two basic forms of mastitis – clinical and subclinical – are distinguished in breeding practice. Infection of the mammary gland can occur in several ways, for instance due to other animal disease (inflammation of the uterus, limb disease, organs disease), wounded skin of the udder, deficiency of close teat closure, poor nutrition, stress (thermal, psychic), breeding management (poor hygiene of breeding). Metabolic disorders play a significant role too. According to the source of infection, they are classified as mastitis from the environment and contagious mastitis.

Immunoregulation is one way how to improve the resistance of the mammary gland to mastitis. Cytokines are water soluble proteins which play key roles in many biological events related with inflammations and immunity (Dinarello 2007). The role of cytokines in pathophysiology has been the subject of many studies in recent years.

The aim of this work was to study inflammatory cytokines – the tumor necrosis factor alpha (TNF-α) and the interleukin 10 (IL-10), which are produced by bovine mammary gland leukocytes.

MATERIAL AND METHODS

Animal selection and trial design

The study procedures were focused on the analysis of the inflammatory cytokines TNF-α and IL-10. The cytokine detections were determined using ELISA method. Eight healthy heifers (16–18 months old) were selected for this study. The heifers were group housed in a tie-stall barn and fed a complete mixed diet. 1, 2 and 18 hours of incubation with LPS (lipopolysaccharide from *Escherichia coli*; concentration 50 μg/ml) or with MDP (muramyldipeptide; concentration 500 μg/ml) was performed in leukocytes obtained under *in vitro* condition (at 37 °C in 5% CO_2). LPS was used as a Gram-negative bacterial toxin. MDP was used as a component of a Gram-positive bacterial cell wall.

Sample collection procedures

Samples of cell population were obtained by lavage of the mammary gland 24 hours following the mammary gland stimulation by sterile buffered saline solution (PBS). In total, 10 ml of PBS was used. Fresh mammary gland leukocytes were adjusted (5 x 10^6 /ml) in medium (RPMI - 1640). The cell concentration was counted in a Bürker chambre in 20 large squares. The cells were smeared on glass slides and stained (Pappenheim). At least 200 leukocytes on each glass slide were counted to determine the differential cell count.

ELISA

Sandwich ELISA was used to determine concentration of TNF-α (Bovine TNF-α Screening; Endogen, Rockford, Illinois, USA) and IL-10 (Recombinant bovine IL-10, AbD Serotec, Bio-Rad Laboratories, USA) using Sunrise reader (Tecan, Austria).

Statistical analysis

The arithmetic mean (x) and ± standard deviation (SD) were calculated from the representation of individual leukocytes types (differential leukocyte count) and from their concentration. Significant differences were detected by pair t-test. Data were analysed with STATISTICA 7.1 (StatSoft CZ, Ltd., Czech Republic) program.

RESULTS AND DISCUSSION

Leukocytes value

Leukocyte count found in the bovine blood was 6.2 x 10^9 /l [±0.33] and in lavage 2.3 x 10^9 /l [±0.21].

Differential leukocyte count

The representation of individual leukocytes is shown in Table 1 and Table 2.

Table 1 Differential number of blood leukocytes after collection

Type of leukocytes	Arithmetic mean (x) [%]	Standard deviation (SD)
Lymphocytes	61.23	4.21
Neutrophil	32.33	2.17
Monocytes	4.27	0.61
Eosinophil	2.17	0.34

Concentration level of TNF-α

Concentration level of TNF-α is shown in Table 3 and Table 4.

Production of TNF-α by leukocytes of bovine mammary gland was higher (LPS:552.57 pg/ml [±27.35]; MDP:680.20 pg/ml [±31.32]) than production of this cytokines in leukocytes blood (LPS:344.87 pg/ml [±23.12]; MDP: 285.56 pg/ml [±19.87]).

Table 2 Differential cell count of mammary gland leukocytes (obtained by the mammary gland lavage 24 hours following stimulation of mammary gland by PBS)

Type of leukocytes	Arithmetic mean (x) [%]	Standard deviation (SD)
Lymphocytes	8.27	1.32
Neutrophil	85.89	7.79
Macrophages	5.32	0.89
Eosinophil	0.52	0.14

Table 3 Mammary gland and blood leukocytes concentration level of TNF-α following incubation with LPS (1, 2, and 18 hours of incubation)

Source of leukocytes	Incubation (hours)	Arithmetic mean (x) [%]	Standard deviation (SD)
Blood	1	0	-
	2	0	-
	18	344.87	23.12
Mammary gland	1	0	-
	2	0	-
	18	552.57	27.35

Table 4 Mammary gland and blood leukocytes concentration level of TNF-α following incubation with MDP (1, 2, and 18 hours of incubation)

Source of leukocytes	Incubation (hours)	Arithmetic mean (x) [%]	Standard deviation (SD)
Blood	1	0	-
	2	0	-
	18	285.56	19.87
Mammary gland	1	0	-
	2	0	-
	18	680.20	31.32

The higher concentration of TNF-α produced by leukocytes of bovine mammary gland is caused by higher neutrophil proportion. Neutrophils are major producers of TNF-α in bovine mammary gland which Sohn et al. (2007) say in their work. They found out higher concentration of TNF-α when clear population of blood neutrophils was used than we have obtained in our study with entire population of leukocytes under the same incubation conditions (medium RPMI, 5% CO_2 and 37 °C).

There is no production of TNF-α cytokines during 1 or 2 hours incubation. Concentration of TNF-α was detected with 18 hours incubation. Riollet et al. (2000) investigated of TNF-α during experimental infection induced by *E. coli* bacteria. Concentration of TNF-α has dramatically increased between 10 and 39 hours after induction of infection. Maximum level of TNF-α (14.1 ± 3.2 ng/ml) was observed between 14 and 18 hours. Concentration of TNF-α has gradually declined to its minimum after 24 hours. Bannerman et al. (2004) observed concentration of TNF-α during experimental mastitis induced by E. coli bacteria. The highest values were measured 16 hours after infection.

Concentration level of IL-10

Concentration level of IL-10 is shown in Table 5 and Table 6.

Table 5 Mammary gland and blood leukocytes concentration level of IL-10 following incubation with LPS (1, 2, and 18 hours of incubation)

Source of leukocytes	Incubation (hours)	Arithmetic mean (x) [%]	Standard deviation (SD)
Blood	1	0	-
	2	0	-
	18	12.67	2.47
Mammary gland	1	0	-
	2	26.02	3.44
	18	36.98	7.78

Table 6 Mammary gland and blood leukocytes concentration level of IL-10 following incubation with MDP (1, 2, and 18 hours of incubation)

Source of leukocytes	Incubation (hours)	Arithmetic mean (x) [%]	Standard deviation (SD)
Blood	1	0	-
	2	0	-
	18	9.45	3.61
Mammary gland	1	0	-
	2	22.33	3.02
	18	33.45	8.28

Production of IL-10 of leukocytes by bovine mammary gland was higher (LPS:26.02 u/ml [±3.44] and 36.98 u/ml [±7.78]; MDP: 22.33 u/ml [±3.02] and 33.45 u/ml [±8.28]) than production of IL-10 by blood leukocytes (LPS:12.67 u/ml [±2.47]; MDP:9.45 u/ml [±3.61]). IL-10 was produced already 2 hours after the beginning of the incubation of leukocytes of mammary gland.

The TNF-α was produced by blood leukocytes and leukocytes of mammary gland after 18 hours incubation. Level of IL-10 was increased after 18 hours incubation in blood leukocytes. Similarly to TNF-α, IL-10 concentration increases several hours after mastitis infection, which can be caused by different pathogens (Bannerman et al. 2004).

CONCLUSION

Mammary gland leukocytes production of TNF-α was higher than production of IL-10 by blood leukocytes. TNF-α was produced in blood and mammary gland leukocytes in 18 hours incubation. IL-10 was produced in 2 hours from inception of incubation by leukocytes of mammary gland. Level of IL-10 was increased in 18 hours of incubation in blood leukocytes. Knowledge of all net cytokines is important for effective using of cytokines during treatment of mastitis. For practical use detection of cytokines is important cheap and effective measured techniques, which can be used in practical application for control of health condition of mammary gland in dairy cows.

ACKNOWLEDGEMENTS

The research has been supported by the project TP 6/2017: Defectoscopic quality assessment of technical and organic materials; financed by IGA FA MENDELU.

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EXPRESSION OF KERATINE 8 AND ATP SYNTHASE SUBUNIT BETA GENES IN RELATION WITH BOAR TAINT

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Abstract: Animal welfare is becoming more and more important for the customers and one of the current topics is to ban the surgical castration of piglets for reducing boar taint. It is important to find new ways of reducing boar taint, one of them is genomic selection with the need of finding candidate genes involved in the skatole and androstenone metabolism. In our study, we have investigated different expression of genes *KRT8* and *ATP5B* in the pig´s liver among groups of surgically castrated and immunocastrated pigs compared to the control group of entire male pigs by qPCR. However, we have found no significant difference of gene expression between investigated groups.

Key Words: boar taint, qPCR, relative expression

INTRODUCTION

Boar taint is an unpleasant odour and flavour characteristic for uncastrated male pigs. It occurs while cooking the meat and has been described as faecal-like odour. The three main substances causing boar taint are androstenone, skatole and indole. There are differences of boar taint levels in tissue among different pig breeds, also the two compounds of boar taint skatole and androstenone contribute differently to the tainted carcass (Xue et al. 1996). According to the international consumer survey in seven European countries the consumers sensitivity to boar taint differs among countries, however there was a greater degree of dislike with the increase in androstenone and skatole level (Matthews et al. 2000).

Androstenone biosynthesis is controlled by the same mechanism as other testicular steroids and during puberty androstenone levels drastically increase simultaneously with other testicular steroids (Gower 1972, Bonneau 1982). Skatole levels also increase at puberty (Babol et al. 2004), probably after an increase of testicular steroids (Zamaratskaia et al. 2004).

Examination of multiple regression relations showed that the level of testicular hormones testosterone, estrone sulphate and free estrone in combination with testes and bulbourethral gland sizes were the best predictors of skatole in fat (Zamaratskaia et al. 2005). The boar is known for its high amounts of estrogens (Claus and Hoffmann 1980), which are produced in the Leydig cells and are positively correlated to skatole levels (Babol et al. 1999). There has been observed a relationship between metabolism of skatole and androstenone in liver which may be explained by the inhibition of enzymes metabolizing skatole by androstenone and other sex hormones (Babol et al. 1999).

To prevent this unpleasant smell of heated meat male piglets are surgically castrated shortly after birth (up to 7 days old), but due to welfare concerns there is a need for another method to prevent the unpleasant odour of meat. One of the methods is genomic selection where crucial part is to determine candidate genes with an influence on boar taint.

According to Gunawan et al. (2013) *ATP5B* and *KRT8* could be candidate genes for boar taint trait and they described significant differential expression of ATP synthase subunit beta (*ATP5B*) and keratin 8 (*KRT8*) in liver tissue of animals with different skatole levels.

KRT8 gene and its function is related to pathological functions in liver where mutation in this gene is involved in human liver disease (Ku et al. 2007), where KRT8 intermediate filaments can

modulate adhesion, size and cell – cycle progression of hepatic cells in association with differential plectin receptor of activated C kinase 1 (Galarneau et al. 2007), but its involvement in metabolism of boar taint is not clear.

ATP5B gene encodes the catalytic subunit of mitochondrial ATP synthesis complex and catalyzes the rate limiting step in formation of ATP in eukaryotic cells (Izquierdo 2006). It also plays role in the porcine skeletal muscle development.

Our aim was to explore whether *ATP5B* and *KRT8* expression levels in liver are affected by castration and if there are changes in the gene expression by comparing expression of these two genes between groups of surgically castrated pigs, immunocastrated pigs and entire male pigs (control group).

MATERIAL AND METHODS

Samples collection and isolation

Samples were collected from 30 male hybrid pigs used for commercial fattening in Czech Republic. The animals were separated into three groups: surgically castrated pigs (SC), immunocastrated pigs (IM) and entire male pigs (EM). Immunocastrated pigs were vaccinated by Improvac (Pfizer Animal Health, S.A., Belgium) according to manufacturer´s recommendations. Samples were collected from the liver and 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 transcribed 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 Power Sybr® Green master mix, 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 consisting of hold at 50 °C/2 min, denaturation at 95 °C/10 min and 40 cycles of 95 °C/10 min and 60 °C/1 min. This was followed by melting curve for verification of specificity of PCR products. Used primers are shown in table 1 and the primers for *ATP5B* and *KRT8* genes were designed for this study using OLIGO 4.0.

Table 1 Details of primers used for analysis

Gene symbol	Oligo sequence (5´→ 3´)	Amp. length	E (%)	Ref. seq.	Author
PPIA	CTGAGTGGTTGGATGGCAAA CCACAGTCAGCAATGGTGATCT	130	93	NM_214353	Svobodová 2011
TOP2B	CTAATGATGCTGGTGGCAAAC CCGATCACTCCTAGCCCAG	100	95	AF222921	Svobodová et al. 2008
TBP1	AACAGTTCAGTAGTTATGAGCCA GA AGATGTTCTCAAACGCTTCG	153	100	DQ845178	Nygart et al. 2007
ATP5B	CCTGTCTCAGCCATTCCAGGT GGTCATATTCACCTGCCAAAATC	113	91	ENSSSCT00000000438	
KRT8	AGGAGAGCAGGCTGGAGTCTG CCACCGGACTGGTAGGAGCT	130	100	ENSSSCT00000000271	

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 analysed by the software REST 2009 V2.0.13 (Qiagen, Hilden, Germany) where *PPIA*, *TOP2B* and *TBP1* are reference genes and *ATP5B* and *KRT8* are genes of interest. Group of entire male pigs was

used as a reference group. The hypothesis test used by REST 2009 software performs a large number of random reallocations of samples and controls between the groups. It then counts the number of times the relative expression of the randomly assigned group is greater than the sample data.

RESULTS AND DISCUSSION

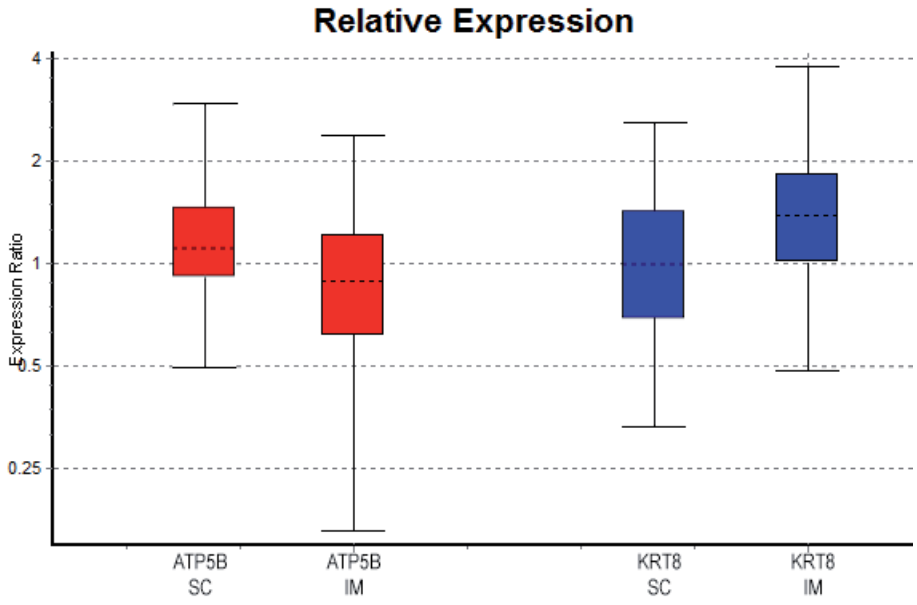
As mentioned above, group of entire male pigs was used as a reference group. The expression, standard error and *P(H1)* values are shown in table 2, where the *P(H1)* values for *ATP5B* gene were 0.225 in SC group and 0.254 in IM group, and for *KRT8* gene were 0.907 in SC group and 0.055 in IM group. Relative expressions with standard errors of *ATP5B* and *KRT8* of different groups are shown in Figure 1.

Table 2 ATP5B and KRT8 values of relative expression, standard error and P(H1) shown for surgically castrated and immunocastrated pigs compared to the control group

Group	Gene symbol	Expression	Standard error	*P(H1)*
Surgically castrated pigs	*ATP5B*	1.175	0.776–1.808	0.225
	KRT8	0.983	0.604–1.584	0.907
Immunocastrated pigs	*ATP5B*	0.760	0.343–1.430	0.254
	KRT8	1.373	0.870–2.207	0.055

Legend: P(H1) – hypothesis test, significant difference

Figure 1 Relative expression of ATP5B and KRT8 genes in different groups



Legend: SC – group of surgically castrated pigs, IM – group of immunocastrated pigs

Gunawan et al. (2013) studied differential gene expression among 448 genes in animals with divergent skatole levels where one of the two up regulated genes in high skatole liver sample was *KRT8*. Gregersen et al. (2012) identified segmental regions on *SSC5* to affect skatole and indole and *KRT8* gene is mapped close to this region (Gunawan et al. 2013). *ATP5B* gene was over expressed in liver from group of high skatole boars and is also functionally related to pathways involved in boar taint. (Gunawan et al. 2013). As far as we know, no other authors have studied these two genes and their different expression in relation to boar taint. Based on these findings, we can consider these genes as candidates for subsequent study of boar taint. However, we have found no significant difference in expression of *KRT8* and *ATP5B* genes between control group, immunocastrated group and surgically castrated group which suggests that the expression of these two genes is not affected by castration, but we did not have relevant data about skatole levels yet, but we would like to study this problem in the near future.

CONCLUSION

In our set of animals, we have found no statistically significant difference in expression of two selected genes (*ATP5B*, *KRT8*) in porcine liver between groups of pigs surgically castrated, immunocastrated and non-castrated.

ACKNOWLEDGEMENTS

The research was financially supported by the NAZV project (QJ1510233) and was carried out under the project CEITEC 2020 (LQ1601) with financial support from the Ministry of Education, Youth and Sports of the Czech Republic under the National Sustainability Programme II.

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NUCLEAR GENES CARBAMOYL PHOSPHATE SYNTHETASE AND ELONGATION FACTOR-1α AS TOOL FOR IDENTIFICATION OF INTRASPECIFIC GENE VARIATION IN CASE OF LIME HAWK-MOTH (*MIMAS TILIAE*)

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Abstract: Within the distributional areal of Lime Haw-Moth (*Mimas tiliae)* probably exist several subspecies, some of them are already described (*M. t. kitchingi)*. The aim of this pilot study was to verify usability of the nuclear genes *CAD* and *EF-1α* by the DNA barcoding method to distinguish between morphologically different subpopulations and that the DNA sequence of this genes corresponds to its geographic distribution. We have demonstrated that these *CAD* and *EF-1α* gene fragments are suitable for species identification, but not for subspecies differentiation. Relation to geographical distribution was not confirmed for *CAD* gene, but in case of *EF-1α* gene such a relationship can be seen.

Key Words: sphingidae, DNA barcoding, biodiversity, hawk-moth, nuclear genes

INTRODUCTION

Biodiversity is an essential element to protect life on the Earth. We know three main categories of biodiversity: gene diversity, species diversity and the complex ecosystem diversity. There are estimated 10 million plant and animal species, but only about 1.5 million are described (and more than 1 million are insects) in the World. DNA barcoding is taxonomical method using a very short DNA sequences from standard part of the genome and alignmenting them with sequencing databases. Can be easily be used even for species description (e.g. first vertebrate discovered via DNA barcoding was *Coryphopterus kuna* Victor 2007). This is method can be useful in cases where species are cryptic and morphologically undistinguishable. For these cases, there are now procedures and methods of molecular analysis to help scientists simplify, accelerate and refine the determination of individual species and possibly discover new species. Molecular taxonomy is a species classification system based on molecular analyses. These are primarily based on the testing of nuclear and mitochondrial DNA sequences in the case of animals and the comparison of sequences of individual genes among the species studied (Mayer et al. 2007). The main goal of molecular taxonomy is to elucidate the molecular relationships between organisms, while DNA barcoding deals primarily with the identification of unknown specimens (Kress et al. 2005). In animals, mitochondrial gene fragments for the cytochrome c oxidase (*COI*) subunit 1 (Krishnamurthy and Francis 2012) are most commonly used for identification, but the purpose of this study was to investigate *CAD* (carbamoyl phosphate synthetase) and *EF*-1α (elongation factor-1α) nuclear genes. To analyse the sequence results of these genes, online accessible bioinformatical tool such as BLAST (Basic Local Alignment Search Tool – available at http://blast.ncbi.nlm.nih.gov/Blast.cgi) were used. We choose *Mimas tiliae* (Linaeus 1758) as a model species for our study because it very large distributional areal (whole Western Europe, most Russia, Turkey, part of Iran) with strange distributional pattern in the east (see Pittaway 1997). There were described one species from the Far East (*Mimas christophi* Staudinger 1887) and one species from Elburz, Iran (*Mimas kitchingi* Melichar and Řezáč 2015), which is by some authors considered as a subspecies of *M. tiliae*. Our aim was to see how looks the genetic diversity within the species distributional areal and make an outlook to the subspecies taxonomy. Although there were published morphological differences, these could be because the species variability within the areal.

MATERIAL AND METHODS

Species

All samples for analysis were provided by staff at the Sphingidae Museum (Orlov, Czech Republic). Samples were divided into group based on morphological structure of the male genitalia: group 1 (F.24, F.27, F.28, F.46, F.82, F.83) and group 2 (F.16, F.31, F.35, F. 37, F.39, F.74, F.75, F.76, F.77, F.78, F.79, F.80, F.81). The distribution of individuals is listed in Table 1. These were the butterfly limbs, supplied with the exact species designation, the sample number, and the capture point of the butterfly that belonged to the limb. Isolation was performed using the Geneaid Genomic DNA Mini Kit designed to isolate DNA from the tissues and proceed according to the enclosed instructions. The amplification of the *CAD* and *EF-1α* nuclear genes was standardized according to protocols available online (www.top-bio.cz/files/1166_pl.pdf).

Table 1 Samples of Mimas tiliae divided into two groups

Group	Sample number	Geographical origin
1	F.24	Greece
1	F.27	Italy
1	F.28	Greece
1	F.46	Switzerland
1	F.82	Greece
1	F.83	Greece
2	F.16	Greece
2	F.31	Russia (Altai)
2	F.35	Kazakhstan
2	F.37	Finland
2	F.39	Russia
2	F.74	Czech republic
2	F.75	Czech republic
2	F.76	Czech republic
2	F.77	Czech republic
2	F.78	Czech republic
2	F.79	Northern Ural
2	F.80	Northern Ural
2	F.81	Northern Ural

DNA amplification

All PCRs were performed in the thermal cycler ABI Verity 96 Well (Applied Biosystems Inc., Foster City, CA, USA) with the following protocol: initial denaturation at 95 °C for 3 min; 30 cycles of denaturation at 95 °C for 40 s, annealing at 59 °C for 40 s and elongation at 72 °C for 1 min; final elongation at 72 °C for 10 min and holding at 4 °C.

DNA Sequencing and Data Analysis

The sequencing reaction mixture was mixed with a commercially available BigDye Terminator v3.1 Cycle Sequencing Kit from Applied Biosystems, and the mixture was mixed to a volume of 10 µl. The temperature cycling profile was 96 °C for 1 min, 25 cycles of 96 °C for 10 s, 50 °C for 5 s, 60 °C for 4 min. We then purified using DNA BigDye® Xterminator ™ Purification Kit from Applied Biosystems according to the instructions from the manufacturer. The data were analysed using and SeqScape Software v2.7 (Applied Biosystems Inc., Foster City, CA, USA) and MEGA7 to compare different bases and phylogenetic trees, which is freely available at http://www.megasoftware.net/. The evolutionary history was inferred using the Minimum Evolution method (Rzhetsky and Nei 1992). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm (Nei and Kumar 2000) at a search level of 1. The Neighbor-joining algorithm (Saitou and Nei 1987) was used to generate the initial tree. The analysis of *CAD* and *EF-1α* genes involved 13 and 19 nucleotide sequences, resp. All positions containing gaps and missing data were eliminated. There were in *CAD* and *EF-1α* genes a total of 675 and 563 positions in the final dataset, resp. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2015).

RESULTS AND DISCUSSION

Amplification of the *CAD* and *EF-1α* gene fragments

Primers were designed using the OLIGO v4.0 software. For CAD amplification, CAD_F1 + CAD_R1 primer combinations proved best. This combination of primers amplified a fragment of 708 bp in size. In the case of the *EF-1α* gene, the most suitable combinations of EF1α-F2 + EF-1α-R2 primers were shown to amplify 602 bp fragments.

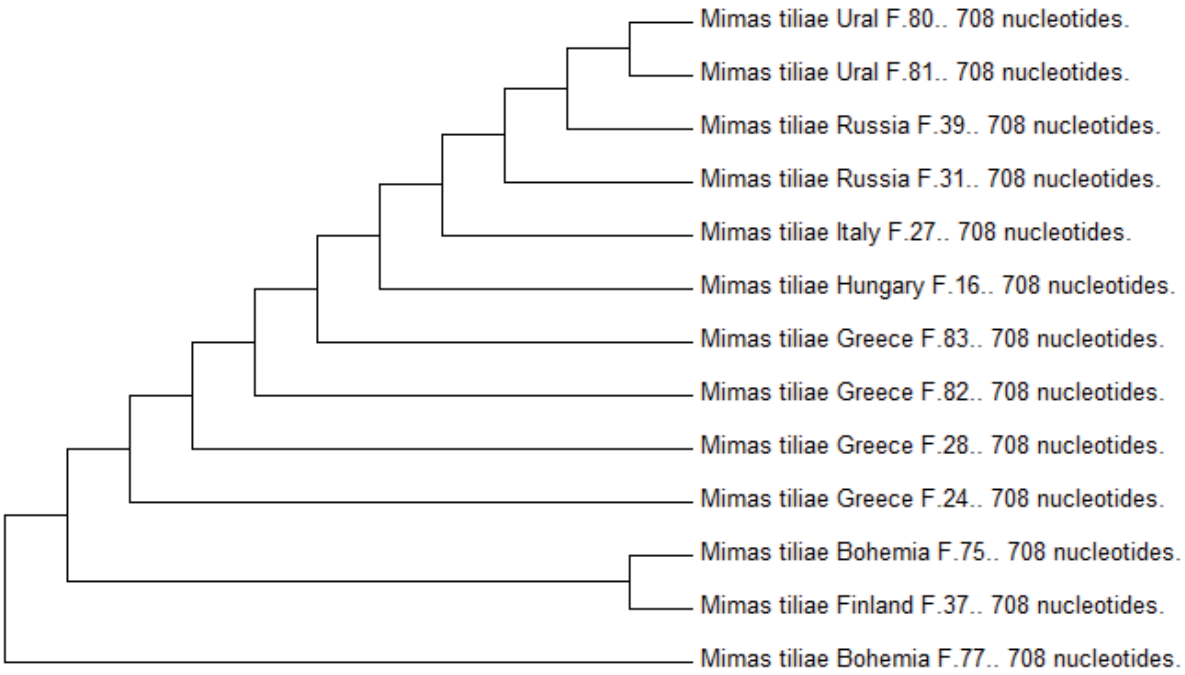
Table 2 Primers used for detection of CAD and EF-1α genes

Gene	Primers	Sequence 5'-3'	Lenght [bp]	GC [%]	PCR fragment [bp]
CAD	CAD_F1	TGG AAG TTC TAT GAA RAG TGT CG	23	43.5	708
	CAD_R1	AAG TAC AAT CTG TCR CTC ATG TC			
EF-1α	EF-1α_F2	CTC CTG GAC ACA GAG ATT TCA TCA A	25	44	602
	EF-1α_R2	CAC AGA CTT GAC TTC AGT GGT GAT G		48	

Phylogenetic analyses

Phylogenetic trees were created using the MEGA7 program and was used a minimal evolution method that divided individual samples into groups according to differences in individual nucleotides.

Figure 1 Evolutionary relationships of studied taxa using the Minimum Evolution method by CAD gene (The optimal tree with the sum of branch length = 0.10648939 is shown).



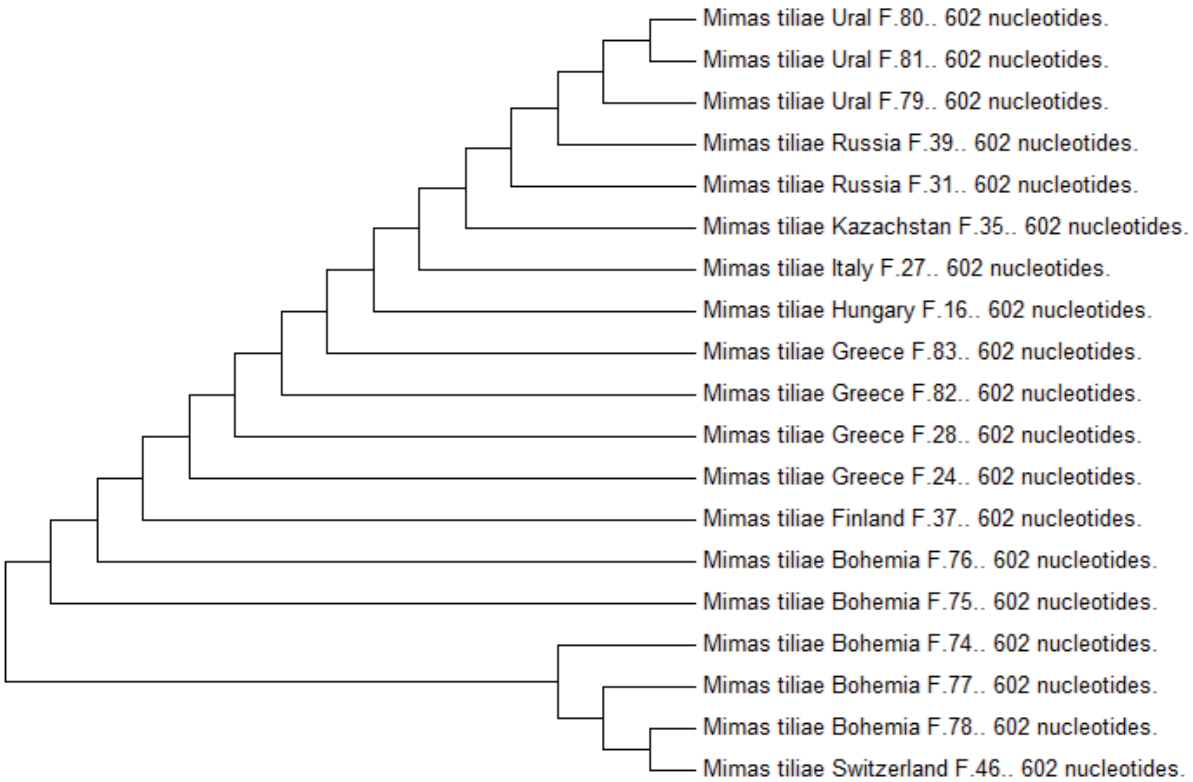
In the case of the *CAD* gene, groups 1 and 2 were not demonstrably split. From this we can assume that nuclear *CAD* gene is not suitable for the type of distinction representatives *Mimas tiliae*. Eastern and Western subpopulation also failed to distinguish. This result, however, is in contradiction with the work of Friedlander et al. which in 1992 introduced this nuclear gene as a candidate and Moulton and Wiegmann 2004, which states that the *CAD* gene (*CPS*) is suitable for phylogenetic examination of the Lepidoptera order. For this reason, it would be advisable to carry out an extended study that would include more species of the genus *Mimas*.

EF-1α gene

There is little bit different situation in case of *EF-1α*. The division into two different morphological groups has not been confirmed by *EF-1α* DNA analysis too, as in the case of *CAD* gene Although we have been able to divide all samples to two clades, but the position of the particular species

is questionable, because we have no this information (if samples from the Czech Republic are from west of east of the country). This is very important information because the position of the country on the boundary of two main populations – eastern and western, according the postglacial re-colonization history confirmed for many other species (e.g. Schmitt and Krauss 2004). The species lives on different broadleaf trees which were affected by glacier during last Ice Age. These trees survived this period in conditions of glacial refugee in the Balkan Peninsula, Pyrenean Peninsula and Apennine Peninsula. But without proper localization we cannot say that this method is/is not useful for this type of study. Although Kim (2010) states that this gene is not useful for our purpose because of the lack of properties needed to solve several levels of taxonomic hierarchy, we found different situation (possibly) prove possible Eastern and Western distributional patterns according historical refugees. Based on it more samples need to be investigated.

Figure 2 Evolutionary relationships of studied taxa using the Minimum Evolution method by EF-1α gene (The optimal tree with the sum of branch length = 0.03344021 is shown).



CONCLUSION

In this pilot study, we investigated whether the *CAD* and *EF-1α* nuclear genes are suitable for species identification of *Mimas tiliae*. Our task was to confirm or disprove this division using the above-mentioned genes and possibly determine the suitability of these genes for species identification. In the case of the *CAD* gene we have concluded that it is not possible to uniquely divide individual *Mimas tiliae* into subpopulations. This may indicate that the *CAD* gene is not entirely suited for the species identification of butterflies of the Sphingidae family. However, to be able to assert this with certainty, it would be necessary to test more samples of several different species for the result to be conclusive. The gene *EF-1α* shows little bit different situation. It didn't help us differ the populations well but we can indicate two main geographical clades according postglacial colonization. But for proper study we need analyse more samples from whole species distributional areal.

ACKNOWLEDGMENTS

We are especially grateful to colleagues at the Sphingidae Museum Orlov for providing samples. The research was carried out under the project CEITEC 2020 (LQ1601) with financial support from the

Ministry of Education, Youth and Sports of the Czech Republic under the National Sustainability Programme II.

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EFFECT OF DIETARY FATTY ACID COMPOSITION ON WEIGHT OF MODEL ANIMALS

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Abstract: The aim of the present study was to compare the effect of diet enriched with 6% fish oil (source of polyunsaturated fatty acids), 6% of safflower oil, 6% of oil from *Schyzichytrium* microalga oil and the effect of diet enriched with 6% of palm oil (source of saturated fatty acids; control) on weight of model animals. Like model animals were used 48 adult male *Rattus norvegicus* Wistar Albino. They were divided into 4 groups with 12 animals each and they were fed for 8.5 weeks. They were weighed every week. There were found no significant differences among all diet in feed intake and final live weight at the day of sacrifice. The only significant variety was observed in total weight gain. It was lower in SF-group in comparison with A-group, which is in contrast with our assumption. The duration of experiment was probably too short to show differences among diet interventions.

Key Words: obesity, safflower oil, fish oil, DHA, palm oil, *Rattus norvegicus*

INTRODUCTION

Obesity is one of the main risk factors contributing on development of many serious diseases. It goes together with higher risc of cardiovascular diseases. This study is focused on important differences among types of fats because of their fatty acid composition. It was compared the influence of safflower oil (source of long chained polyunsaturated fatty acids n-6 – LC-PUFA n-6, SF-diet), palm oil (source of LC-PUFA n-6 which was used as a control group, P-diet), fish oil (source of LC-PUFA n-3, respectively of eicosapentoenoic fatty acid, EPA, 20 : 5n-3) and oil from *Schizochytrium* microalga oil (source of LC-PUFA n-3, mainly of dokosahexaenoic fatty acid, DHA, 22 : 6n-3). EPA and DHA are important parts of cell membranes, they influence their fluidity and behavior of the integral membrane proteins (Schitz and Ecker 2008). If it comes to our eating habits there is high difference in intake of PUFA n-6 and PUFA n-3. It is recommended to consump them in ratio 1 : 1–1 : 2, but the reality is on the other side and we eat them in ratio 1 : 15–1 : 20, it is called western type of diet. Mainly founded fatty acid in this diet are PUFA n-6, the key metabolite is arachidonic acid (AA, 20 : 4n-6). This ratio can contribute to higher risc of chronical degenerative diseases. EPA and DHA are metabolite by the same pathway but it leads to different products. Eicosanoids which are produced from AA have pro-inflammatory effect, they increase cluttering of blood plates and they have vasoconstrictor effect. The products from EPA have in many cases opposite effects (Das, 2006). Current studies also suggest that the right ratio of PUFA n-6 and n-3 improves obesity-linked inflammation and insulin resistance (Liu et al. 2013, Glass and Olefsky 2012).

MATERIAL AND METHODS

Animals, dietary interventions, analysed tissues

A total of 48 adult male rats of the laboratory strain Wistar Albino (Bio Test Ltd., Konárovice, Czech Republic) at the age of 10 weeks (the mean live weght of 230 ± 12 g) were used. Rats were

housed in plastic boxes (53.5 cm × 32.5 cm × 30.5 cm) of 4 animals each in a room temperature at 23 ± 1 °C, humidity 60%, and 12/12 h light/dark cycle (maximum intensity of 200 lx). The experiment was performed in compliance with 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 (Experiment Project Number 2017/4).

Basic feed mixture, pelletized complete chow for mice and rats (Biokron, Blučina, Czech Republic), composed of wheat, oat, wheat sprouts, soybean meal, extruded soybean, maize, dried milk, dried whey, dried yeast, grounded limestone, monocalcium phosphate, salt, L-lysine hydrochloride, and a premix of vitamins and minerals, was fed to all animals for the whole experiment with addition of different types of oils.

The rats were randomly divided into 4 groups (12 animals per group) and following dietary interventions were applied for 8.5 weeks: basic feed mixture with fish oil (commercial *oleum jecoris aselli*, 60 g/kg, 12 animals), basic feed mixture with palm oil (60 g/kg, 12 animals), basic feed mixture with oil extracted from *Schizochytrium* microalga (60 g/kg, 12 animals) and basic feed mixture with safflower oil), all of the diets also included extra vitamins/minerals premix (1 g/kg, 48 animals). The mixtures were prepared at the laboratory this way: pelletized chow was ground, homogenized with an appropriate amount of required oil and premix. It was weighted 150 g, 200 g, respectively 50 g of the mixture (dependent on the part of our project), mixed with small amount of water and feeding dose was prepared. The animals were fed daily *ad libitum* and had free access to tap water. Feed consumption was measured daily and animals were weighed each week.

After 8.5 week of feeding all animals were sacrificed (after 12 h fasting) under anesthesia with isoflurane; blood samples were collected from the heart and samples of different tissues were taken.

Fat determination

The sample of feeding mixture was homogenized in Moulinex blender (model D56, Moulinex, France) and subsequently transferred to 150 ml Erlenmeyer flask. The mixture of hexane/2-propanol 3 : 2 (*v/v*) – HIP 1 was added and the sample was sonicated for 15 min using PS10000 apparatus (Notus-Powersonic, Vráble, Slovakia). The extract was filtered using Büchner funnel, then 24 ml of NA_2SO_4 was added. After shaking and separation of the layers in the separation funnel, n-hexane layer was filtered through anhydrous NA_2SO_4 to 50 ml volumetric flask. The water layer was re-extracted with 10 ml of HIP 2 (7 : 2, *v/v*). Layer of n-hexan was filtered through anhydrous NA_2SO_4 after re-extraction. Then it was transferred to 50 ml volumetric flask. Combined extracts were taken to 100 ml round-bottom flask and the content was evaporated on a rotary vacuum evaporator (RV 05-ST 1P-B model; IKA Labortechnik, Staufen, Germany) at 40 °C. Evaporation was finished under nitrogen and total lipids were gravimetrically determined.

Statistical evaluation

One-way analysis of the variance ratio test, including *post-hoc* Tukey's test, was used for evaluation of the differences among the dietary intervations. For all evaluations STATISTICA 12 package (StatSoft, Tulsa, OK, USA) and MATLAB were used.

RESULTS AND DISCUSSION

The composition of the diets is shown in Table 1. As it is shown all of diets were isocaloric.

Feed intake, daily weight gain and final live weight of rats are presented in Table 2. No significant differences in feed intake between groups were found. Daily weight gain was significantly decreased in rats with SF-diet in comparison with other diets, among P-, F- and A-diet were not found any significant differences.

Table 1 The composition of the diets

		Diet			
		P	**SF**	**F**	**A**
Components in the diet (g/kg)	**Basic feed mixture**	939	939	939	939
	Maize starch	-	-	-	-
	Palm oil	60	-	-	-
	Oil extracted from *Schizochytrium microalga*	-	-	-	60
	Fish oil	-	-	60	-
	Safflower oil	-	60	-	-
	Extra premix of vitamins/minerals	1	1	1	1
Nutrients (g/kg)	**Crude Protein**	228	228	228	228
	Fat	56	56	56	56
	Crude fibre	49	49	49	49
	Nitrogen-free extractives	667	667	667	667
Metabolizable energy (MJ/kg)		15.2	15.2	15.2	15.2

Legend: P – basic feed mixture with 6% of palm oil; SF – basic feed mixture with 6% of safflower oil; F – basic feed mixture with 6% of fish oil (commercial oleum jecoris asseli); A – basic feed mixture with 6% of oil extracted from the Schizochytrium microalga.
Basic feed mixture – pelletized complete chow for mice and rats; composition [g/kg]: wheat 475; extruded soybean 180; oat 50; maize 50; wheat sprouts 50; maize sprouts 40; potato protein 22.5; dried whey 53; flax meal 20; dried yeast 12; grounded limestone ($CaCO_3$) 17; monocalcium phosphate 15; common salt (NaCl) 2.5; L-lysine hydrochloride 3; premix of vitamins + minerals 10 (premix composition [mg/kg]: vitamin A 900; vitamin D3 8; vitamin E 10 000; vitamin K3 250; vitamin B1 800; vitamin B2 1000; vitamin B6 500; $FeSO_4$ H_2O 8300; $Mn(x)_{1-3}$ nH_2O + MnO 6500; $Zn(x)_{1-3}$ nH_2O + ZnO 8500; Cu(x) nH2O + $CuSO_4$ 5H2O 1200; KI 100; Na_2SeO_3 16.5; $CoSO_4$ 7 H_2O 51.5; butylhydroxytoluene 3400; propyl gallate 1400; carrier: wheat flour + $CaCO_3$ added to 1 kg).
Crude was protein determined using KD 310-A-1015 Kjel ROC Analyser (Furulund, Sweden).
Fat was determined gravimetrically as hexane/2-propanol extract (see fat determination).
Crude fiber was determined using ANCOM^{220} Fiber Analyser (Ancom Technology, Macedon, NY, USA).
Nitrogen-free extractives were calculated as a remainder to 100% after subtracting the content of crude protein, fat, crude fibre and ash (determined gravimetrically after incinerating an aliyuot at 550 °C.
Metabolizable energy was calculated from nutrient contents.

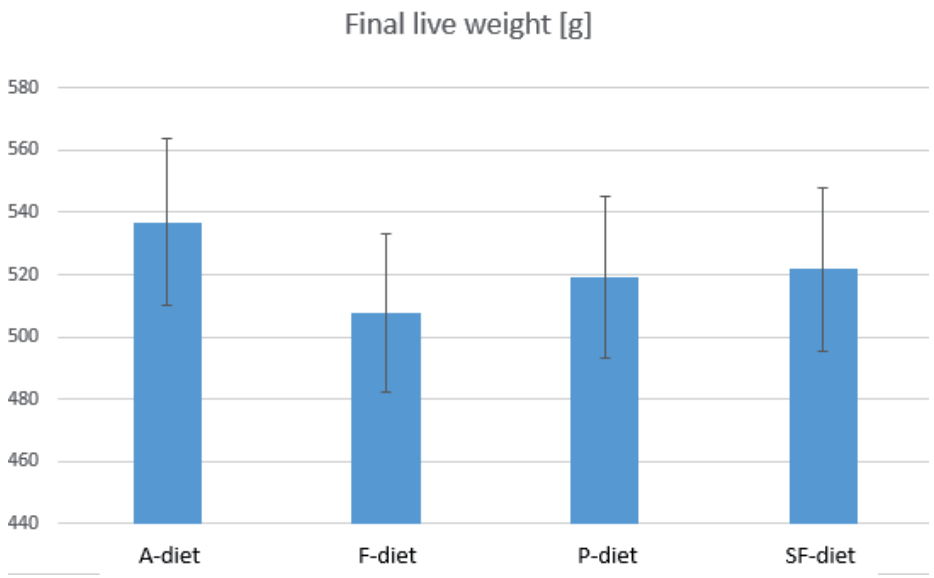
Table 2 Feed intake, daily weight gain and final live weight of rats

Diet	Feed intake[1] [g/day]	Daily weight gain[2] [g] (mean ± SEM)	Final live weight[3] [g] (mean ± SEM)
P	45.6 ± 0.05	5.3 ± 0.5	536.8 ± 30.1
SF	45.1 ± 0.05	4.6 ± 0.3	507.5 ± 16.9
F	46.1 ± 0.05	4.9 ± 0.3	519.1 ± 21.5
A	46 ± 0.05	5.1 ± 0.2	521.7 ± 13.6

Legend: [1]The mean of whole feeding period; daily feed intake of individual rats was calculated as ¼ of the total daily intake per a given box (four animals). [2]The mean of whole experiment. [3]At the day of sacrifice.

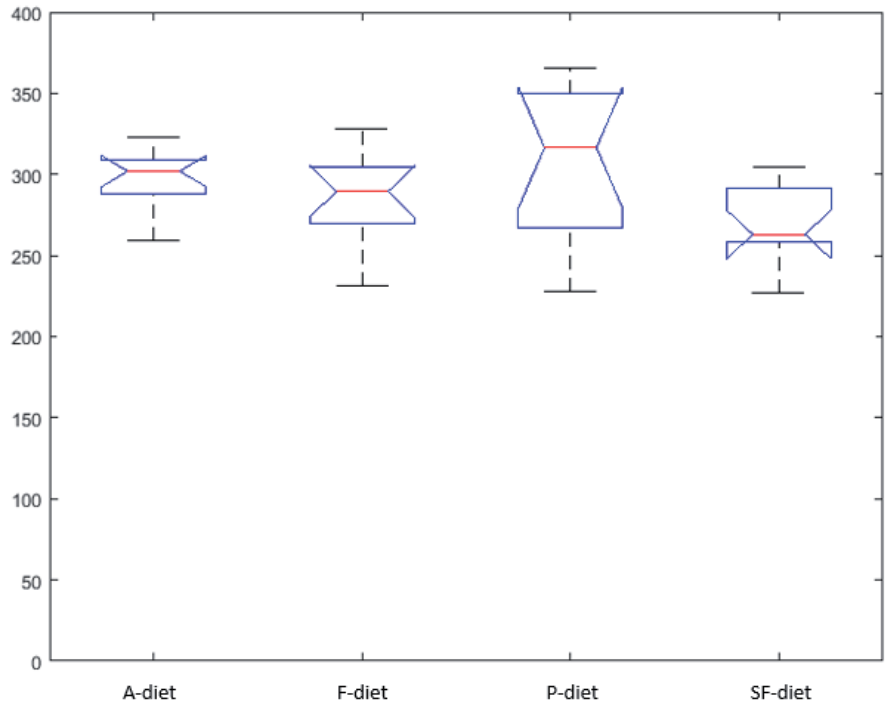
When final live weights are compared, there were found no significant differences (Figure 1). But if it comes to total weight gain it was observed significant difference between A- and SF-diet. Animals which were fed with SF-diet had lower weight gain during whole experiment. The highest weight gain was observed in A-diet. It was probably caused by lower live weight at the beginning of our experiment. There were not found any differences among F-diet and P-diet compared with other groups (Figure 2).

Figure 1 Final live weight [g] ± SEM at the date of sacrifice



Legend: P – basic feed mixture with 6% of palm oil; SF – basic feed mixture with 6% of safflower oil; F – basic feed mixture with 6% of fish oil (commercial oleum jecoris asseli); A – basic feed mixture with 6% of oil extracted from the Schizochytrium microalga. Tested by one-way ANOVA with post-hoc Tukey´s test ($p < 0.05$). There were not observed any significant differences among diets.

Figure 2 Final weight gain [g] during whole experiment



Legend: P – basic feed mixture with 6% of palm oil; SF – basic feed mixture with 6% of safflower oil; F – basic feed mixture with 6% of fish oil (commercial oleum jecoris asseli); A – basic feed mixture with 6% of oil extracted from the Schizochytrium microalga. Tested by one-way ANOVA with post-hoc Tukey´s test ($p < 0.05$).

CONCLUSION

The purpose of the present study was to determine the effect of the diet enriched with 6% of fish, palm, alga and of safflower respectively on weight, gain of weigt and feed intake of animal models (*Rattus norvegicus*). It was analysed composition of feed mixtures, all of them were isocaloric.

There were found no significant differences among all diet in feed intake and final live weight at the day of sacrifice. The only significant variety was observed in total weight gain. It was lower in SF-group in comparison with A-group, which is in contrast with our assumption The duration of experiment was probably too short to show differences among diet interventions.

ACKNOWLEDGEMENTS
The experiment was supported by the Internal Grant Agency of the Mendel University in Brno, project No TP4/2017.

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BITES BETWEEN DOMESTIC DOGS

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Abstract: Canines may sometimes exhibit behaviors that are not acceptable for their owner. Among the most undesirable behavior for owners is dog bites. A little is known about factors that may influence the frequency of a dog bite another dog. Therefore, the objective of the present study was to examine whether the use of a leash and dog breed may affect a dog bite another dog. Two hundred and seven dog bites were examined in the City of Brno, Czech Republic. A dog off a leash bit another dog four times more often than a dog on a leash. A dog off a leash was bitten by another dog two times more often than a dog on a leash. Dog breeds did not affect dog bites. Further research will be necessary to fully understand a dog bite another dog.

Key Words: dog, bite, breed, leash

INTRODUCTION

Dogs live with humans for thousands of years. People utilize the remarkable skills of dogs for many purposes (Coren 2010). However, dogs may sometimes exhibit behaviors that are not suitable for their owner. Among the most problematic behavior for owners is dog bites (Rezac et al. 2011).

So far, most studies report the factors that may affect the frequency of dog bites to the human body (Cornelissen and Hopster 2010, Rezac et al. 2015). On the other hand, a little is known about factors that may influence the frequency of a dog bite another dog. Therefore, the objective of the study was to assess whether the use of a leash and dog breed may affect a dog bite another dog.

MATERIAL AND METHODS

Two hundred and seven dog bites were examined in the City of Brno. The observation was conducted by focal- animal and all-occurrences sampling methods (Altmann 1974). For each observation on- and off-leash dog and breed were recorded. Dogs were classified based on the Federation Cynologique Internationale (FCI) breed standard categories. The other dogs were classified as crossbreeds. Data were stored in the Excel database. Frequencies of occurrence of dog bites were expressed as percentages.

RESULTS AND DISCUSSION

The effect of the use of a leash on the frequency that dog bit another dog is shown on Figure 1. A dog off a leash bit another dog in 173 cases (83.57%). A dog on a leash bit another dog in 34 cases (16.43%). The reason for the increased incidence of bites in dogs without a leash may be the fact that they are not restricted by their owners, unlike walking on a leash when the owner has more control over the dog.

The effect of the breed on the frequency of dog bites is shown on Figure 2. A pure breed dog bit another dog in 171 cases (82.61%). A crossbreed dog bit another dog in 36 cases (17.39%). The reason for the increased incidence of bites in pure breed dogs may be in a greater incidence of pure breeds compared to crossbreeds.

Figure 1 The effect of the use of a leash on the frequency of dog bites

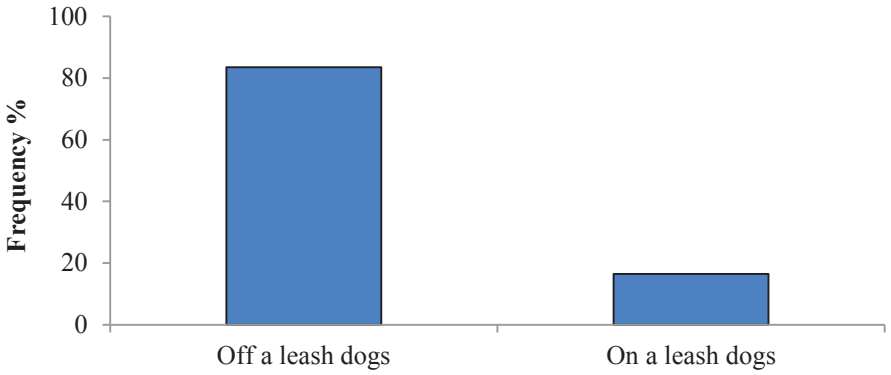
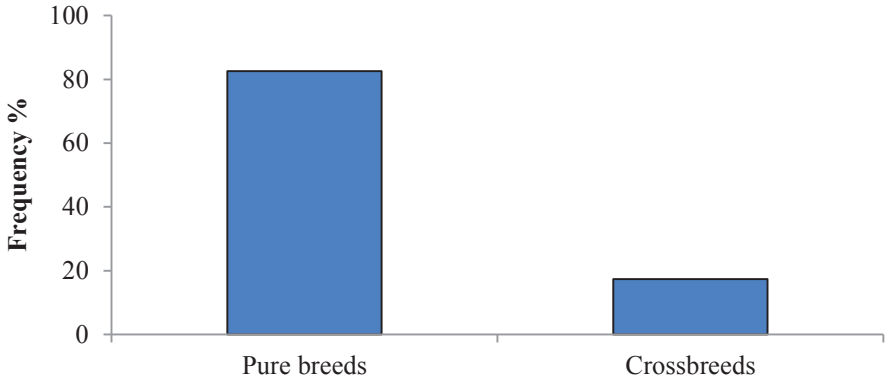
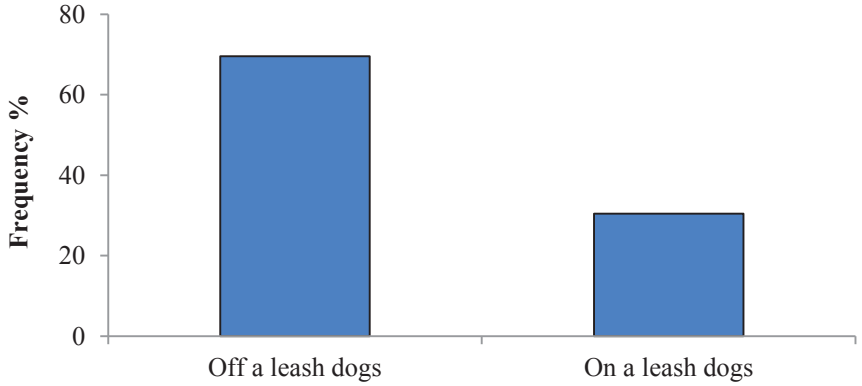


Figure 2 The effect of the breeds on the frequency of dog bites



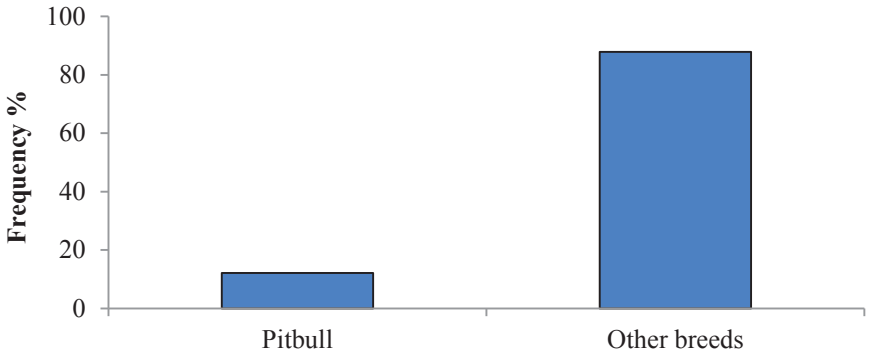
The effect of the use of a leash on the frequency that a dog was bitten is shown on Figure 3. A dog off a leash was bitten by another dog in 144 cases (69.57%). A dog on a leash was bitten by another dog in 63 cases (30.43%). The reason may be the fact that the dog off a leash may move away from his/her owner on a longer distance, and therefore, the owner fails to react in time on possible threat behavior of dogs that bit.

Figure 3 The effect of the use of a leash on the frequency that dogs were bitten



The effect of the pitbull breed on the frequency of dog bites is shown on Figure 4. The pitbull breed bit another dog in 25 cases (12.08%). Other breeds bit dog in 182 cases (87.92%). The reason may be the proportional representation of the pitbull breed in the dog population.

Figure 4 The effect of the pitbull breed on the incidence of bites



CONCLUSION

Our results indicate that dogs off a leash bite more often than dogs on a leash. Further research will be necessary to understand factors that may affect dog bites.

ACKNOWLEDGEMENTS

The research was financially supported by a grant IP 2017/049 from IGA AF MENDELU.

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THE ASSESSMENT OF OCCURRENCE OF DRUG-RESISTANT STRAINS OF *ESCHERICHIA COLI* IN THE POULTRY

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Abstract: The aim of the study was the assessment of occurrence of drug-resistant strains of *Escherichia coli* in the poultry. In the study we used of 30 strains *E. coli* isolated from poultry from a private, non-industrial shed. 20 different antibiotics were applied and drug-resistance analysis was carried out by disc-diffusion method. The strains *E. coli* resistant to at least one antibiotic constituted 23.33%. The drug-resistance analysis showed that tetracycline (16.67%) was the most effective antimicrobial antibiotic. In addition, in the analyzed material two strains of the MDR (multi-drug resistant) were identified.

Key words: antimicrobial agents, *Escherichia coli*, poultry manure

INTRODUCTION

Bacteria *Escherichia coli* are gram-negative rods that the length does not exceed 1–3 μm and the width is an average of 0.4–0.8 μm. *E. coli* have the ability to move, due to the presence of ciliary parenchymas. They are relatively anaerobic microorganisms and their optimum temperature for growth is 37 °C (Thairu et al. 2014, Pappelbaum et al. 2015). *E. coli* naturally live in the digestive tract of vertebrates. They participate in the synthesis of vitamins B and K and they are necessary for the proper functioning of digestive processes. However, some strains have pathogenic properties and after secondary ingestion of the vertebrate digestive tract, they become harmful and may cause a number of diseases of the digestive and urinary tract (Libudzisz 2009).

E. coli is also an avian pathogen because it causes infectious disease, colibacillosis, which is considered to be one of the main causes of morbidity and mortality of the poultry. As a result of *E. coli* infection in the poultry such diseases as yolk sac, colitis and intestinal infections, respiratory infections, sepsis occur. They cause high economic losses in the poultry industry because of high mortality of animals (Lutful Kabir 2010). The development of colibacillosis is conducive to biological and environmental stresses, viral infections and high concentrations of ammonia in overcrowded and poorly ventilated poultry houses. Although biosafety regulations have been very strictly respected in recent years, the reduction of colibacillosis is difficult due to the prevalence of pathogenic strains of *E. coli* bacteria (Ghunaim et al. 2014).

Poultry farms are an environment, in which different and often not fully known virulence factors of pathogenic *E. coli* strains interact. It makes colibacillosis treatment very problematic (Tonu et al. 2011). High hopes were placed in antibiotic therapy, which leads to the reduction of bacterial infections. Unfortunately, the use of drugs for therapeutic and preventive purposes, as feed additives and growth promoters in households and industry has led to uncontrolled overgrowth and the spread of the phenomenon of antibiotic resistance (Benameur et al. 2014). The resistance of bacteria, especially *E. coli*, to antibiotics is a serious risk to the poultry industry, environment and public health (Hammerum and Heuer 2009). Especially dangerous are multi-drug resistant strains (MDR), so strains resistant or intermediate-sensitive to at least one antibiotic in each of the three classes of drugs active against a particular species of microorganism (Maddox et al. 2012, Magiorakos et al. 2012).

Therefore, in response to the free and often unreasonable use of antimicrobial drugs, the European Union introduced in 2006 the prohibition of preventive application of antibiotics for livestock (Horigan et al. 2016).

MATERIALS AND METHODS

Purpose of the experiment

The purpose of the experiment was an assessment of occurrence of drug-resistant *E. coli* strains in poultry obtained from a private, non-industrial shed. It was planned to determine the presence of drug-resistant *E. coli* strains in the poultry from hens not treated with antibiotics.

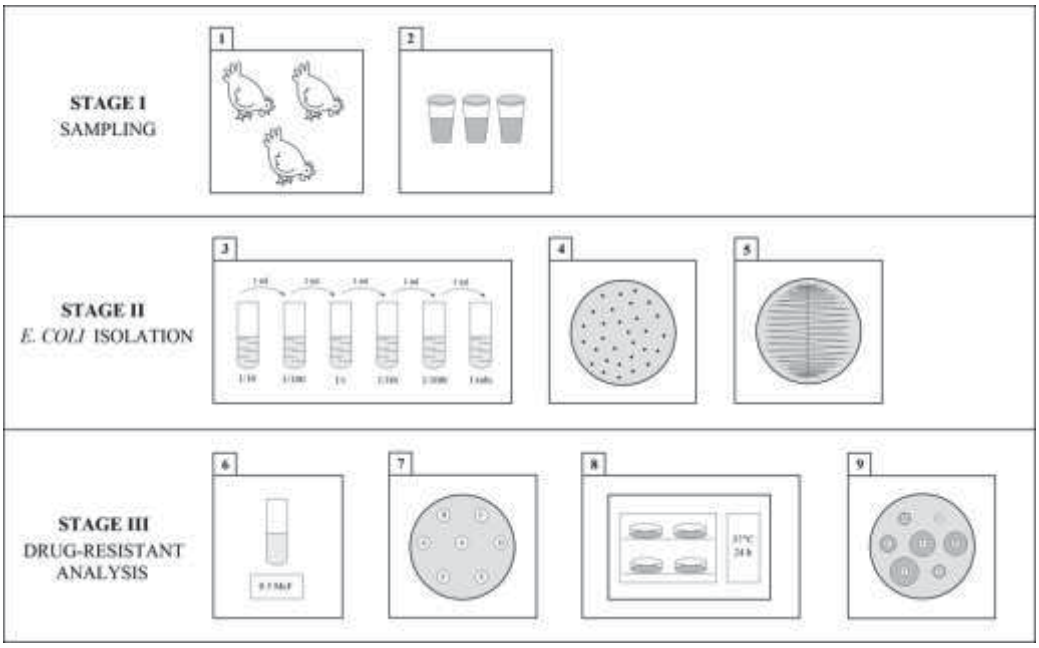
Sampling site

Samples of poultry were taken from a shed of about 120 broilers located in Polanka Wielka in the Lesser Poland (Poland). Animals are kept in a closed, non-ventilated room of 9 m^2. Animals are fed with standard feed with cereal grains added and without the addition of antimicrobial agents. After 8–9 weeks and reaching a weight of about 3 kg animals are slaughtered.

Methods

Stages of the studies are shown in the diagram below (Figure 1).

Figure 1 Diagram of research methodology



STAGE I – Poultry was collected in sterile containers and transported to a laboratory.

STAGE II – Isolation of *E. coli* strains was performed by serial dilution according to Koch using TBX medium, incubating the cultures for 24 h at 44 °C. The blue-green colonies of *E. coli* were transplanted to further TBX medium and multiplied (24 h, 44 °C) to be used in the next step of the study.

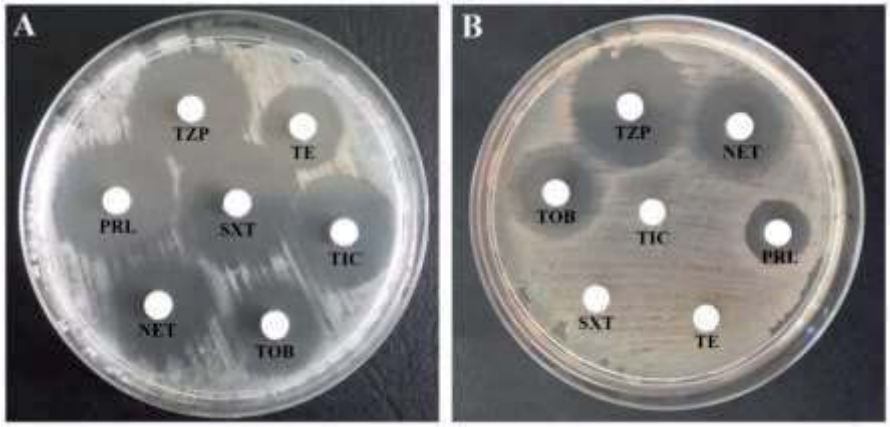
STAGE III – For the determination of drug-resistance of *E. coli* isolates the disc-diffusion method recommended by the European Committee on Antimicrobial Susceptibility was used (EUCAST 2017), for antibiotics not included in the list information contained in other authors' publication was used (Kronvall et al. (1984) – cefalotine, Turnidge (2011) – cefazol, Barry et al. (1983) – cefamandol, Sader et al. (2007) – tetracycline). MHA medium (Mueller-Hinton agar, BTL, Poland) was dispensed quantitatively (15 ml) on sterile Petri dishes. Then, from 18–24 h, pure cultures of *E. coli* were taken of sterile swabs and placed in saline tubes (0.9% NaCl), thoroughly mixed and set a concentration of 1.5 x 10^8 CFU/ml (0.5 McFarland Standard) with a densitometer (DEN-1, Biosan, Poland). With a sterile swab the bacterial suspension was evenly seeded on previously prepared Petri dishes

with MHA medium. Sterile antibiotic discs (Oxoid, Ireland) were applied on the prepared Petri dishes. Cultures were incubated for 24 h at 37 °C. After this time the growth inhibition zones around the discs (mm) were read. *E. coli* strain ATCC 25922 was used as a control of the quality of the disc-diffusion method.

RESULTS AND DISCUSSION

Based on the statement of the breeder, it is known that these animals do not receive antimicrobial feeds in the feed. 30 *E. coli* strains were isolated from the poultry samples. Strains sensitive to all analyzed antibiotics accounted for 76.67% (23 strains). 16.67% of the isolates were resistant to 1 antibiotic (5 strains) and 6.67% (2 strains) – were resistant to 6 antibiotics, including 2 MDR strains. The highest resistance was found for tetracycline (16.67%) and ciprofloxacin (10%). The obtained data is shown below (Figure 2 and Table 1). The highest resistance to tetracycline is not surprising, because it is an antibiotic that has been used long in medicine and veterinary, and therefore increased resistance to microorganisms is often encountered (Chopra and Roberts 2001).

Figure 2 Zones of growth inhibition of E. coli caused by the application of antibiotics



Hanon et al. (2015) investigated drug-resistant *E. coli* strains from broiler flocks, which accounted for more than 50% of all analyzed isolates. The highest resistance of *E. coli* was found with antibiotics such as ampicillin, ciprofloxacin, nalidixin acid and sulfamethoxazole. Strains were isolated from the industrial farm, so the results obtained by the researchers should not be surprising. Hering et al. (2016) analyzed the efficacy of cefotaxime against *E. coli* strains isolated from poultry manure from an industrial shed. They found that there is 81.9% of *E. coli* resistance to this drug.

Álvarez-Fernández et al. (2013) analyzed *E. coli* strains isolated from the poultry carcases of industrial farms. They found that 91.7% of the analyzed strains were resistant to two or more antibiotics. The highest resistance was observed with nalidixic acid (85% of the tested strains), ampicillin (75%), ciprofloxacin (73.3%) and tetracycline (61.7%). Skočková et al. (2015) evaluated the microbiological quality of poultry meat obtained from various supermarkets. *E. coli* strains, which were resistant to at least one antimicrobial agent, constituted 82.8%. 51.7% isolates showed multi-drug resistance. The percentage of drug-resistant strains was the biggest in the relation to ampicillin and nalidixic acid (55.2%). In addition, 37.9% of the strains were resistant to ciprofloxacin, 34.5% – to tetracycline and 31% – to trimethoprim.

Dou et al. (2016) isolated the organs (liver, spleen, lungs) from dead animals that showed typical symptoms of *E. coli* infection before death. 60% strains were resistant to antibiotics such as ampicillin, streptomycin, tetracycline, nalidixic acid, trimethoprim, sulfamethoxazole antibiotics and sulfisoxazole. In addition, of all analyzed strains, 80.25% were MDR strains.

The problem of widespread drug resistance is primarily related to isolates obtained from large scale industrial farms. Despite the ban on administering medicinal products for therapeutic and preventive purposes in the EU, breeders often break the law by using drugs in unwarranted situations. This is the main reason for acquiring resistance by bacteria that with improperly processed manure, can be fertilized in the environment (Obeng et al. 2012, Álvarez-Fernández et al. 2013).

Table 1 The profile of the drug-resistance of E. coli isolated from the poultry

Antibiotic (symbol, µg), class	Limit values (mm)	Resistant strains		Intermediate-sensitive strains		Sensitive strains	
		No. of isolates	No. of isolates (%)	No. of isolates	No. of isolates (%)	No. of isolates	No. of isolates (%)
Amikacin (AK, 30), Aminoglycosides	18/15 (EUCAST 2017)	0	0	0	0	30	100
Amoxicillin/ clavulanic acid (AMC, 30), β-lactams	19 (EUCAST 2017)	0	0	0	0	30	100
Ampicillin (AMP, 10), Aminoglycosides	14 (EUCAST 2017)	**2**	**6.67**	0	0	28	93.33
Aztreonam (ATM, 30), β-lactams	26/21 (EUCAST 2017)	0	0	0	0	30	100
Cefamandol (MA, 30), β-lactams	18/14 (Barry et al. 1983)	0	0	0	0	30	100
Cefepim (FEP, 30), β-lactams	27/21 (EUCAST 2017)	0	0	0	0	30	100
Cefotaxime (CTX, 30), β-lactams	20/17 (EUCAST 2017)	0	0	0	0	30	100
Cefoxoxine (FOX, 30), β-lactam	19 (EUCAST 2017)	0	0	0	0	30	100
Ceftazidime (CAZ, 30), β-lactams	22/19 (EUCAST 2017)	0	0	0	0	30	100
Cephalotin (KF, 30), β-lactams	13 (Kronvall et al. 1984)	**1**	**3.33**	0	0	29	96.67
Cefazolin (KZ, 30), β-lactams	23/19 (Turnidge 2011)	0	0	0	0	30	100
Ciprofloxacin (CIP, 5), Chinolones	26/24 (EUCAST 2017)	**3**	**10**	1	3.33	26	86.67
Gentamics (CN, 10), Aminoglycosides	17/14 (EUCAST 2017)	0	0	1	3.33	29	96.67
Netilmicin (NET, 30), Aminoglycosides	15/12 (EUCAST 2017)	0	0	0	0	30	100
Piperacillin (PRL, 100), β-lactams	20/17 (EUCAST 2017)	**2**	**6.67**	0	0	28	93.33
Piperacillin/tazobactam (TZP, 110), β-lactams	20/17 (EUCAST 2017)	0	0	0	0	30	100
Tetracycline (TE, 30), Tetracyclines	15/11 (Sader et al. 2007)	**5**	**16.67**	1	3.33	24	80
Ticarcillin (TIC, 75), β-lactams	23 (EUCAST 2017)	**2**	**6.67**	0	0	28	93.33
Tobramycin (TOB, 10), Aminoglycosides	17/14 (EUCAST 2017)	0	0	4	13.33	26	86.67
Trimethoprim/sulfamethoxazole (SXT, 25), Sulfonamides	14/11 (EUCAST 2017)	**2**	**6.67**	0	0	28	93.33

CONCLUSION

The study allowed for the isolation, identification and evaluation of the drug resistance profile of 30 isolates of *E. coli* isolated from poultry gained from a private, non-industrial shed. Among the collected strains were found resistant isolates for tetracycline, ciprofloxacin, ampicillin, piperacillin, ticarcillin, trimethoprim/sulfamethoxazole and cefalotin. In addition, 2 MDR have been reported.

It should be remembered, that all *E. coli* strains were from hens that did not receive antibiotics, so it can be considered that bacteria involved in the study presented a natural resistance that was not caused by selective pressure. Thus, it can be considered that the hens are a natural source of drug-resistant *E. coli*.

Although, little resistance to the tested antibiotics was found in the overall pool of strains, the presence of MDR strains is particularly disturbing. It is therefore advisable to conduct further studies to identify risk factors for the spread of drug resistance in poultry. This is particularly important because the manure is processed and then used in agriculture as a fertilizer. If, during the treatment process, the drug-resistant *E. coli* strains are not removed from it, uncontrolled release of dangerous isolates may appear in the environment. People working in sheds and staff dealing with animal waste processing are particularly at risk. Therefore, it is important to continually monitor the abundance of drug-resistant *E. coli* strains, because if they are the released to environment, dangerous *E. coli* strains pose a serious threat to public health.

ACKNOWLEDGEMENTS

This study was funded by the statutory measures of the Department of Microbiology, University of Agriculture in Cracow, Poland – DS 3157/KM.

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THE AGE EFFECT ON SELECTED BLOOD BIOCHEMICAL PARAMETERS OF YOUNG DWARF LOP RABBITS

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Abstract: The aim of the study was to evaluate the age effect on selected biochemical parameters in young rabbits of the Dwarf Lop breed. In the experiment, a total of 16 rabbits (8 males and 8 females) were used. The blood samples were taken at 8th and 15th week of the age. The basic biochemical profile was determined using the blood plasma. All of the examined parameters were in physiological reference ranges. With respect to the age effect, we found increase in the concentration of total protein ($P < 0.05$), creatinine ($P < 0.01$) and activity of alanine aminotransferase ($P < 0.05$). On the other hand, the age-related decrease was found in the level of glucose and activity of alkaline phosphatase ($P < 0.01$). In addition, we found a significant ($P < 0.05$) positive correlation between the live weight and values of the albumin, albumin/globulin ratio and creatinine while the negative correlation was found between the live weight and the level of glucose, alkaline phosphatase and inorganic phosphorus levels ($P < 0.05$). It can be concluded that the age of the Dwarf Lop rabbits had a significant effect on the selected biochemical parameters. These findings can be useful for clinical examination of the dwarf rabbits.

Key Words: pet rabbit, physiology, plasma, biochemistry, normal values

INTRODUCTION

Recently, the rabbit become a favourite companion animal. With respect to many aspects, mainly dwarf rabbits are reared as pets (Snook et al. 2013), while the Dwarf Lop and the Netherland Dwarf breeds show the highest popularity (González-Redondo and Contréraz-Chacón 2012). This interest has been accompanied also in veterinary sphere (Harcourt-Brown 2002, Meredith 2014).

The blood examination can provide valuable insights into the rabbit health and has an impact to the veterinarian´s decision. Currently, there is a lack of studies dealing with the dwarf rabbits physiology (Meredith 2014). Concerning the rabbit physiology, the researchers interest is mainly focused on laboratory and meat-type rabbits. The most of published normal reference values originate from medium-sized rabbits. Besides that, some physiological reference ranges are too wide and so almost any result fall within them (Harcourt-Brown 2002). These undesirable phenomena can complicate diagnosis in the pet rabbits examined in the veterinarian practices. Therefore, Wesche (2014) recommends to take in consideration also the effects of rabbit´s breed, age and sex in evaluating the laboratory results.

In general, the rabbit blood parameters were recently intensively studied with regard to the effects of nutrition (Trebušak et al. 2014), sex (Özkan et al. 2012), welfare conditions (Ondruška et al. 2011), genotype (Abdel-Azeem et al. 2010, Martinec et al. 2012) and age (Jeklová et al. 2009). Our previous research in the dwarf rabbits revealed that their blood parameters can be affected also by the dwarf rabbit breed (Šimek et al. 2017). Furthermore, we found that selected haematological parameters in the Dwarf Lop breed were affected by age of the rabbits (Šimek et al. 2016). Therefore, we assume that this age effect might exist also on the biochemical parameters.

The aim of present study was to evaluate the age effect on selected biochemical indicators in the blood of young the Dwarf Lop rabbits.

MATERIAL AND METHODS

Animals

Experimental procedures were approved by the Animal Welfare Committee of the University of Veterinary and Pharmaceutical Sciences Brno (no. 66/2016/2230/FVHE). The study was conducted on a total of 16 (8 males and 8 females) rabbits belong to the Dwarf Lop breed. The rabbits originated from common pet stock.

Experimental design

The young rabbit kits were housed together with the lactating does until their 8th week of age; subsequently the weaning was performed. The rabbits received a commercial pelleted feed (Berkel-Futter Light 6008, Coesfeld, Germany) in course of the entire experimental period. Ingredient composition of feed and the chemical composition of feed in dry matter (DM) is displayed in Table 1. All rabbits were fed once a day (approx. 30 g of the feed per kg of live weight per day). The meadow hay was offered three–times a week and animals had unlimited access to drinking water.

Blood sampling and biochemical examination

The blood samples were taken at the age of 8 and 15 weeks. Always one day before the samplings, all the rabbits were given their last pelleted feed while the meadow hay and water were available during the night. The blood samplings were performed between 9–10 a.m. With respect to the ear length, the blood samples were taken using *vena saphaena lateralis*. Obtained blood samples were relocated to sample tubes with heparin. The biochemical examination was carried out using a DPC Konelab 20i Analyzer® (Thermo Fisher Scientific, Finland). In blood plasma, following parameters of the biochemical profile were determined: total protein (TP), albumin (Alb), glucose (Glu), total cholesterol (Chol), triacylglycerids (TAG), creatinine (Crea), urea, alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), calcium (Ca) and inorganic phosphorus (P_i). The globulin (Glob) and albumin/globulin (Alb/Glob) ratio were calculated.

Statistical analysis

Statistical analyses were performed using the STATISTICA CZ version 10 software. One-way ANOVA was used to determine differences in the biochemical parameters. When ANOVA showed significant differences between the age groups, Tukey´s HSD test was used. Pearson´s correlation was used to determine the correlation coefficients between the live weight (LW) and biochemical parameters.

Table 1 Ingredient and chemical composition of the pelleted feed (g/kg)

Ingredient	Pelleted diet	Chemical composition in 1 kg of DM	
Alfalfa meal	417.0	Crude protein	160.5
Barley	85.0	Crude fibre	173.2
Wheat bran	226.0	ADF	233.6
Oat bran	60.0	NDF	420.0
Malt sprouts	151.0	ADL	53.0
Sugar beet pulp	29.00	Ether extract	26.8
Mollasses	19.00	Crude starch	151.9
Monocalcium phosphate	1.0	Ash	86.2
Calcium carbonate	8.5	Ca	11.20
Sodium chloride	3.5	P	5.7

RESULTS AND DISCUSSION

Results of the biochemical examination are presented in Table 2. The results of the correlation analysis between the LW and biochemical parameters are given in Table 3.

Table 2 Live weight and biochemical parameters of the Dwarf Lop rabbits in relation to the age

Parameter	Age 8 weeks n = 16			Age 15 weeks n = 16			Sign.
	Mean ± standard error						
Live weight (g)	772.50^{A}	±	31.75	1211.25^{B}	±	45.26	**
TP (g/l)	51.32^{a}	±	0.90	54.19^{b}	±	1.04	*
Alb (g/l)	29.47	±	1.08	32.28	±	1.11	ns
Glob (g/l)	21.86	±	0.78	21.91	±	1.10	ns
Alb/Glob	1.39	±	0.09	1.55	±	0.11	ns
Glu (mmol/l)	7.29^{B}	±	0.23	6.37^{A}	±	0.19	**
Chol (mmol/l)	1.20	±	0.12	1.04	±	0.10	ns
TAG (mmol/l)	1.17	±	0.13	0.96	±	0.12	ns
Crea (µmol/l)	63.18^{A}	±	4.65	86.06^{B}	±	4.70	**
Urea (mmol/l)	5.26	±	0.33	5.38	±	0.31	ns
ALP (µkat/l)	3.85^{B}	±	0.31	2.18^{A}	±	0.18	**
ALT (µkat/l)	0.71^{a}	±	0.08	1.09^{b}	±	0.17	*
AST (µkat/l)	0.57	±	0.07	0.79	±	0.10	ns
Ca (mmol/l)	2.94	±	0.06	3.03	±	0.04	ns
P_i (mmol/l)	1.74	±	0.13	2.00	±	0.10	ns

^{A,B}: $P < 0.01$, ^{a,b}: $P < 0.05$

Concerning the LW, it can be noted that our values correspond with the Dwarf Lop breed standard (Zadina 2003). Regarding the total protein concentration, we found its significantly higher level in 15 – week old rabbits as compared to the 8 – week old rabbits (+2.87 g/l). This finding is in agreement with previous studies, when older rabbits showed also the higher TP content than young rabbits (Archetti et al. 2008, Kishk 2010, Ondruška et al. 2011). The increasing tendency of TP content corresponds with our previous findings (Šimek et al. 2017) where we found the higher TP level in Dwarf Lop females at the age 6 months (64.07 g/l). Moreover, our results are in physiological ranges for healthy rabbits according to Wesche (2014). However, we found slightly lower levels of TP in our study than Martinec et al. (2012) in 3 – month old medium-sized rabbit breeds (range of 60.28–70.66 g/l) and Ondruška et al. (2011) in 8 – week old New Zealand White rabbits (56.10–57.51 g/l) which can be associated with the dwarf breed. No significant differences were recorded in content of the albumin, globulin and in Alb/Glob ratio while their values in our study fell into reference ranges established by Wesche (2014). As for the correlation, we found positive correlation (0.36) between the albumin concentration and the LW of the rabbits ($P < 0.05$), which is consistent with findings of Abdel-Azeem et al. (2010) in medium-sized rabbits (0.50) and also Šimek et al. (2017) in 6 – month old Dwarf Lop females (0.54). Furthermore, we found also the positive correlation in the Alb/Glob ratio, which is associated with the above stated correlation between the albumin level and LW. The glucose level was highly significantly affected by the rabbit age while the older rabbits showed lower glycaemia than younger rabbits (-0.92 mmol/l). On the basis of the significant negative correlation between the glucose and LW ($P < 0.05$) in our study, it seems that the glucose level decreases with the ongoing growth of raising rabbits. This decreasing tendency is consistent with findings of Ondruška et al. (2011) which described in laboratory rabbits a lower glycaemia level in breeding individuals as compared to 8 – week old young kits. On the other hand, Kishk (2010) found an opposite age-related changes in young New Zealand White rabbits from their 3rd up to 10th week of age. In our study, the concentration of total cholesterol and TAG showed a non-significant decrease with the advancing age. Total cholesterol level found in our study was within the physiological range published by Wesche

(2014), while the TAG level in our study (1.17, resp. 0.96 mmol/l) is out of the mentioned range. However, in our previous study in clinically healthy 6 – month old Dwarf Lop females we found the TAG level as 0.87 mmol/l (Šimek et al. 2017). Regarding the creatinine concentration, we found the higher creatinine level in 15 – week old rabbits as compared to the 8 – week old rabbits ($P < 0.01$). Moreover, we found also the significant positive correlation between the creatinine content and the LW (0.62). Our results correspond with the statement of Harcourt-Brown (2002) that plasmatic level of the creatinine is directly proportional to the individual´s body mass. Similar age-related tendency in the creatinine level was described in previous rabbit study, where this correlation was found to be 0.79 (Kishk 2010).

Table 3 Correlation coefficients between the LW and biochemical parameters in the Dwarf Lop breed

Biochemical parameter	Correlation coefficient	Biochemical parameter	Correlation coefficient
TP	0.17	Crea	0.62*
Alb	0.36*	Urea	0.25
Glob	-0.25	ALP	-0.44*
Alb/Glob	0.37*	ALT	0.15
Glu	-0.38*	AST	0.24
Chol	-0.04	Ca	0.11
TAG	-0.28	P_i	-0.40*

**(P < 0.05).*

The urea content found in our study was in physiological reference range (Wesche 2014). Also observed ALP, ALT and AST levels were in physiological ranges for healthy rabbits (Harkness et al. 2010, Wesche 2014). The age of rabbits in our study had the significant effect on the activities of the ALP and ALT enzymes. We found highly significant decrease in the ALP activity in 15 – week old rabbits as compared to the 8 – week old rabbits (-1.67 μkat/l). Campbell (2004) notices that the ALP activity varies with age, and Harcourt-Brown (2002) states that higher ALP are found in young animals with the high osteoblastic activity; results of our study in pet rabbits are consistent with both of the above mentioned statements. Moreover, we found a negative correlation between the ALP activity and LW which is in agreement with our previous study in the Dwarf Lop breed (Šimek et al. 2017). Regarding the ALT, we found significant increase in the ALT activity in relation to advancing age (+0.38 μkat/l). Further, in our study, we found no significant age-related changes in calcium and inorganic phosphorus concentrations, while we recorded the significant negative correlation between the P_i and LW. The observed values of Ca and P_i were in physiological ranges (Harkness et al. 2010, Wesche 2014).

CONCLUSION

Results of the present study have confirmed our assumption that selected blood biochemical parameters can be affected by the age of dwarf rabbits. All of the examined parameters were in physiological reference ranges. The significant age-related increase in content of the total protein, creatinin and in activity of ALT was recorded. With ongoing rabbit´s age, we found a significant decrease in plasmatic levels of the glucose and ALP. Furthermore, we found that the albumin level, albumin/globulin ratio and creatinine level showed significant positive correlation with the LW of rabbits, while the glucose, ALP and P_i level displayed a negative correlation with their LW. The observed data can contribute to the more accurate assessment of rabbit physiology and can serve both of the veterinarian practices and further research on the dwarf rabbit physiology.

ACKNOWLEDGEMENTS

The research was financially supported by the internal project of the University of Veterinary and Pharmaceutical Sciences Brno, IGA no. 207/2017/FVHE.

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LIFE STRATEGIES OF JUMPING SPIDERS (ARANEAE: SALTICIDAE) OF GENUS *PELLENES* – A POSSIBLE EXPLANATION OF THE UNUSUAL SOCIABILITY

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Abstract: The unusual sub social behaviour of spiders of the genus *Pellenes* spp. has not yet been reliably explained. In our study, we infer that relatively common gregarious wintering in one land snail shell (especially for species *Pellenes tripunctatus* and *Pellenes nigrociliatus*) can be conditioned by the collective maturing of offspring in shells which are hanging on vegetation. Observation was carried out on two steppe locations. Altogether, eight shells (*Caucasotachea vindobonensis*) hanging on vegetation were found. In each of these shells have been discovered one female of spider species *Pellenes tripunctatus*. This is an interesting finding because this way of caring for offspring has not yet been confirmed for this species (only old records of *Pellenes nigrociliatus*). In the course of our research, we also found the use of another species of gastropods *Helicigona lapicida* by spider *Pellenes tripunctatus*, which was never reported before.

Key Words: Araneae, Salticidae, shells, *Pellenes tripunctatus,* sociability, breeding of offspring

INTRODUCTION

Spiders represent the most abundant group of invertebrates using empty land snail shells. As the shells are primarily occupied during the period when the air temperature begins to decrease, shells are mentioned mainly in connection with the overwintering of spiders. Besides of overwintering, empty shells are used also as a hiding place or for reproduction. Spiders there also create specific nests for establishment of next generation (mainly family Salticidae). Shells offer advantageous microclimate which ensures protection during hot summer days or protection from various predators (Moreno-Rueda et al. 2008).

The most common inhabitant (in terms of overwintering) is the Salticidae family with specific species of *Pellenes tripunctatus* (Walckenaer, 1802), *P. nigrociliatus* (Simon, 1875) and *Sitticus penicillatus* (Simon, 1875), as reported by several authors (Horn 1980, Bauchhenss 1995, Szinetár et al. 1998, Bellmann 1999, Moreno-Rueda et al. 2008, Hula et al. 2009, Michálková 2012, Niedobová et al. 2013, Štempáková 2014, Štempáková 2016). The most occupied are the shells of *Xerolenta obvia* (Menke, 1828), *Caucasotachea vindobonensis* (Férussac, 1821), *Helicigona lapicida* (Linnaeus, 1758), *Arianta arbustorum* (Linnaeus, 1758), *Zebrina detrita* (O. F. Miller, 1774) or less occupied shells of larger species of gastropod – *Helix pomatia* (Linnaeus, 1758). In addition, for some spider species there is a strong affinity to certain species of gastropods (Mikulska 1961, Horn 1980, Szinetár et al. 1998, Hula et al. 2009, Niedobová et al. 2013, Štempáková 2016). The strongest and most frequently mentioned is the affinity of *P. nigrociliatus* to the shells of *X. obvia*.

It confirmed that *Pellenes nigrociliatus* use these shells for reproduction and breeding of offspring. Spiders hang up empty shells by spider web on stalks of grass. Then will create a nest for establishment of a new generation inside the shell (Mikulska 1961). After hatching cubs, the female will ensure the protection of their offspring. The young spiders remain in the shells until they are able to reproduction (Horn 1980). Probably similar strategies are also used by *P. tripunctatus*, but in the

case of the shell of the gastropod *C. vindobonensis*. *Pellenes tripunctatus* use this shell for hibernation very often and even was confirmed also affinity to the mentioned shell (Hula et al. 2009, Niedobová et al. 2013), but in the case of an unusual life strategy in the form of hanging shells, there are almost no records. It is possible to mention the case when this behaviour was observed and slightly different from species *P. nigrociliatus*. The breeding and protection of offspring were ensured by both female and male [(Horn 1980) cited by (Braun 1956)]. This statement, respectively this form of relatively weaker sub sociability can explain a gregarious overwintering of predatory species within a single shell.

MATERIAL AND METHODS

The survey took place on June 13, 2017 and June 14, 2017 at pre-selected sites. The first site (visited on June 13, 2017, about 400 m southeast of the protected area – Malhotky) is a step, lighted locality with low and sparse vegetation and calcareous subsoil. This site was selected for a large number of empty shells, confirmed from earlier records. A slightly different site is protected area – Švařec, which was visited on June 14, 2017. It is an acidic environment with the admixture of calcareous rocks of anthropogenic origin, also with the presence of sparse and low vegetation. After arriving at the site, a survey of the area were starting and then the searching for the individual shells hanging on the vegetation was carried out. When we found a shell, photo-documentation took place and the individual shells were collected. The recording of environmental conditions was also carried out. Determination of spiders and detection of all available information related to this life strategy were carried out in the laboratory.

RESULTS AND DISCUSSION

Altogether, eight shells hanging on vegetation were found. At the first site (a locality next to the Malhotky site), six shells of *C. vindobonensis* were found. At the second locality Švařec, two shells of *C. vindobonensis* were found. One female (without offspring) of *P. tripunctatus* was present in each shell, which contradicts the theory that the male remains with the female (Braun 1956).

Young spiders inside the hanging shells create for a period of time a group in which individuals are relatively tolerated to each other. In the case of *P. nigrociliatus*, the young individuals leave the shell after the first molting (Mikulska 1961). It is likely that a certain affinity or form of sociability is formed between the individuals during this period. It is possible that a group of these young spiders living together in shells can influence to a certain extent by the subsequent gregarious overwintering, which is very noticeable for *P. tripunctatus*. Regarding to group overwintering, total of six adults individuals of *P. tripunctatus* were recorded in one shells (Štempáková 2016). This is an interesting phenomenon, because no form of social behaviour is mentioned in connection with the Salticidae family. These are typical predators that attack each other (Buskirsk 1981, Herberstein 2011).

It is interesting that the species *P. tripunctatus* also uses the shells of *H. lapicida*, as it was seen on the Švařec site. At the recent time, the overwintering of this species is associated with shells of *C. vindobonensis* (Hula et al. 2009, Niedobová et al. 2013, Štempáková 2016). There are records which are also confirmed by wintering in the shells of *Xerolenta* sp. (Bellmann 1992) or *Z. detrita* (Bauchhenns 1995).

The issue of coexistence of spider offspring in shells or group overwintering in shells enriches previously acquired knowledge of life and unusual life strategies of these species. By continuing with similar research, we may supplement or refute the previously unsubstantiated facts as well as the previously unknown conditions leading to the formation of these unusual groups.

Figure 1 Red dot – site near the Malhotky locality. Green dot – Švařec site

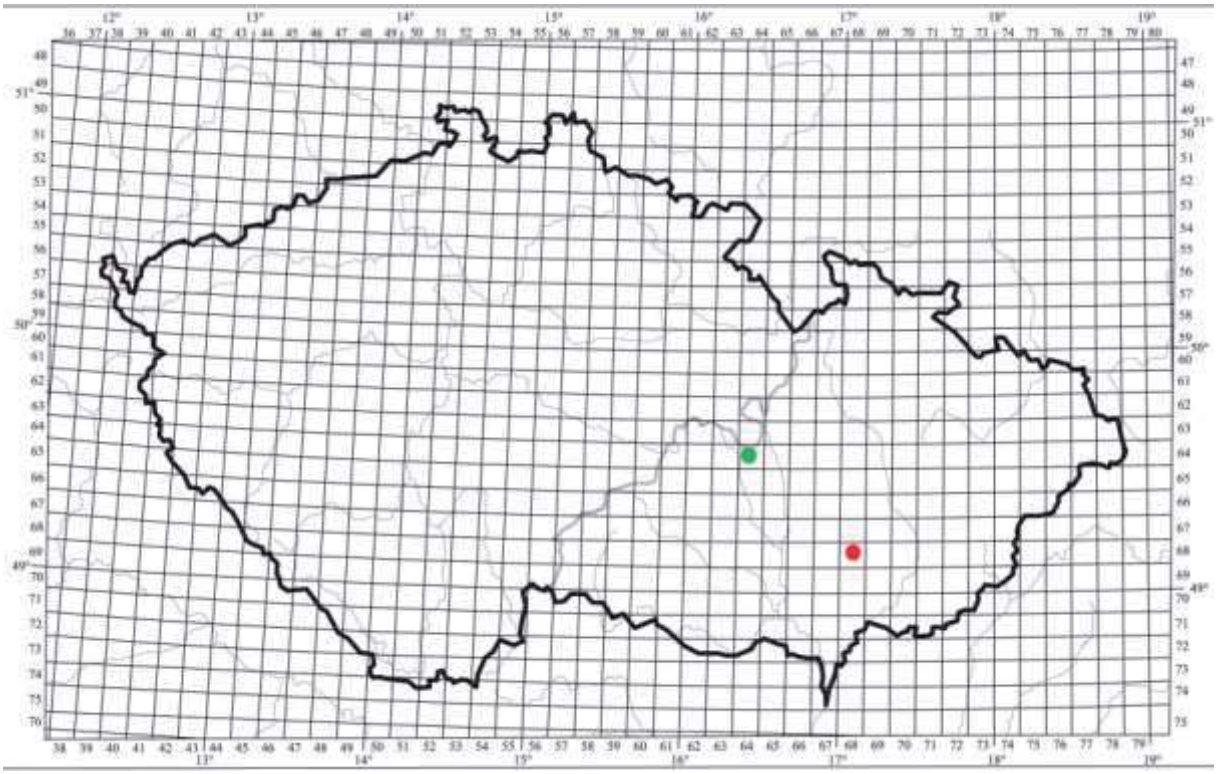

Figure 2 Caucasotachea vindobonensis inhabited by Pellenes tripunctatus (locality near the Malhotky)

Figure 3 Caucasotachea vindobonensis inhabited by Pellenes tripunctatus (locality Švařec)

Figure 4 Helicigona lapicida inhabited by Pellenes tripunctatus (locality Švařec)

CONCLUSION

The results brought interesting insights into the behaviour of *Pellenes tripunctatus* and the unexplained form of social behaviour of these predatory species. The detected coexistence of the individual young spiders in the hanging shells has probably an impact on the formation of groups within the gregarious overwintering. Interestingly, there is also evidence that *P. tripunctatus* uses *Helicigona lapicida* shells.

In general, shells are important refuges for various, very often rarely or endangered species of invertebrates.

ACKNOWLEDGEMENTS

The research was financially supported by the grant IGA FA MENDELU No. IP_8/2017.

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DIETARY SUPPLEMENTATION OF *RHUS CORIARIA* (SUMACH) MODERATELY AFFECTS THE RABBIT SPERMATOZOA MOTILITY

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Abstract: Appropriate nutrition and feeding belong among the factors that ensure successful and beneficial animal production. The most suitable forage for the rabbits as herbivores is the feed of crop origin. It is important that the feed contains all the essential nutrients, fiber, mineral substances and vitamins. In present study, the effect of sumach addition into feeding ration of rabbits was observed with special interest in spermatozoa motility properties. Male rabbits (n = 25) of New Zealand white breed were used in this experiment. The rabbits were divided into five different groups. One control group (C) and four experimental groups (S1, S2, S3, S4). Sumach (*Rhus coriaria*) fruit was added to the complex feeding ration in milled and parched form in four different concentrations: 0.50%; 0.75%; 1.00% and 1.50% and fed for the period of 90 days. Results of the CASA analysis show that sumach dietary supplementation affects the spermatozoa motility, however with no statistical significance. Based on the results of this study, moderate effect of sumach on male reproduction depends on additive concentration and duration of intake. The first 20 days of the experiment showed very homogeneous results. Analyses conducted at the Day 40 showed both positive and negative effect of sumac. Concentrations 0.50% and 1.00% seemed beneficial. At the Day 60 and the Day 80 of the experiment decreased values of CASA parameters in all concentrations were monitored. Evaluations carried out at the terminal collection day (Day 90) reported enhanced motility, progressive motility and velocity curved line in rabbit groups administrated with addition of sumach in concentrations of 0.75% and 1.00%.

Key Words: rabbit, *Rhus coriaria*, sumach, spermatozoa motility, CASA

INTRODUCTION

The intensification of rabbit breeding puts the emphasis on physiological, health and behavioural demands due to high sensitivity of rabbits to environmental conditions (Casamassima et al. 2017). There is a worldwide trend in administration of antioxidants with natural origin to the farm animals in order to improve the animal welfare and possibly to create a functional food (Vizzarri and Corino 2016). It is highly believable that the way to achieve this goal leads through the use of phytogenics, feed additives derived from herbs and plants with natural growth promoting factor (Gálik et al. 2013).

Sumach is the commonly used name for genus *Rhus*, the plant recognized due to its wide range of beneficial properties as follows: antimicrobial (Nasar-Abbas and Halkman 2004), antioxidant (Kosar et al. 2007), antimutagenic (Chakraborty et al. 2009), antidiabetic (Mohammadi et al. 2010), antifungal (Mccutcheon et al. 1994), antiinflamatory (Fourie and Snyckers 1983), etc. These plants originate from temperate and tropical regions and are able to adapt to non-agricultural conditions in marginal regions (Rayne and Mazza 2007). Sumach found its use also in traditional Arabic Palestinian

medicine for the treatment of variety of diseases and has been used since the antiquity (Kizil and Turk 2010). Sumach is nowadays extensively used alone or in combination with other spices as a condiment (Sagdıc and Ozcan 2003). Abu-Reidah et al. (2015) analyzed sumach for the content of phenols and other phytochemicals and report that *Rhus coriaria* is rich in tannins, (iso)flavonoids and terpenoids and in total is composed of 211 constituents.

Target of this study was to evaluate addition of sumac in the rabbit feeding mixture on spermatozoa motility parameters in various time periods.

MATERIAL AND METHODS

Experimental design

Six months old adult male rabbits (n = 25) of New Zealand White breed were subjected to this study. Animals were divided in four experimental groups (S1, S2, S3, S4) and one control group (C). The animals contained within the experiment were housed in air-conditioned halls in individual one-storey metal cages. The light mode was set to 14 hours. All groups were administrated complex granular feed KK V1 *ad libitum* and the drinking water of optimal temperature was unlimitedly supplied via automatic drinker. The feed was administered in form of pellets. The complete feed mixture was composed of: 23% lucerne fodder, 18% sunflower scrap, 18% sugar beet, 10% wheat, 9% wheat bran, 5% molasses, 5% olive starch, 3.5% 3.2%, soybean oil 1.7%, lignobond 1%, monocalcium phosphate 0.3%, sodium chloride 0.3%. The chemical composition is provided in Table 1.

Table 1 Composition of feed mixture

Compound	Amount	Units
Dry matter	926.26	g/kg
Crude protein	192.06	g/kg
Fat	36.08	g/kg
Fibre	135.79	g/kg
Non-nitrogen compounds	483.56	g/kg
Ash	78.78	g/kg
Organic matter	847.49	g/kg
Calcium	9.73	g/kg
Phosphorus	6.84	g/kg
Magnesium	2.77	g/kg
Sodium	1.81	g/kg
Potassium	10.94	g/kg
Metabolizable energy	12.35	MJ/kg

Commercial feed dedicated for experimental groups was enriched with sumach (*Rhus coriaria*) in various concentrations: 0.50% in S1 group, 0.75% in S2 group, 1.0% in S3 group and 1.50% in S4 group. The same diet was applied for the whole duration of experiment (90 days). Control group received feed without sumach addition (Table 2).

In this animal study, institutional and national guidelines for the care and use of animals were followed, and all experimental procedures involving animals were approved by The State Veterinary and Food Institute of Slovak Republic, no. SK CH 29004.

Table 2 Overview of the feed treatments with Rhus coriaria

Sample	Concentration of *Rhus coriaria* (%)	Number of animals	Duration of experiment (days)	Amount of *Rhus coriaria* added (g/100kg)
C	-	5	90	0
S1	0.50	5	90	600
S2	0.75	5	90	850
S3	1.00	5	90	1000
S4	1.50	5	90	1600

CASA analysis

Motility analyses were realized using the CASA method with SpermVision software (Minitub, Tiefenbach, Germany) and the microscope Olympus BX 51 (Olympus, Japan). After semen collection the samples were pipetted into Makler counting chamber (10 μm, Sefi-Medical Instruments, Germany). Measurements of spermatozoa motility were performed immediately after the semen collection. The following spermatozoa characteristics were assessed: motility (MOT), progressive motility (PRO) and velocity curved line (VCL). Every single output of the CASA system is the result of 7 diverse sub-measurements of 7 different fields of Makler Counting Chamber (Tirpák et al. 2015).

Statistical analysis

For the determination of the effect of feed additive on spermatozoa motility, 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 $P < 0.001$.

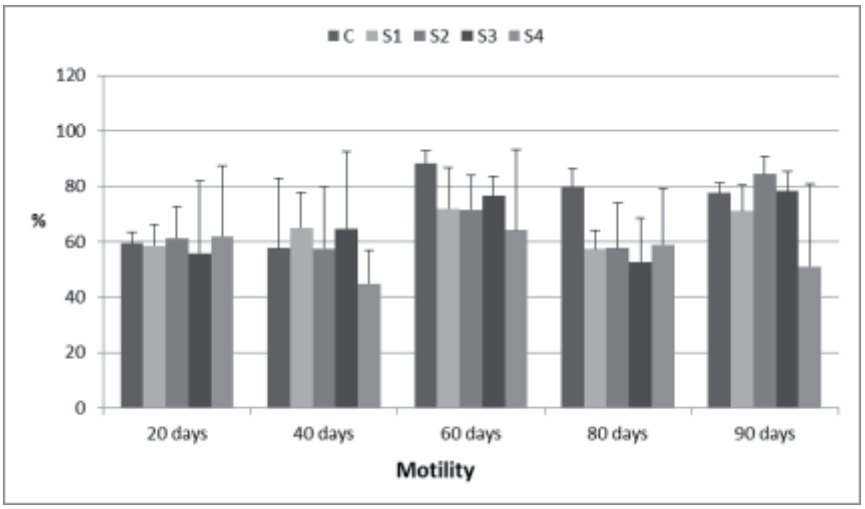
RESULTS AND DISCUSSION

Inceptive evaluation of spermatozoa motility was realized 20 days since the beginning of the experiment. Animals in experimental groups S1 and S3 had decreased spermatozoa motility while the groups S2 and S4 had higher motility than those in the group C. Results obtained after 40 days suggest slightly positive effect of sumach supplementation on spermatozoa motility in rabbits fed with concentrations 0.50% (S1) and 1.00% (S3) of additive. Notable differences were observed at testing days 60 and 80 where the experimental groups showed lower motility compared to the control. The last sampling (Day 90) revealed elevated motility in experimental groups S2 and S3. All the differences noted during the experiment were insignificant ($P > 0.05$).

Twenty days of sumach administration caused the enhanced percentage of progressively motile spermatozoa in all experimental groups, rising along with higher additive concentration. Spermatozoa assessed on the Day 40 of present research showed higher amount of spermatozoa with progressive movement in groups S1 and S3. In the case of progressive motility, the control groups emerged to be the most efficient when evaluated at Day 60 and 80. Percentage of progressively motile spermatozoa was monitored at the end of the experiment in rabbits within groups S2 and S3. Statistical significance this was not detected ($P > 0.05$) in this parameter.

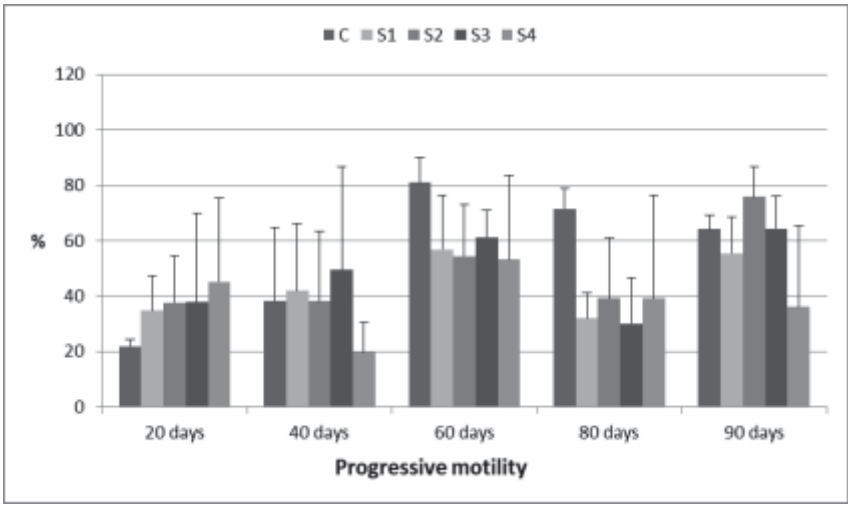
Rabbits fed with an additive-free feeding ration provided semen with spermatozoa of higher velocity during first 60 days of testing. Sumach concentrations (0.75% and 1.00%), represented by groups S1 and S3, demonstrated the positive effect of dietary supplementation on the velocity of the spermatozoa at the end of the trial. As for the velocity curved line, all observed differences absented the statistical significance ($P > 0.05$).

Figure 1 Spermatozoa motility (MOT; %) in different semen extenders at various stages of the experiment



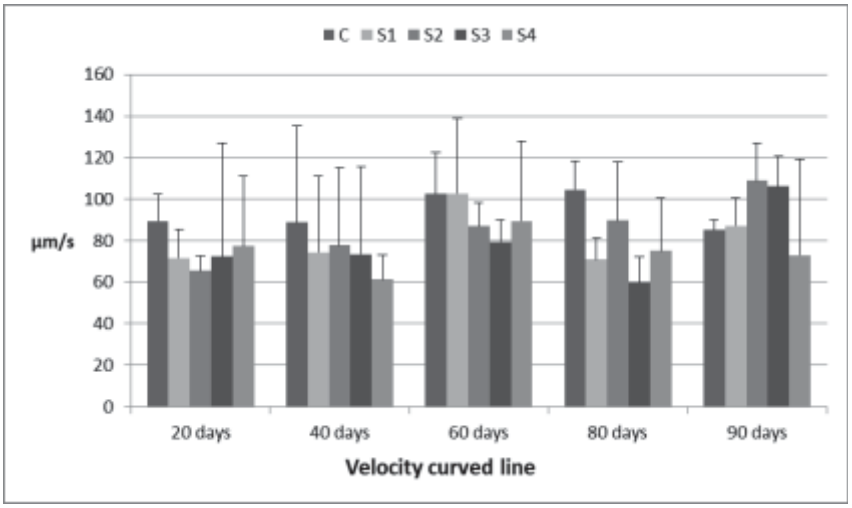
*Legend: C – control group (without the sumach addition); S1 (0.50%), S2 (0.75%), S3 (1.00%), S4 (1.50%) – experimental groups with sumach addition. Significant difference: *P < 0.05; **P < 0.01; ***P < 0.001.*

Figure 2 Spermatozoa progressive motility (PRO; %) at various stages of the experiment



*Legend: C – control group (without the sumach addition); S1 (0.50%), S2 (0.75%), S3 (1.00%), S4 (1.50%) – experimental groups with sumach addition. Significant difference: *P < 0.05; **P < 0.01; ***P < 0.001.*

Figure 3 Velocity curved line (VCL; µm/s) of spermatozoa at various stages of the experiment



*Legend: C – control group (without the sumach addition); S1 (0.50%), S2 (0.75%), S3 (1.00%), S4 (1.50%) – experimental groups with sumach addition. Significant difference: *P < 0.05; **P < 0.01; ***P < 0.001.*

Vizzarri and Corino (2016) and Casamassima et al. (2017) claim that growing interest in dietary supplementation of natural extracts shows promising outcomes in various aspects of rabbit breeding. The literature lacks the information about sumach effect on reproduction. However, there is a lot of information which help to explain and backup our findings. Capcarová et al. (2010) studied the effect

of sumach supplementation on internal milieu of adult male rabbits. Performed blood analysis revealed enhanced antioxidant activity and decreased level of cholesterol. Oral administration of sumach to type 2 diabetic rats showed mild antihyperglycemic properties coming out of several mechanisms. Concentration of low density lipoproteins was rapidly decreased while the high density lipoproteins were strongly increased. Glutathione peroxidise activity was not affected, however the superoxide dismutase and catalase activity was found positively significant following sumach application (Mohammadi et al. 2010). Sumach was administrated also to rats and humans in order to monitor DNA damage in treated and untreated individuals. Reduction of DNA damage along with antioxidant activity in experimental groups indicates direct scavenging effect of sumac. Induction of glutathione S-transferase (GST), composed of two isozymes (GST-α and GST-π) suggest sumach protective properties against genotoxic carcinogens which are detoxified by these enzymes (Chakraborty et al. 2009).

CONCLUSION

Addition of *Rhus coriaria* as dietary supplement to rabbit feed did not show any significant effect on spermatozoa motility properties in any tested concentration (0.50%, 0.75%, 1.00%, 1.50%). Administration of this substance resulted in decrease of motility parameters (motility, progressive motility and velocity curved line) after 20, 40, 60 and 80 days of treatment. CASA assessment revealed the improvement of all motility parameters in rabbits fed with sumach additive (0.75% and 1.00% of active compound) for 90 days. Based solely on these results, it is not convenient to declare neither positive nor negative effects of sumach on male reproduction.

ACKNOWLEDGEMENTS

The authors are thankful to Ing. Tomáš Marcinka for skillful technical assistance. The research was financially supported by the VEGA 1/0760/15, VEGA 1/0857/14, APVV-16-0289, APVV-15-0544, KEGA 006/SPU-4/2015 and AgroBioTech Research Centre built in accordance with the project Building „AgroBioTech" Research Centre ITMS 26220220180.

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MICROSATELLITE DETECTION FOR VARIABILITY STUDY OF MHC GENES REGION IN CAMELS

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Abstract: The major histocompatibility complex (MHC) is a genome region contains genes of immune response and genes causes host and pathogen interactions. MHC class I and II genes encode antigen presenting molecules responsible for detection of peptide on cell surface. This is the reason of the great selection pressure on MHC genes includes both positive and negative selection. Microsatellites are used for variability study of specific genome region. The aim of this study is to find and test some microsatellites located in MHC region and describe its variability and suitability for future testing and usability for multiplex PCR. The model organisms for this study are Bactrian camel (*Camelus bactrianus*) and the dromedary (*Camelus dromedarius*) for their MHC variability and specific resistance to diseases. Camel's MHC region was already not study for microsatellites.

Key Words: camel, MHC, microsatellite, variability, PCR

INTRODUCTION

MHC class I and II genes belong the most polymorphic genes studied in vertebrates. There are more than 100 different alleles for different animal species including humans (Bontrop et al. 1999, Robinson et al. 2013). The large number of functionally important polymorphisms MHC class II genes are located in exon 2. This exon encodes the site for binding of antigen molecule (antigen-binding site) (He et al. 2014, Alcaide et al. 2014). Diversity in this region related with the diversity and variability of pathogens.

Infectious diseases of cattle have significant economic impact on livestock breeding and can affect human health too. This can be caused directly or through the food chain. Camel is economically and culturally important animal because of its long–term adaptation to live in areas of dry climate and its ability to transport important commodities consequential to the development of human culture in these areas (Bulliet 1975, Burger 2012, Wu et al. 2014).

Original camels (*Camelus*) are used as interesting biomodel in the field of infectious diseases because of their resistance against serious infections attacking other species of livestock in the same geographical areas (Wernery and Kaaden 2004, Dirie and Abdurahman 2003).

Currently camels have been identified at a potential vector for Middle East Respiratory Syndrome (MERS). MERS is coronavirus disease attacking humans as known as „Camel flu" (Azhar et al. 2014, Hernida et al. 2015).

MHC region has approximately 4 Mb (million bases) and contains hundreds of various genes with various functions including antigen presentation and non-immune processes.

The crucial aim of this study is to detect microsatellite repeats in MHC region and to verify using fdNTP method instead of fluorescent labelled primers.

MATERIAL AND METHODS

Sequences, animals, DNA samples, PCR reaction mix, fragment analysis reaction mix

For microsatellite primers designing were chosen genomic sequences of *Camelus dromedarius* from NCBI database (National Center for Biotechnology Information) NW_011591952.1 (3.29 Mb), NW_011591249.1 (0.33 Mb) and NW_011591121.1 (1.05 Mb).

The total of 21 DNA samples were analysed (11 samples *Camelus dromedarius*, 10 samples *Camelus bactrianus*). The samples were provided by the Veterinary university in Brno.

For the visualization of amplified fragments were used fluorescent labelled nucleotide (cytosine – fdCTP) which was incorporated to the amplicons during PCR. This method is much cheaper than fluorescent labelled primers and for is sufficient optimalization of method.

The total volume of PCR reaction mix was 10.0 µl and contains of 7.7 µl PCR H_2O (Top-Bio, ltd., Prague, Czech Republic), 1.0 µl 10 × Taq Buffer (Top-Bio, ltd., Prague, Czech Republic), 0.5 µl CombiTaq polymerase (Top-Bio, ltd., Prague, Czech Republic), 0.2 µl dNTP mix (Thermo Fisher Scientific Inc., Waltham, USA), 0.2 µl fdCTP, 0.2 µl forward primer. 0.2 µl reverse primer and 0.2 µl DNA sample. dCTP labelled by R110 fluorescent dye (fdCTP) was used in concentration 10 µM. This solution incorporates labelled cytosine into the amplicon and emits blue signal after laser excitation in genetic analyser.

For DNA amplification was used thermal cycler ABI Veriti 96 Well (Applied Biosystems™) and DNA was amplified according following protocol: initial denaturation at 95 °C for 3 min; 30 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and elongation at 72 °C for 30 s; final elongation at 72 °C for 30 min and holding at 7 °C.

For the fragment analysis of our samples genetic analyser ABI PRISM 3500 (Applied Biosystems™) was used and composition of reaction mix was: 11.3 µl Formamide, 0.2 µl LIZ500 size standard (Thermo Fisher Scientific Inc., Waltham, USA) and 0.2 µl PCR product. Total volume of reaction mix was 11.7 µl.

The reaction mixture was denaturated 5 min/95 °C than was cooled 5 minutes on the ice and was transfered to genetic analyser plate. The fragment analysis was run in POP7 polymer.

To evaluate the results, the GeneMapper 5 software (Applied Biosystems™) was used. The genomic sequences were analysed using WebSat (http://www.wsmartins.net/websat/) to detect the repetitive microsatellite sequences.

RESULTS AND DISCUSSION

Microsatellites locations

There was detected and located total of 58 microsatellites in sequence NW_011591121.1 (Wu et al. 2014), 31 in NW_011591952.1 (Wu et al. 2014) and 33 in sequence NW_011591249.1 (Wu et al. 2014). From these potential microsatellites, total of 27 microsatellites was chosen to first screening. Firstly, were preferred dinucleotide microsatellites with CA and TG motive with perfect repetitions and at least seven motive repeats. These parameters were chosen based on the past experiences.

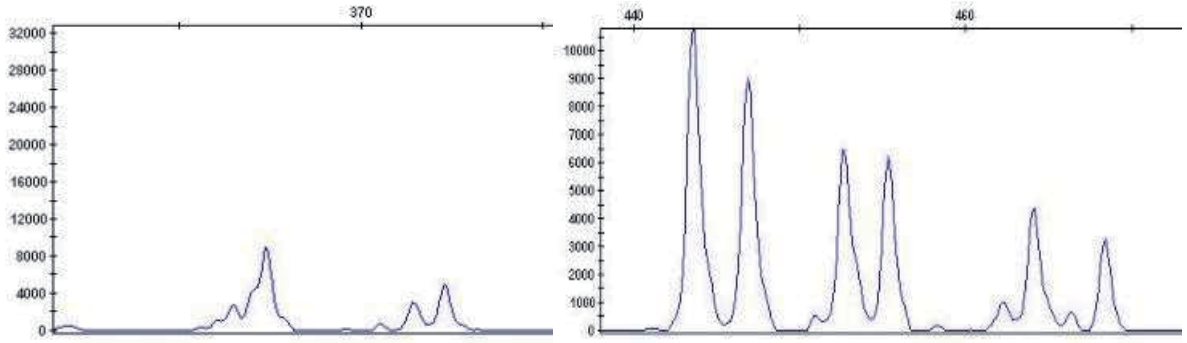
We obtained specific PCR product of all analysed loci. Presence of polymorphism was verified using different samples of animals. Only 8 of them were chosen for testing. The rest of tested microsatellites were not specific enough or were monomorphic.

In a result, there were 3 suitable microsatellites in NW_011591121.1 sequence, 1 in NW_011591952.1 sequence and 4 in NW_011591249.1 sequence found.

It was found that fdNTP labelling method is effective method to polymorphism screening and comparable to primer labelling (Figure 1 and 2). Labelled nucleotides were incorporated into the specific amplicon and played the same role as the fluorescent labelled primers. In the first phase of testing this method is very useful because it can be tested high number of primers for a low price. Labelled nucleotides can substitute labelled primers as well and when the primer is found to be suitable for testing can be synthetized labelled one. In following figures is shown the comparison between fdNTP and labelled primer fragment analysis result.

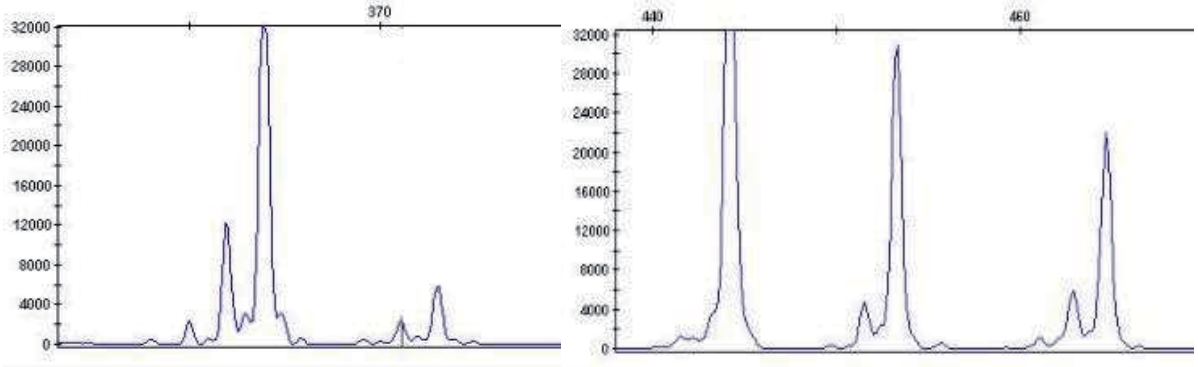
The next phases of research will be focused on possible multiplexing of all these microsatellites into one PCR reaction and to optimize the reaction conditions.

Figure 1 Fragment analysis results using fdNTP



Example 1 Example 2

Figure 2 Fragment analysis results using labelled primers



Example 1 Example 2

CONCLUSION

Important result of this study proves that using fluorescent nucleotides, to verify designed primers for PCR and fragment analysis, can substitute end labelled primers in first phase of testing. This method is cheaper and brings comparable results.

In this study, a group of eight microsatellites was obtained. In future study, this group will be expanded by additional microsatellites and condition of multiplex PCR will be optimized. Eventually this microsatellites panel can contribute to MHC region diversity study in camels.

ACKNOWLEDGEMENTS

The research was financially supported by the Internal Grant Agency of Mendel university in Brno, project No IP 21/2017.

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EFFECT OF DOCOSAHEXAENOIC (DHA) AND EICOSAPENTAENOIC ACID (EPA) FEEDING ON SELECTED MARKERS EXPRESSION IN RATS

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Abstract: The objective of the present study was to compare gene expression of selected markers depending on the type of diet with additional oil in feeding mixture. The experiment was analysed by the real-time polymerase chain reaction method (RT-PCR). As a housekeeping gene with stabile expression was selected β-actin gene (*Actb*) and analysed markers were peroxisome proliferator-activated receptor gamma (*PPARγ*), G-protein coupled receptor-sensor (*GPR120*), adiponectin receptor 1 (*Adipor1*) and adiponectin receptor 2 (*Adipor2*). Forty-eight adult male rats (Wistar Albino) were divided into four groups and were fed 10 weeks the diet containing specific oil for each group. Group R was fed by fish oil added to basic feeding mixture, group P was fed by palm oil added to basic mixture, group S was fed by safflower oil added to basic mixture and group D was fed by *Schizochytrium* microalga extract added to basic mixture. After 10 weeks of feeding, rats were sacrificed by isoflurane overdosing and liver samples were taken and expression of the liver genes coding four selected markers was measured. The main hypothesis of this project was if there were any differences in expression levels among groups depending on the type of diet.

Key Words: rat, DHA, EPA, fatty acids, RT-PCR

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 signalling 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 rats as a model organism for testing a hypothesis that rats fed with specific oils (fish oil, extract of *Schizochytrium* microalga, safflower oil and palm oil) added to the feeding mixture will have increased or decreased level of expression of selected markers in liver tissue.

MATERIAL AND METHODS

Animals, feeding, samples collecting, real-time PCR

The total of 48 rats of the laboratory strain Wistar Albino (Bio Test, Konárovice, Czech Republic) were divided into 4 groups – R, D, S and P (12 animals for each group). The rats were housed in the plastic boxes (53.5 9 32.5 9 30.5 cm) of four animals in a room maintained at 23 ± 1 °C, humidity of 60% and 12/12 h of light/dark cycle (maximum intensity of 200 lx). 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.

Each group was fed the specific feed mixture. Group R was added fish oil (EPA), group D was added extract of *Schizochytrium* microalga (DHA), group S was added safflower oil (LA) and group P was added palm oil (saturated fatty acids) to the basic feed mixure. Feeding mixture was added by vitamin premix. Total amount of EPA, DHA and LA in mixture was 200 mg/kg live wight/day.

The animals were fed daily *ad libitum* for ten weeks and had free access to the drinking water.

After ten weeks the rats were sacrificed by isoflurane overdosing and the liver were removed.

Two samples were collected from each animal. Every sample from another part of liver. Samples were immediatelly put into the RNA*later*™ stabilization solution (Thermo Fisher Scientific) to preservation current level of RNA in tissue.

Total RNA was isolated from the liver aliquot (30 mg) using RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA, USA). The quality of isolation was checked on the 1.2% RNA gel visualized by ethidium bromide. The concentration of isolated RNA was measured on spectrophotometer NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA). Isolated RNA was stored at 80 °C. One microgram of the isolated RNA was reverse transcribed using Omniscript RT Kit (Qiagen) and oligo-dT primers.

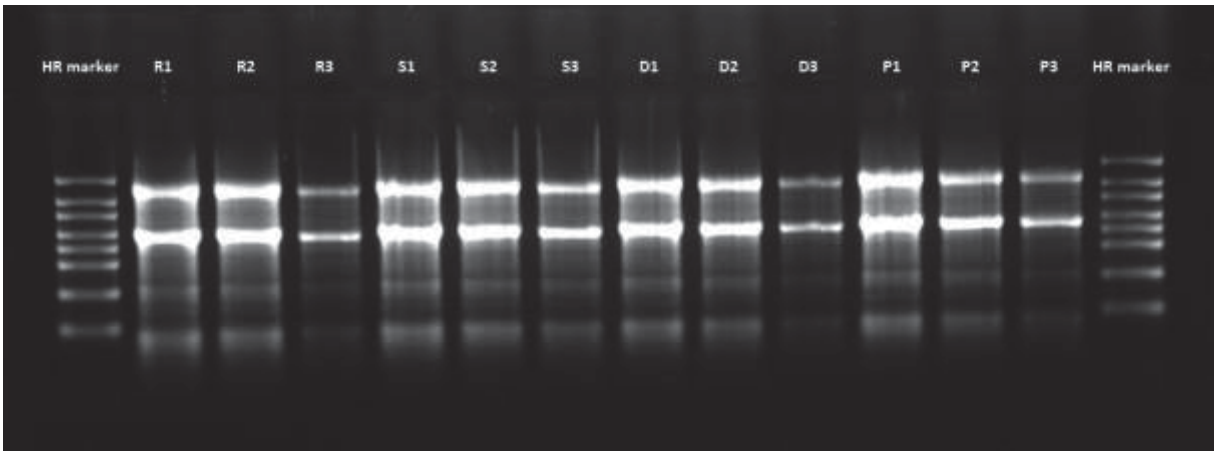
The obtained cDNA was used for quantitative PCR with specific primers for the rat PPARγ (fw CCCTGGCAAAGCATTTGTATG, rev ATCGAAACTGG CACCCTTGA); GPR120 (fw CCCAACCGCATAGGAGA AATC, rev ACACTCGGATCTGGTGGCTCT); Adipor1 (fw GATGTTCTTCCTGGGTGCAGTG, rev CAGAGATG CCCAGGACACAGAC); Adipor2 (fw ATCTGTGTGCTG GGCATTGC, rev AGCCAGCCTATCTGCCCTATG); and housekeeping gene Actb (fw AGAGGGAAATCGTG CGTGAC, rev GTTTCATGGATGCCACAGGATT). The reaction mixture was as follows: 1 µl of cDNA; 0.2 µl of AmpErase® Uracyl N-glycosylase (Applied Biosystems, Carlsbad, CA, USA); 10 µl of Power SYBR® Green PCR Master Mix (Applied Biosystems); 0.2 µl of each primer (mol/µl); and 8.4 µl of H_2O. All analyses were carried out on the 7500 Real Time PCR System (Applied Biosystems) under the following conditions: PPARγ, GPR120, Adipor1, Adipor2: 2 min at 50 °C, 10 min at 95 °C, 40 cycles consisting of 15 s at 95 °C + 1 min at 60 °C; Actb: 2 min at 50 °C, 10 min at 95 °C, 40 cycles consisting of 15 s at 95 °C + 30 s at 65 °C + 30 s at 60 °C. Melting programme consisted of one cycle of 15 s at 95 °C + 1 min at 60 °C + 30 s at 95 °C + 15 s at 60 °C. The effectivity of each reverse transcription reaction was calculated according to the standard curve method using decimal dilution of the input cDNA. The measured CT data were analysed by considering the basal condition as the reference value for relative amount of the gene expression determined under each condition. Results were analysed using REST 2009 software (Qiagen).

RESULTS AND DISCUSSION

RNA isolation verification

RNA isolated from liver tissue was verified by 1.2% agarose gel electrophoresis in FA buffer and formaldehyde was added to the gel and buffer. RNA fragments were visualized by ethidium bromide under UV transilluminator (Figure 1). For fragments sizing was used High Range RNA sizing standard. Electrophoresis conditions was 110 V/30 min.

Figure 1 1.2% agarose gel for verification of RNA isolation



Samples concentrations measured od NanoDrop 2000 and transcription volumes

All samples were measured on spectrophotometer NanoDrop 2000 for total of RNA concentration (Table 1). Reverse transcription of samples was based on concentration measured data. 1 µg of RNA was transcribed to cDNA. Volume of transcribed RNA to cDNA depends on the sample concentration of RNA. In Table 2 are shown volumes of samples for transcription.

Table 1 RNA concentration data measured on NanoDrop 2000

R1	1742.55 ng/µl
R2	1708.85 ng/µl
R3	572.60 ng/µl
S1	2030.15 ng/µl
S2	1556.25 ng/µl
S3	1022.55 ng/µl
D1	1505.25 ng/µl
D2	1172.55 ng/µl
D3	483.00 ng/µl
P1	1882.75 ng/µl
P2	1177.65 ng/µl
P3	583.85 ng/µl

Table 2 Volume of RNA to trasncription based on measured concentrations

Sample	RNA	
	c (in ng/µl)	1µg (in µl)
R1	1742.55	**0.57**
R2	1708.85	**0.59**
R3	572.60	**1.75**
S1	2030.15	**0.49**
S2	1556.25	**0.64**
S3	1022.55	**0.98**
D1	1505.25	**0.66**
D2	1172.55	**0.85**
D3	483.00	**2.07**
P1	1882.75	**0.53**
P2	1177.65	**0.85**
P3	583.85	**1.71**

Comparison of expression of selected markers

Results of RT-PCR were analysed using REST 2009 V2.0.13 software (Qiagen). Comparison of results between tested groups showed there was not differences in expression of selected markers ($p > 0.05$). There was not significant difference between R. P. S and D group (Table 3, 4 and 5). P group fed by palm oil added to basic feeding mixture was selected as control group.

Table 3 Comparison of expression between P and D group

Gene	Expression	Std. Error	P (H1)
Actb	0.714	0.529–0.918	0.030
GPR120	0.887	0.595–1.202	0.685
PPARγ	0.859	0.613–1.115	0.589
Adipor1	3.045	1.026–14.090	0.187
Adipor2	0.803	0.635–1.134	0.483

Table 4 Comparison of expression between group P and R

Gene	Expression	Std. Error	P (H1)
Actb	0.719	0.517–0.895	0.057
GPR120	0.754	0.563–1.164	0.460
PPARγ	0.609	0.402–0.902	0.052
Adipor1	4.075	1.143–26.018	0.000 (UP)
Adipor2	0.916	0.575–1.338	0.605

In group fed by fish oil added to basic feeding mixture was upregulated *Adipor1* gene. in comparison with P group (control group).

Table 5 Comparison of expression between group P and S

Gene	Expression	Std. Error	P (H1)
Actb	0.867	0.657–1.180	0.665
GPR120	0.770	0.556–1.099	0.479
PPARγ	1.099	0.841–1.538	0.775
Adipor1	4.500	1.597–22.530	0.041 (UP)
Adipor2	1.023	0.713–1.448	0.701

In group fed by safflower oil added to basic feeding mixture was upregulated *Adipor1* gene. in comparison with P group (control group).

CONCLUSION

The tested hypothesis was the influence of fish oil. palm oil. safflower oil respectively *Schizochytrium* extract. on expression of selected markers in liver tissue in rats. In our study this hypothesis wasn´t proved.

Expression of *Adipor1* gene was slightly upregulated in group R and S in comparison with control group P.

ACKNOWLEDGEMENTS

The research was financially supported by the Internal Grant Agency of Mendel University in Brno. project No Tym004/2017.

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TECHNIQUES AND TECHNOLOGY

DESIGN AND VERIFICATION OF COMPOST PILES FORMULAS WITH VARIOUS PROPORTIONS OF GRAPE POMACE

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Abstract: The winemaking industry produces large amounts of waste products of which grape pomace is the most important in terms of quantity. The properties and composition of grape pomace depend mainly on the technologies used in the processing of grapes and on a number of environmental factors as well as the technologies applied while wine is growing such as application of foliar and soil fertilizers and phytosanitary treatment. The presence of grape pomace in various proportions in compost piles can influence the composting process and the final quality of the compost produced. This paper therefore focuses on the design and verification of compost piles formulas with different proportions of grape pomace content for composting in strip piles. Simultaneously, it evaluates selected parameters influencing the course of the process and the quality of the final compost. The aim of this paper is to support technology for production of high-quality organic fertilizer that will be used for agricultural land.

Key Words: composting, grape pomace, strip pile, windrow turner, compost piles

INTRODUCTION

Grapevine is the world's most cultivated fruit species grown on an area of 8 million hectares (Faostat 2014). Grapes, the main raw material, can contain up to 25 per cent of skin, seeds and stems (Yu and Ahmedna 2013) that remain unutilized after they have been pressed for wine production. This remaining material is commonly known as grape pomace, or grape marc, and is generated in significant quantities in wine-producing countries. A global grape production of 67 million tonnes (Mt) leads to the generation of 5 million tonnes (Mt) of fresh grape pomace every year (Corbin et al. 2015). The exact composition of pomace depends on the grape variety, the relative proportions of its constituents and the used pressing method (Mäkelä et al. 2017).

The amount of grape pomace produced and its quality is influenced by a number of factors (Mäkelä et al. 2017). In addition to the variety, the method of harvesting and processing in the intake part of the line, the quantity of pomace is influenced by the selected pressing method (Baydar et al. 2007). The proportion of pomace is usually 20 to 30 per cent of the weight of the processed grapes.

Grape pomace is made up of remaining pulp, skin, seeds or stems that represent about a quarter of the weight of grapes. In terms of properties, the grape pomace is a structural material with a bulk density between 360 and 420 kg/m^3. In terms of substance content, the ratio of the main nutrients N : P : K : Ca is 4 : 1 : 4 : 4. This is a raw material with a high organic acid content, which is involved in a low pH range of 3.5 to 3.8, while the C : N ratio is ranging between 15 and 35 : 1 (Yu and Ahmedna 2013).

In line with the latest waste management rules that are implemented within the EU, waste-free technologies are preferentially sought for the use of waste products. Taking into account the total annual volume of production, the focus is, inter alia, on the use of grape pomace for the production of compost.

The basis of successful composting is the creation of a suitable ratio of input materials or the so-called compost pile formula, with subsequent creation and maintenance of optimal conditions, including temperature, humidity, sufficient aeration and pH for sufficient development

of microorganisms throughout the composting process (Diaz et al. 2007). Gea et al. (2005) stated that the optimization of input raw material ratios can affect not only the course of the composting process but also its overall duration.

The aim of this paper was to design and verify the formulas of compost piles with different proportions of grape pomace while evaluating selected parameters influencing the course of the decomposition process and the quality of the resulting compost.

MATERIAL AND METHODS

Experimental piles and site

Experimental measurements were carried in the year 2016 at the Lednice, Czech Republic. For the purpose of experimental measurements, input raw materials were formed into strips of triangular profile with a base width of 1.5 m and height of 0.9 m. The length of each pile according to the evaluated variants was 15 m. Aeration of piles was accomplished using the Euro Bagging 2.5 HP windrow turner in combination with the Zetor Crystal 8011 tractor.

Raw material composition of piles

The pile formulas were designed in four variants with different grape pomace contents. These variants were suitably supplemented with other components in different ratios in order to achieve an optimal ratio C : N = 35 : 1 as indicated in Table 1. The content of the substances was determined in a laboratory.

Table 1 Raw material composition of piles

Raw material	Content of substances				Raw material ratios by variant			
	Moisture (%)	Organic matter (% of dry matter)	N (% of dry matter)	P_2O_5 (% of dry matter)	I	II	III	IV
Grape pomace	75.2	91.5	1.8	0.4	40	30	25	15
Vegetable waste	88.4	89.2	1.7	0.9	36	40	46	55
Grass matter	60.5	90.4	1.0	0.6	10	16	15	16
Straw	14.3	94.5	0.5	0.1	10	10	10	10
Wood chips	44.7	98.3	0.1	0.1	4	4	4	4
Ratio C : N	-	-	-	-	35.1 : 1	35.5 : 1	35.3 : 1	35.4 : 1

The measurement and evaluation of composting process actors

During the composting process, temperature was always measured at the same location using the SANDBERGER GTH 1150 thermometer. Since the pile was established, the temperature was monitored on a weekly basis. The temperature was always measured at the centre of the profile at depths of 0.25 m, 0.50 m and 0.75 m from the ridge of the pile.

Monitoring of moisture

The moisture content of the compost was scanned at a depth of 0.5 m from the ridge of the pile using the TESTO 310 probe. The probes in individual piles were connected to the data recorder. When the moisture content in piles dropped below 40 per cent, the moisture was adjusted by shedding with water.

Nutrient analysis

Determination of the nutrient content was carried out according to the standard for industrial composts ČSN 46 5735 and in accordance with the Decree of the Central Institute for Supervising and Testing in Agriculture of Czech Republic No. 475/2000 Coll. The actual analysis was due to the large number of organic substances carried out according to Morgan. To determine the nitrogen (N) content, a fresh 10 g compost sample was soaked with 30 ml of concentrated sulphuric acid. Then hydrogen peroxide was added and set on fire on the mineralizer to discolour the sample. It was then transferred to 250 ml volumetric flasks, supplemented with distilled water, and measured by Kjeldahl water vapour distillation on the Vapodest system. The determining of other nutrients, due to the large number of organic substances, was carried out according to Morgan. Analyses were carried out of mixed samples weighing 10 g in combination with Gohler's solution (sodium acetate and acetic acid) and activated carbon. After dilution, the solution was measured on an atomic absorption spectrometer (Mg, K) and a spectrophotometer (P). The following tools were used: vapodest 300 by Gerhardt GmbH & Co. KG, Germany; atomic absorption spectrometer Agilent 240 AA by Agilent Technologies, Inc., USA; spectrophotometer Genesys 10S UV-Vis by Thermo Fisher Scientific, Inc., USA.

Determination of pH

To determine the pH, 10 g of the compost sample and 50 ml of $CaCl_2$ was mixed together. Then, the compound was mixed on a mechanical shaker for 60 minutes and the leachate was then measured with a pH meter. This method was used due to the working procedures common for Agrochemical soil testing in the Czech Republic.

Determination of C_{ox} and humus

To determine the components, 10 ml of 0.4 M chromosulfuric mixture was added to a 150 ml beaker 0.2 g of a ground compost. This content was mixed together with a slightly swirling motion so that the compost did not settle on the walls. The compost was crushed into 3 mm. The Ika MF 10 basic Microfine grinder drive by the Ika Works, Inc., USA was used. At the same time, 10 ml of 0.4 M chromosulfuric mixture was added into three beakers as blind samples. All four beakers were covered with hourglass slides and placed on a tray for 45 minutes in an oven at 125 °C. After being removed from the oven, the beakers were allowed to cool for about 10 minutes and the contents of each were subsequently diluted with distilled water to a volume of about 70 ml. The diluted sample and the blind samples were then titrated with a 0.1 M of Mohr salt solution to a grey colour.

Formula for calculating C_{ox}:

$$\boldsymbol{C_{ox}} = \frac{(\boldsymbol{a-b})\cdot \boldsymbol{f}\cdot \boldsymbol{0{,}03}}{\boldsymbol{m}} \quad (\%) \qquad (1)$$

Legend: a – Consumption of 0.1 M Mohr salt solution in a blind sample - mean of three values (ml); b – Consumption of 0.1 M Mohr salt solution in the studied sample (ml); f – Factor of Mohr salt; m – Average length of control roots (mm)

Formula for calculation of humus content:

$$\%\ \boldsymbol{humus} = \boldsymbol{1.724}\ \cdot \boldsymbol{C_{ox}} \qquad (2)$$

Statistics

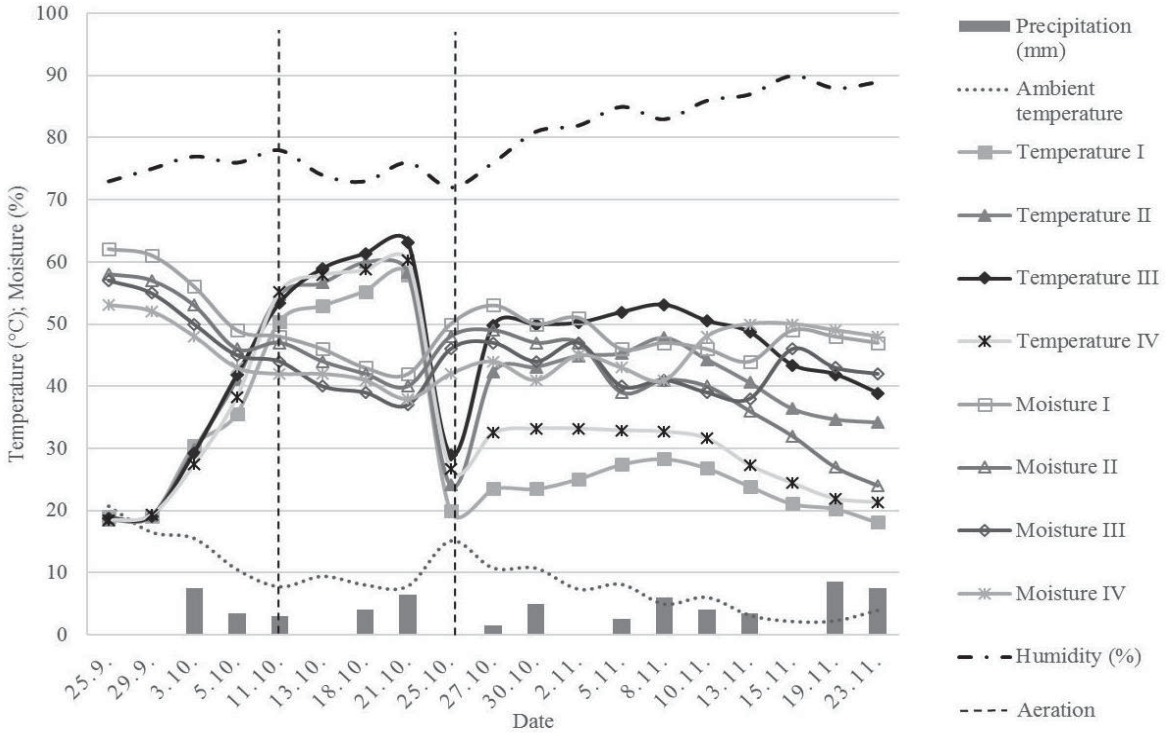
In order to evaluate conclusive differences between the evaluated variants, basic statistical indicators were used, including arithmetic mean, standard deviation and analysis of variance at the significance level of $\alpha = 0.05$. As a follow-up method, the Tukey test was used at the significant level of $\alpha = 0.05$. These statistical evaluation methods were applied using the computer software package "Statistica 12.0" (StatSoft Inc., USA).

RESULTS AND DISCUSSION

The course of temperature and moisture content in the assessed piles is shown in Figure 1. Temperature is one of the most important parameters to monitor a composting process, because it is an indicator of the development of an active thermophilic microbial population, which is required for compost sanitation (Ruggieri et al. 2009a).

A positive standard for industrial composts "ČSN 46 5735" requires the temperature above 45 °C of input raw materials for more than five days (that is called hygienisation). From the values shown in Figure 1, it is clear that for all variants these requirements were met, specifically temperature that had been above 50 °C for 11 days. Kollárová (2006) stated that with the increasing proportion of grape pomace in the compost piles, the maximum temperature reached was lower. However, in variant II, that reached the second highest temperature of 60.2 °C, this claim was not confirmed. Considering the seasonality of the raw material, which is most available in the grape processing period, the course of the composting process can also be significantly influenced by ambient temperatures. Gorodnij et al. (1990) stated that the temperature at composting of grape pomace should not exceed 70 °C, otherwise useful microorganisms may be killed. This requirement has been met for all variants.

Figure 1 The course of the main parameters of composting process



Legend: The line indicates date of aeration of piles.

Table 2 Lists of the resultant values of the selected parameters and the content of main nutrients contained in the evaluated variants of the compost piles, gained from statistical analysis measured during 2016.

Variant	K (mg/kg)	Mg (mg/kg)	P (mg/kg)	N_c (%)	C_{ox} (%)	Dry matter (%)	pH (-)
I	9,716.5 ± 4.95^{b}	1,222.5 ± 3.53^{b}	771 ± 1.41^{a}	1.16 ± 0.01^{b}	8.0 ± 2.8^{a}	52.75 ± 0.63^{a}	7.3 ± 0.14^{a}
II	6,948 ± 11.31^{a}	944 ± 2.82^{a}	759.5 ± 6.37^{a}	0.08 ± 0.01^{a}	4.0 ± 0.14^{a}	76.20 ± 0.28^{d}	7.3 ± 0.28^{a}
III	10,335.5 ± 4.95^{c}	1,562 ± 2.83^{c}	846.5 ± 2.12^{b}	1.15 ± 0.35^{b}	6.5 ± 0.14^{a}	57.55 ± 0.35^{c}	7.3 ± 0.0424^{a}
IV	15,273.5 ± 2.12^{d}	2,213.5 ± 4.95^{d}	858 ± 1.14^{b}	0.2 ± 0.01^{a}	6.5 ± 0.14^{a}	52.00 ± 1.4^{b}	7.6 ± 0.03^{b}

Legend: Values are mean ± SD, n = 3; in each column, mean values of different letters are significantly different at P < 0.05

The moisture seemed to be at higher values with several increases that coincided with rainy periods. However, the overall trend is of moisture decrease, because of water evaporation, as it is typical for composting processes (Ruggieri et al. 2009b, Zhang et al. 2014). Gea et al. (2005) stated that grape pomace is a good absorb material which has beneficial effects in maintaining the desired moisture content in the pile. With regard to the compost piles structure, the moisture ranged between 40 and 58 per cent.

The results of nutrient analysis show that compost produced with a certain amount of grape pomace is a good organic fertilizer. That is a significant element in the circulation of substances and nutrients in nature. This fact is demonstrated by the relatively high nitrogen content, between 0.20 and 1.16 per cent. The results of statistical evaluation suggest that the proportion of grape pomace as input raw material influences the differences in the resulting nutrient content. According to the results of compost analyses, variant II does not meet the requirements of ČSN 46 5735 "Industrial compost" for minimum moisture content of 40 to 65 per cent. This might be caused by the ration of input raw material. Although the conditions for the correct C: N ratio were met, the higher proportion of dry matter in Variant II could influence the texture of the deposit, which differed comparing to Variant I by a higher proportion of mowed grass, but also a lower proportion of vegetable waste compared to variants III and IV. By the variants II and IV, the content of nitrogen was not reached in terms of norm (minimum is at least 0.6 per cent). Plíva et al. (2016), for these cases mentions the possibility of re-processing the raw material by increasing the proportion of raw materials with high nitrogen content. Mahimairaja et al. (1995) stated that the nutrient content in composts depends on the input raw materials, from which the compost was made. Patti et al. (2009) stated that high-quality compost should normally contain between 0.1 and 0.2 per cent of phosphorus, 0.5 to 1.3 per cent of potassium, and more than 0.5 per cent of magnesium. The analyses of compost samples for individual variants show that the contents of the main elements are approaching these values, most notably at variant IV. The optimum pH according to ČSN 46 5735 for industrial composts should range from 6.0 to 8.5 with respect to the microflora. In the experimental compost piles, laboratory analyses revealed pH values ranging between 7.3 and 7.6, which correspond to optimal values. Other evaluated results were C_{ox} content and subsequent conversion to humus content in formula variants composted in strip piles. The results obtained show that all variants of the compost piles achieve Cox values in the range of 4 to 8 per cent. Subsequent conversion of C_{ox} to the percentage of humus has found out that this compost was very high in humus, as the resulting values are higher than 5 per cent.

CONCLUSION

The composting of grape pomace is a process by which we can obtain good organic fertilizer used in viniculture practice. Despite the fact that authors regularly evaluate positively the process of vineyards waste composting, there is a significant lack of information regarding the right composition of substances, degree of composting process and its features. In this study, grape pomace composts, varying in four different formulas, were evaluated for selected parameters (temperature and moisture) and subjected to detailed chemical analysis. The results indicate that when the grape pomace ratio is between 15 and 40 per cent, it is possible to achieve the required hygienisation temperature of more than 45 °C for more than five days. The chemical analysis also revealed that all the grape pomace composts contained levels of free potassium, ranging between 6,948 and 15,273 mg/kg. Plant macronutrients such as Mg were present at levels between 944 and 2,213.5 mg/kg, while phosphorus ranged between 759 and 846 mg/kg and nitrogen between 0.08 and 1.16 per cent. All grape pomace composts provided some benefits in returning the nutrients into the vineyard and all reached C_{ox} values in the range between 4 and 8 per cent, which means that they are humus-rich composts. When optimizing formulas and for ensuring a smooth course of the composting process as well as the composition of the compost produced, it is necessary to keep the C : N input ratio at 35 : 1.

ACKNOWLEDGEMENTS

The research was financially supported by the project IGA - ZF/2017 - DP005 - Verify the effectiveness of biostimulators on the decomposition of the BRO in composting.

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CHARACTERISTICS OF INPUT MATERIALS AND ITS INFLUENCE ON THE OPERATION OF THE BIOGAS PLANT

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Abstract: Detailed monitoring of input material quality is underestimated at biogas plants. However, dry matter and organic dry matter content of material are changeable and have considerable influence on organic loading rate, biogas and methane production and thus on financial balance of biogas plant. The aim of this study is to monitor dry matter and organic dry matter content of input materials, organic loading rate, biogas quality and quantity at chosen agriculture biogas plant, which processes maize silage and pig manure, over long-term period (26 weeks). Samples of raw maize silage and liquid pig manure were collected weekly. Hypothesis predicted changeability of input material characteristics and its influence on biogas quality and quantity and on organic loading rate is confirmed. Determined dry matter content of maize silage ranges from 31.58 to 41.55% and pig slurry reaches dry matter content from 0.95 to 3.49%. Determined organic dry matter content of maize silage ranged from 95.47 to 97.15% of dry matter (30.45–39.86% organic dry matter). Pig slurry reaches organic dry matter content from 60.32% to 81.87% of dry matter (0.57–2.6% organic dry matter). Based on real organic dry matter content of input material and information from operating diaries, organic loading rate is determined. Theoretical organic loading rate is calculated with assumed organic dry matter (32% maize silage and 5.5% pig manure). Confirmation of determined and theoretical organic loading rate shows that monitored biogas plant is usually overloaded. Higher organic loading rate does not lead to an increase of biogas and methane production, but rather to its reduction. So overloading represent wasting of input material and cause lower biogas production and quality which have negative influence on cash flow. This study is unique because of long-term monitoring of input material characteristics which are compared with calculated data.

Key Words: dry matter, organic dry matter, organic loading rate, biogas production

INTRODUCTION

In the last decades, rising prices of fossil fuels have led to worldwide increased research and usage of renewable resources and technologies, including biogas. Biogas is produced during anaerobic fermentation, which is a complex microbial process. Optimal conditions for microbial community are very important to achieve the highest possible quantity and quality of biogas. Therefore, regular monitoring and control of the anaerobic fermentation process are necessary for correct operation of biogas plant. The operator of biogas plant records many parameters into operating diary every day. These records usually include electricity and heat produced and consumed, quality of biogas (content of methane, oxygen, hydrogen, and hydrogen sulfide), pH and temperature in fermenter, consumption of input material. But detailed monitoring of input material quality is still underestimated. The operators of biogas plants usually calculate amount of input material based on dry matter (DM) and organic dry matter (ODM) content determined once at start of plant operating. However, DM and ODM content of input material are changeable and have considerable influence on organic loading rate (OLR), biogas and methane production and thus on financial balance of biogas plant.

The aim of this study is to monitor DM and ODM content of input materials, OLR, biogas production and methane concentration in biogas at chosen agriculture biogas plant over long-term period (26 weeks). Hypothesis predicts changeability of input material characteristics and its influence

on biogas quality and quantity and on OLR. This study is unique because of long-term monitoring of input material characteristics which are compared with calculated data.

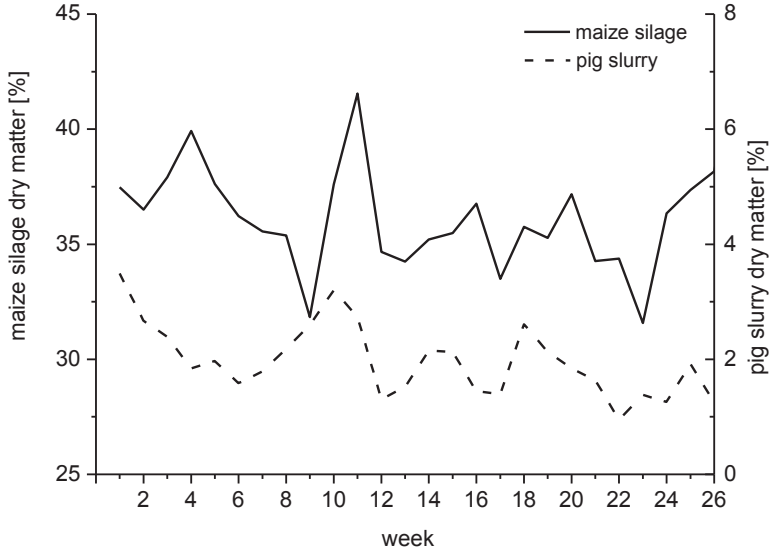
MATERIAL AND METHODS

For this research, the two-stage biogas plant with electric output of 999 kW and a thermal output of 1177 kW has been chosen. The biogas plant processes maize silage and pig manure at mesophilic temperature of 38 °C. The loading is continuous and the hydraulic retention time is about 50 days. The daily dose of input materials is 60 Mg (85% maize silage and 15% liquid pig manure). The volume of primary fermenter is 2640 m^3 and volume of secondary fermenter is 1400 m^3. Generated biogas is accumulated in gasholder with the volume of 860 m^3. Storage tank for finally fermented material has the volume of 4247 m^3. Samples of raw maize silage and raw liquid pig manure, were collected weekly for time period of 26 weeks. DM content was determined according to Czech Standard Method CSN EN 14346 by drying samples at 105 °C ± 5 ºC in the laboratory oven EcoCELL 111 (BMT Medical Technology Ltd., Czech Republic) to constant weight. ODM content was determined by incineration of the samples at 550 °C ± 5 °C in a muffle furnace LMH 11/12 (LAC Ltd, Czech Republic) according to Czech Standard Method CSN EN 15169. All measurements were done in triplicates. Based on the ODM content and information from operating diaries (volume of fermenters, amount of input materials) OLR was calculated.

RESULTS AND DISCUSSION

Very important parameter of input materials is DM content. According to Deublein and Steinauser (2011), DM content of maize silage usually ranges in interval 20–40% and liquid pig manure reaches DM content from 2.5 to 13%. According to Friehe et al. (2010), maize silage reaches DM content from 28 to 35%. According to Al Seadi et al. (2008), DM content of pig slurry ranges between 3 and 8%. Determined DM content of maize silage and pig slurry is shown in Figure 1. DM content of maize silage ranges from 31.58 to 41.55% with median 35.99% and pig slurry reaches DM content from 0.95 to 3.49% with median 1.88%.

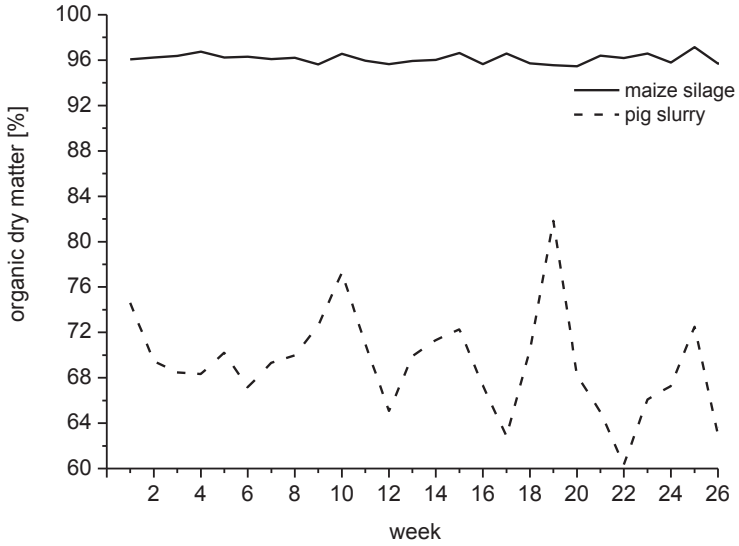
Figure 3 Determined dry matter content of maize silage and pig slurry



ODM content is parameter which represents how many % of DM is theoretically possible convert into biogas. Determined ODM content of maize silage and pig slurry is shown in Figure 2. ODM content of maize silage ranged from 95.47 to 97.15% of DM (median 96.14% of DM), which represents 30.45–39.86% ODM (median 34.55%), but operator calculates amount of input silage with 32% of ODM. Pig slurry reaches ODM content from 60.32% to 81.87% of DM (median 69.40% of DM), which represents 0.57–2.6% ODM (median 1.32%), despite ODM used for calculation of input slurry amount is 5.5%. According to Deublein and Steinauser (2011), maize

silage reaches ODM content 94–97% of DM and ODM content of liquid pig manure ranges in interval 77–85% of DM. Maize silage reaches ODM content in interval 85–98% of DM according to Friehe et al. (2010). According to Al Seadi et al. (2008), ODM content of pig slurry ranges between 70 and 80% of DM.

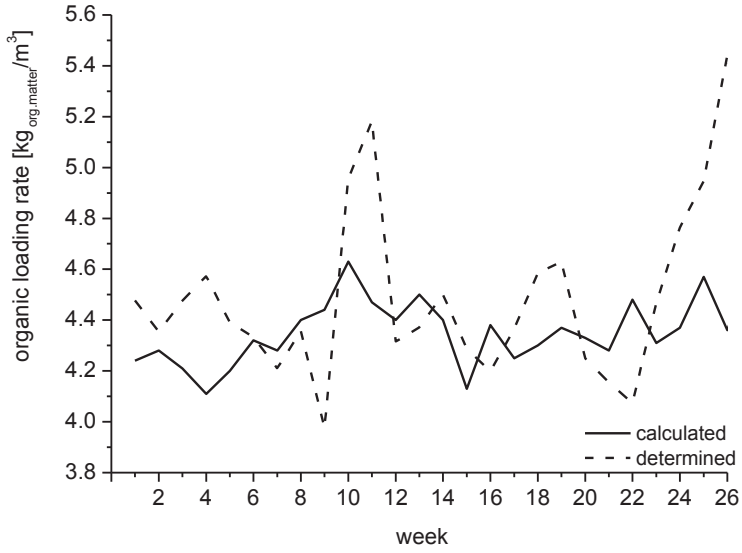
Figure 2 Determined organic dry matter content of maize silage and pig slurry



OLR is important parameter for the stability of anaerobic fermentation, which represents the amount of ODM fed into a fermenter per day per unit volume under continuous feeding (Mao et al. 2015). Typically, this value ranged in interval 0.5–3.0 kg of ODM per m^3 and day (Trávníček et al. 2015). According to study performed by Gou et al. (2014), mesophilic systems showed the best stability at ORL lower than 5 kg of ODM per m^3 and day. The ORL should be optimized for maximum production of biogas. Biogas yield increases with increasing ORL. However, when ORL is significantly higher than normal, the fermentation process becomes unbalanced due to the excessive production of short-chain fatty acids to inhibitory concentrations (Kiely et al. 1997 in Naik et al. 2014). Carbon dioxide produces under these conditions often causes foaming of the fermenter and contributes to operating problems (Naik et al. 2014).

Based on real ODM content of input material and information from operating diaries, OLR is determined. Theoretical ORL is calculated with assumed ODM (32% maize silage, 5.5% pig manure). Both values are compared in Figure 3.

Figure 3 Organic loading rate for whole biogas plant



Monitored biogas plant is usually overloaded, see Figure 5. Overloading can cause operating problems outlined above, but the biogas plant does not have any serious problems. However, overloading always represents wasting of input material and money. Moreover, as is shown in Figures 7 and 8, higher OLR does not lead to an increase of biogas and methane production, but rather to its reduction.

Figure 4 Dependence of biogas production on organic loading rate

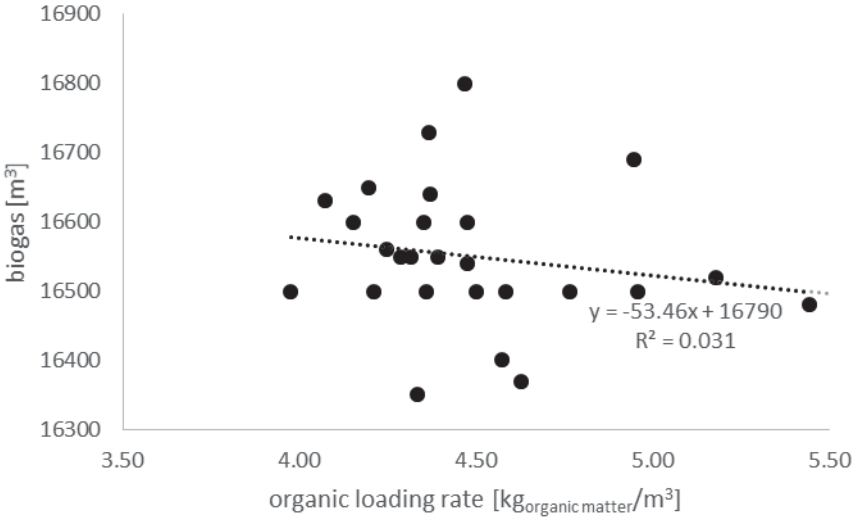
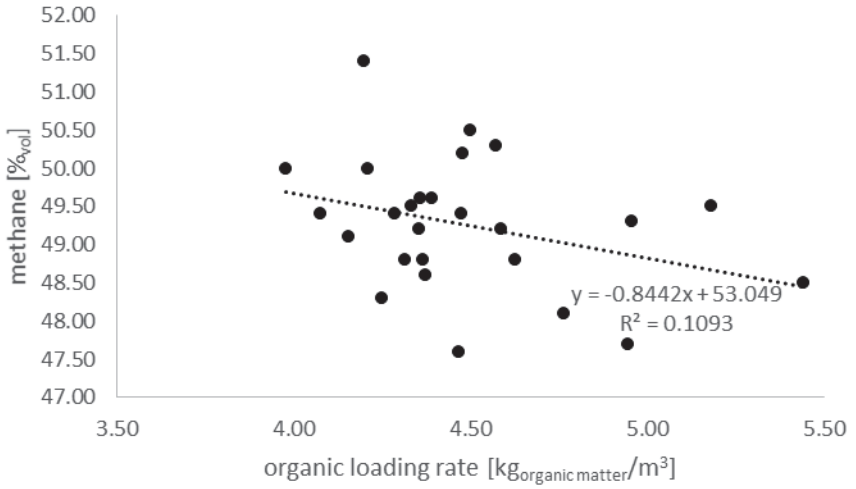


Figure 5 Dependence of methane content in biogas on organic loading rate



CONCLUSIONS

The monitoring of in input material quality is important for general operation of biogas plants. Hypothesis predicted changeability of input material characteristics and its influence on biogas quality and quantity and on OLR is confirmed. Characteristics of input material are changeable. Determined DM content of maize silage ranges from 31.58 to 41.55% with median 35.99% and pig slurry reaches DM content from 0.95 to 3.49% with median 1.88%. Determined ODM content of maize silage ranged from 95.47 to 97.15% of DM (median 96.14% of DM), which represents 30.45–39.86% ODM (median 34.55%), but operator calculates amount of input silage with 32% of ODM. Pig slurry reaches ODM content from 60.32% to 81.87% of DM (median 69.40% of DM), which represents 0.57–2.6% ODM (median 1.32%), despite ODM used for calculation of input slurry amount is 5.5%. Based on real ODM content of input material and information from operating diaries, OLR is determined. Theoretical ORL is calculated with assumed ODM (32% maize silage, 5.5% pig manure). Confirmation of determined and theoretical ORL shows that monitored biogas plant is usually

overloaded. Charts show that higher OLR does not lead to an increase of biogas and methane production, but rather to its reduction. So overloading represent wasting of input material and money and cause lower biogas production and quality which have negative influence on cash flow too.

ACKNOWLEDGEMENTS

The research was financially supported by the Internal Grant Agency of the Faculty of AgriSciences, Mendel University in Brno, IP 13/2017.

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EFFECTS OF BRONOPOL ON ANAEROBIC STABILIZATION OF SEWAGE SLUDGE AND BIOGAS PRODUCTION

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Abstract: Antimicrobial preservatives (as a bronopol) are widely used in cosmetics and toiletries to prevent the spoilage of products due to microbial contamination than can be presented in wastewaters and in sewage sludge and may inhibit the process of sludge anaerobic stabilization. The aim of this study is to specify inhibitory effect of bronopol on anaerobic stabilization of sewage sludge and biogas production. Anaerobic fermentation test was carried out in 24 batch fermenters (the volume of 5 dm^3) for 21 days at 38 ± 0.2 °C. Bronopol was used as toxic substance in 7 different concentrations; 25, 50, 75, 100, 125, 150 and 175 mg/l. The quantity and quality of produced biogas were monitored during hydraulic retention time. Hypothesis, which predicts presence of inhibitory effect of bronopol on anaerobic microorganisms, mainly on methanogenic Archaea, was confirmed. The lowest concentration of bronopol which causes significant inhibition of biogas production is 75 mg/l. This concentration leads to reduction of 5.8 ± 2.3% in the biogas yield. The lowest concentration of bronopol which causes significant inhibition of methane production is 75 mg/l. The reduction in biogas and methane production after addition of the highest bronopol concentration (175 mg/l) is 51.5 ± 0.9% and 76.9 ± 2.9%, respectively. Which means that methanogens are more inhibited by bronopol than other groups of anaerobic microorganism.

Key Words: 2-bromo-2-nitropropan-1,3-diol, anaerobic fermentation, municipal wastewater sludge, inhibitory effect, methane yield

INTRODUCTION

Sewage sludge is produced as the by-product of wastewater treating at every wastewater treatment plant. The anaerobic stabilization of sewage sludge reduces its volume, stabilizes organic matter and reduces the quantity of pathogenic microorganisms. Anaerobic stabilization consists of 4 microbial steps (hydrolysis, acidogenesis, acetogenesis and methanogenesis) that are carried out by different groups of microorganism. Methanogens are the most sensitive and final group of anaerobic microorganism which convert organic matter to biogas, the mixture of methane and carbon dioxide. One of the main disadvantages of anaerobic stabilization is its lower resistance to toxicants than aerobic treatment. Many different toxicants can be presented in wastewaters than in sludge and may inhibit the process of sludge anaerobic stabilization (Chen et al. 2014). For example, antimicrobial preservatives (as a bronopol) are widely used in cosmetics and toiletries to prevent the spoilage of products due to microbial contamination (Kajimura et al. 2008).

Bronopol (2-bromo-2-nitropropan-1,3-diol) is a white odourless crystalline substance well soluble in water, lower alcohols, diethyl ether, ethylacetate and acetic acid. Bronopol is used as preservative and antiseptic agent in liquid medication forms, cosmetic creams, gels and aerosols, deodorants, foot powders, liquid soap, shampoo, household detergents, cleaning pastes, polishing compounds, toilet water deodorants ect. (Legin 1996). Bronopol is also used as a biocide in industrial processes, for example textiles, paper mills and cooling water systems (USEPA 2005). Bronopol is toxic for a wide spectrum of microorganisms, including Gram-negative species, resistant to lots of antibacterial agents (Kajimura et al. 2008). The antimicrobial behaviour of bronopol is caused by electron-deficient bromine atoms in the molecules, which have oxidation properties.

The antimicrobial mechanism of bronopol in the cross-linking of sulfohydrids of enzymes existing on the microbial cells surface. Adaptation to bronopol is theoretically impossible because of its nonspecific mechanism. The main advantage of bronopol is high antimicrobial activity combinated with relatively low toxic effect to hot-blooded animals (Legin 1996). Dissolution of bronopol in aqueous solution at warm temperature and higher pH may lead to release of formaldehyde that is converted into formic acid (Trivisano et al. 2015). In aqueous solutions, bronopol rapidly degrades to many transformation products like 2-bromo-2-nitroethanol, bromonitromethane, tri(hydroxymethyl)nitromethane, nitromethane, 2-bromoethanol, formaldehyde ect. However, its antimicrobial behaviour is still acceptable (Cui et al. 2011).

The aim of this study is to specify inhibitory effect of bronopol on anaerobic stabilization of sewage sludge and biogas production. Hypothesis predicts presence of inhibitory effect of bronopol on anaerobic microorganisms, mainly on methanogenic Archaea. This study is the first evaluation of inhibitory effect of bronopol on anaerobic stabilization of sewage sludge and biogas production.

MATERIAL AND METHODS

According to Czech Standard Method CSN EN ISO 5667-13, sludge sample was taken directly from the anaerobic stabilization tank at the WWTP Brno - Modřice, 513 000 PE, Czech Republic and transported to the laboratory immediately.

According to Czech Standard Method CSN EN 15934, fresh sample was dried at 105 ± 5 ºC in the laboratory oven EcoCELL 111 (BMT Medical Technology Ltd., Czech Republic) to determine the sludge total solids (TS) content. According to Czech Standard Method CSN EN 15169, volatile solids (VS) content was determined by incineration of the samples in a muffle furnace (LMH 11/12, LAC, Ltd., Czech Republic) at 550 ± 5 ºC. In accordance with Czech Standard Method CSN EN 12176, pH, conductivity and redox potential of sludge were determined by using pH/Cond meter 3320 (WTW GmbH, Germany).

In order to measure biogas quantity and quality, anaerobic fermentation test was hold at temperature 38 ± 0.2 ºC, according to German Standard VDI 4630. Three systems, which each consists of eight batch fermenters of volume 5 dm^3, were used. All 24 batch fermenters were filled up with 3 dm^3 of anaerobic sludge. In this study, 8 ml of glycerine were used as a carbon and energy source for microbial growth. One fermenter was used as a blank in each system. To achieve 8 different concentrations of bronopol (25, 50, 75, 100, 125, 150, 175 mg/l), bronopol stock solution was added into remaining fermenters. All concentrations were tested in triplicate.

Hydraulic retention time was 21 days. The biogas was collected in wet gas meters and was measured daily over this period. Biogas quality (content of methane, carbon dioxide, hydrogen and hydrogen sulphur) was analysed during the test 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 were converted to biogas and methane yield, by expressing them as m^3 per kg of VS of the substrate. All measurements were done in triplicate. All measured values are expressed as arithmetic mean ± standard deviation.

RESULTS AND DISCUSSION

Anaerobic fermentation is a complex process in which many mechanisms such as antagonism, synergism, acclimation and complexing could affect the inhibition level. This is reason why the inhibition levels reported in literature show significant variation (Chen et al. 2008). The potential toxicity of chemical compounds is significantly affected by the physical and chemical conditions in which they are present, e. g. pH, redox potential, conductivity, DM, ODM of sludge. Therefore, the characteristics of used sewage sludge are shown in Table 1.

Table 1 Sewage sludge sample characteristics

Sample	pH [-]	Redox potential [mV]	Conductivity [S/m]	Dry matter [%]	Organic dry matter [%]
Sewage sludge	7.21 ± 0.01	-17.20 ± 0.43	0.75 ± 0.01	3.16 ± 0.01	60.06 ± 0.12

The curve of cumulative biogas production during 21 days hydraulic retention time is shown in Figure 1. Specific biogas yield after same time is shown in Table 2. The lowest concentration of bronopol which causes significant inhibition of biogas production is 75 mg/l. This concentration leads to reduction of 5.8 ± 2.3% in the biogas yield. The reductions of 35.2 ± 4.6%, 53.4 ± 1.1%, 53.2 ± 1.5, 51.5 ± 0.9% in biogas production can be observed after addition of 100, 125, 150 and 175 mg/l, respectively. There are no significant differences among inhibitions caused by bronopol in concentrations 125, 150 and 175 mg/l. As can be seen in Figure 1, anaerobic microorganisms need longer adaptation time after addition of bronopol in higher concentration. Thus, curves of biogas production are affected after addition of bronopol in all tested concentrations. However, there are no significant differences in final biogas yield among blank and bronopol in concentrations till 50 mg/l.

Figure 1 Cumulative biogas yield

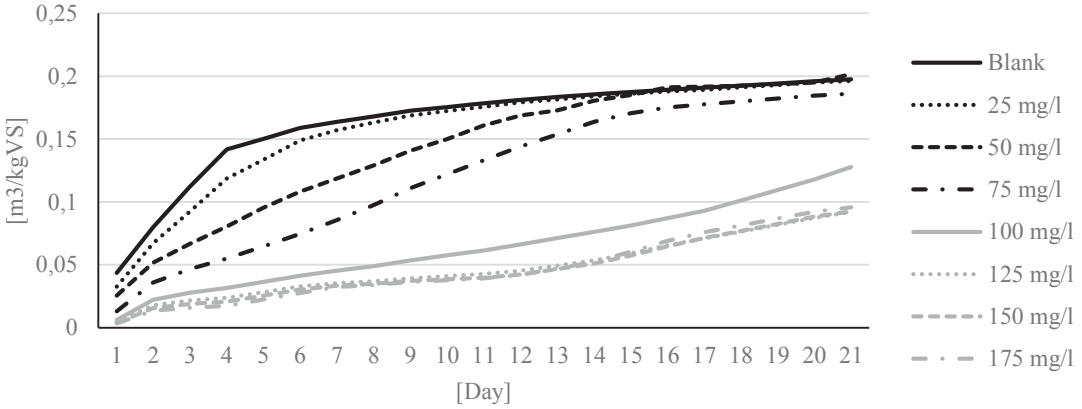

Table 2 Biogas yield after 21 days hydraulic retention time

Sample	Specific biogas production [m^3/kg_{VS}]	Relative biogas production [%]
Blank	0.197363 ± 0.003079	100.0 ± 1.60
25 mg/l	0.196141 ± 0.006142	99.4 ± 3.1
50 mg/l	0.201557 ± 0.014229	102.1 ± 7.20
75 mg/l	0.185954 ± 0.004507	94.2 ± 2.3
100 mg/l	0.127830 ± 0.008997	64.8 ± 4.6
125 mg/l	0.091880 ± 0.002196	46.6 ± 1.1
150 mg/l	0.092294 ± 0.003022	46.8 ± 1.5
175 mg/l	0.095805 ± 0.001845	48.5 ± 0.9

The composition of biogas generated during 21 days hydraulic retention time is shown in Figure 2. The curve of cumulative methane production during same time is shown in Figure 3. Specific methane yield after 21 days is shown in Table 3. The lowest concentration of bronopol which causes significant inhibition of methane production is 75 mg/l. This concentration leads to reduction of 10.2 ± 4.5% in the methane yield. The reductions of 54.8 ± 5.1%, 79.4 ± 4.4%, 79.6 ± 5.6%, 76.9 ± 2.9% in biogas production can be observed after addition of 100, 125, 150 and 175 mg/l, respectively. There are no significant differences among inhibitions caused by bronopol in concentrations 125, 150 and 175 mg/l. As can be seen in Figures 2 and 3, methanogens need longer adaptation time after addition of bronopol in higher concentration. The curves of biogas production are affected after addition of bronopol in all tested concentrations. However, there is no significant difference in cumulative

methane production after 21 days after addition of bronopol in concentration 25 mg/l and blank. The methane yield after addition of bronopol in concentration 50 mg/l seems to be the highest, but there is the highest standard deviation, so the difference between blank and 50 mg/l is not significant, see Table 3.

Figure 2 Methane concentration in biogas

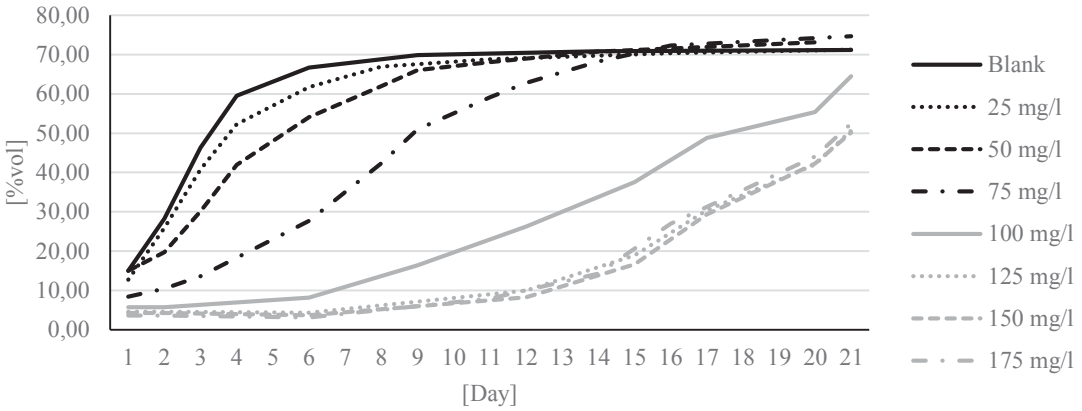

Figure 3 Cumulative methane yield

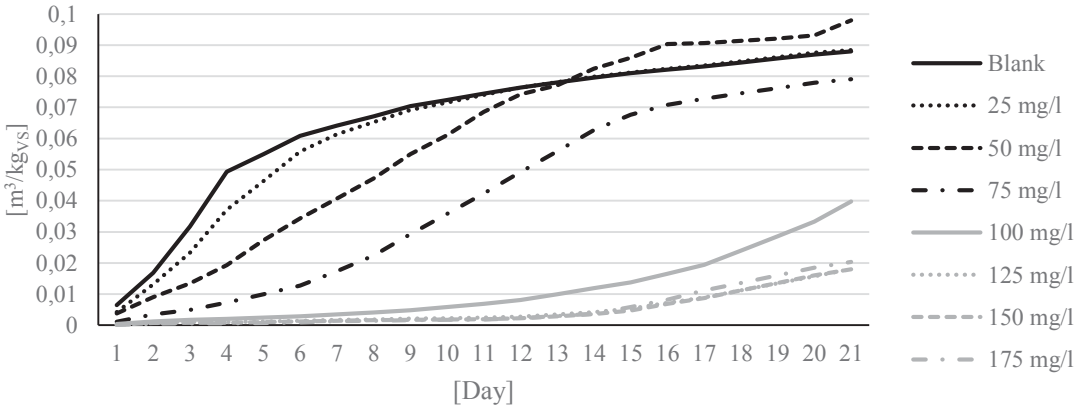

Table 3 Methane yield after 21 days hydraulic retention time

Sample	Specific methane production [m^3/kg_{VS}]	Relative methane production [%]
Blank	0.087934 ± 0.002940	100.0 ± 3.30
25 mg/l	0.088425 ± 0.004310	100.6 ± 4.90
50 mg/l	0.097912 ± 0.012311	111.3 ± 14.0
75 mg/l	0.078984 ± 0.003919	89.8 ± 4.5
100 mg/l	0.039739 ± 0.004498	45.2 ± 5.1
125 mg/l	0.018076 ± 0.003878	20.6 ± 4.4
150 mg/l	0.017967 ± 0.004895	20.4 ± 5.6
175 mg/l	0.020331 ± 0.002566	23.1 ± 2.9

The comparison of Tables 2 and 3 shows that reduction in biogas and methane production after addition of the highest bronopol concentration (175 mg/l) is 51.5 ± 0.9% and 76.9 ± 2.9%, respectively. Which means that methanogens are more inhibited by bronopol than other groups of anaerobic microorganism.

The lowest concentration of bronopol which causes significant inhibition of biogas and methane production is 75 mg/l. This concentration leads to reduction of 5.8 ± 2.3% and 10.2 ± 4.5% in the biogas and methane yield, respectively. Which is higher than minimum effective concentration of bronopol

reported by Legin (1996) that represents 25 mg/l to Gram-negative bacteria 50 mg/l to Gram-positive bacteria, 50 mg/l to yeast-like fungi. On the other hand, the minimum effective concentration of bronopol effective against mold-like fungi is 200 mg/l (Legin 1996). Study by Cui et al. (2011) presents following EC50 values for bronopol to *Chlorella pyrenoidosa*; 5.76 mg/l, 2.18 mg/l, 4.84 mg/l and 5.17 mg/l, after 24, 48, 72 and 96 hours, respectively. The toxicity potential (IC50) of bronopol to *Vibrio fischeri* reported by Wang et al. (2008) in Cui et al. (2011) is 19.19 mg/l. Previous reported concentration of bronopol are significantly lower that concentrations which causes reduction of 50% in biogas and methane yield, 125 and 100 mg/l, respectively.

CONCLUSION

The inhibitory effect of bronopol on anaerobic stabilization of sewage sludge was studied using 24 batch anaerobic fermenters at temperature 38 °C ± 0.2 ºC during hydraulic retention time 21 days. Bronopol (2-bromo-2-nitropropan-1,3-diol) was used as toxic substance in 7 different concentrations; 25, 50, 75, 100, 125, 150 and 175 mg/l. Cumulative biogas and methane production were used as the comparative parameters of bronopol inhibitory effect. Hypothesis, which predicts presence of inhibitory effect of bronopol on anaerobic microorganisms, mainly on methanogenic Archaea, was confirmed. The lowest concentration of bronopol which causes significant inhibition of biogas production is 75 mg/l. This concentration leads to reduction of 5.8 ± 2.3% in the biogas yield. The reductions of 35.2 ± 4.6%, 53.4 ± 1.1%, 53.2 ± 1.5, 51.5 ± 0.9% in biogas production can be observed after addition of 100, 125, 150 and 175 mg/l, respectively. The lowest concentration of bronopol which causes significant inhibition of methane production is 75 mg/l. This concentration leads to reduction of 10.2 ± 4.5% in the methane yield. The reductions of 54.8 ± 5.1%, 79.4 ± 4.4%, 79.6 ± 5.6%, 76.9 ± 2.9% in biogas production can be observed after addition of 100, 125, 150 and 175 mg/l, respectively. The reduction in biogas and methane production after addition of the highest bronopol concentration (175 mg/l) is 51.5 ± 0.9% and 76.9 ± 2.9%, respectively. Which means that methanogens are more inhibited by bronopol than other groups of anaerobic microorganism.

ACKNOWLEDGEMENTS

The research was financially supported by the Internal Grant Agency of the Faculty of AgriSciences, Mendel University in Brno, IP – 13/2017.

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THE EVALUATION OF GREENERY COVER INFLUENCE ON THE SOIL COMPACTION IN THE INTER-ROWS OF GRAPEVINE

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Abstract: This paper focuses on the evaluation of penetrometer measurements carried out in the years 2015–2017, in the area of South Moravia, on the side called Popice/Gotberg with degraded black soil and different greenery. Four mixtures with varied species composition and work designation – Yearling mixture, Perlennial mixture–diverse, Perennial mixture-dry and Yearling mixture-pollinators were used for the planting of the experimental vineyard. Penetrometer measurements were conducted using a manual penetrometer "Eijkelkamp P1.25". The measurements were made in the space between the intervals at depths of 100–300 mm. The water content in the soil at time of measurement, expressed in weight percent, was determined by the gravimetric method. The obtained results show that the highest average values of soil penetrometric resistance were measured in the year when experiment began (2015) and were in the range of 2.27–2.48 MPa. Thanks to the evaluated variants of the greenery, the penetrometric soil resistance was reduced to 0.91–1.06 MPa over the next two years. The largest decrease of soil resistance was obvious at the variant A (yearling mix 58.8%) as well as the variant B (perennial mixture varied 56.7%). Based on the results obtained, these mixtures can be recommended for wine-growing practice and, at the same time, they might be used as a preventive and corrective tool for solving problems with soil compaction.

Key Words: grapevine, vegetation cover, soil compaction, penetrometers, penetration resistance

INTRODUCTION

Cropping systems in viniculture are increasingly focusing on technologies using greenery cover in between rows of vines. A suitable solution is the use of grassland systems with the use of diverse species of plant mixtures, which are the main tool in maintaining soil fertility and partially also the tool of quality management in viniculture (Linares et al. 2014).

Vegetation cover fulfils the whole range of significant functions. An important role is represented by the ability to dampen travel of mechanization vehicles and protection of soil against erosion effects (Göblyös et al. 2011, Ferrero et al. 2005, Boone and Veen 1994, Šimon and Lhotský 1989). From the perspective of ensuring good soil properties, legume or legume-cereal mixtures appear to be highly beneficial. The most valued are especially fabaceous plants of the family *Fabaceae*. It is a huge family, which contains of variety of plants, of which the most important are legumes (beans, peas, broad bean, etc.), and after them follow fodder crops (clover, alfalfa, etc.). These plants can produce a very rich root system, which may penetrate to a greater depth, in many cases up to 3 m (Judit et al. 2011). Due to the higher content of humus caused by the activity of microorganisms, there is simultaneously improved the soil structure and the retention capacity of soil is increased as well (Bauer et al. 2004). Similar to that, King and Berry (2005) state that greenery cover in the inter-rows of grapevine, especially in organic farming due to cover crops, might be able to enhance soil microbial activity, fertility and soil structure. The greenery cover in the inter-rows of grapevine reduces the need for frequent soil tillage in agroecosystems. It protects the soil like an umbrella from heavy rainstorms, thus considerably reduces erosion (Varga et al. 2007, Rinaldi et al. 2000). A key role is played by the type and density of vegetation, which influence the effective hydraulic conductivity (Zhang et al. 1995).

The objective of the work was to evaluate the influence of vegetation cover in various species composition on the penetrometric resistance of soil in selected vineyards in area conditions in the South Moravia.

MATERIAL AND METHODS

Experimental Site

Experimental measurements were carried out in the years 2015–2017 at site Popice/Gotberg. The basic geographical and soil characteristics is claimed as black soil on loess, sandy-loam to loam-sandy sediment. Influenced by the anthropogenic activity (terracing of vineyard). The region - the outer Carpathians and The Pieniny Klippen Belt, coordinates 48°56´06,0´´ North Latitude 16°41´20,0´´ East Longitude, soil typology: CEc: CEcp – carbonate black soil (pelic).

Equipment and Soil Penetration Resistance Measuring Methods

Soil penetration resistance in individual layers in the soil horizon was measured by the penetrometer type "Eijkelkamp P1.25" (Eijkelkamp Agrisearch Equipment, Netherlands). The device consists of a measuring needle tip, tensometric load cell sensor, optical sensor for depth measuring and electronics evaluation with a microprocessor and battery. Actual penetrometer measurements were performed in the area between the rows of vines with the evaluated variants of vegetation cover. For each experimental variant, there were 30 measurements carried out in the depth range 100–200, 200–300 and 300–400 mm. The measured values were corrected based on the determined soil humidity according to Lhotský (2000). The water content in percentage of weight in soil was determined by the gravimetric method.

Experimental Variants of Vegetation Cover

At the site, there were evaluated 4 variants of vegetation cover. The species composition is stated in Table 1.

Table 1 Designation and composition of evaluated mixtures

Variant	Working designation of the mixture	Species composition (% representation of species in the mixture)
A	Yearling mixture	*Lolium multiflorum* (25%), *Phalaris canariensis* (15%), *Phacelia congesta* (5%), *Phacelia tanacetifolia* (5%), *Trifolium alexandrinum* (10%), *Camelina sativa* (10%), *Fagopyrum esculentum* (5%), *Sinapis arvensis* (5%), *Trifolium resupinatum* (10%), *Lotus ornithopodioides* (5%), *Trifolium campestre* (5%)
B	Perennial mixture - diverse	*Festuca ovina* (20%), *Festuca rubra* (10%), *Festuca arundinacea* (10%), *Trifolium repens* (5%), *Medicago lupulina* (15%), *Trifolium pannonicum* (5%), *Lotus corniculatus* (5%), *Onobrychis viciifolia* (10%), *Securigera varia* (5 %), *Anthyllis vulneraria* (15%)
C	Perennial mixture - dry	*Festuca ovina* (40%), *Trifolium repens* (20%), *Festuca rubra* (20%), *Medicago lupulina* (20%)
D	Yearling mixture - pollinators	*Fagopyrum esculentum* (30%), *Phacelia congesta "Fiona"* (20%), *Calendula officinalis* (20%), *Camelina sativa* (10%), *Phalaris canariensis* (10%), *Lolium multiflorum* (10%)

Statistical Analysis

Results were reported as means and standard deviation. Analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) tests were conducted to determine the differences among which means that the statistical significance was declared at $p \leq 0.05$. These statistical evaluation methods were applied using the computer software package "Statistica 12.0" (StatSoft Inc., USA).

RESULTS AND DISCUSSION

In the Table 2 are written average values of penetrometric soil resistance, which have been measured at the test point in three years period. The soil humidity at the time of measurement was 10.5% of its weight (2015); 16.9% of its weight (2016) and 12.8% of its weight (2017).

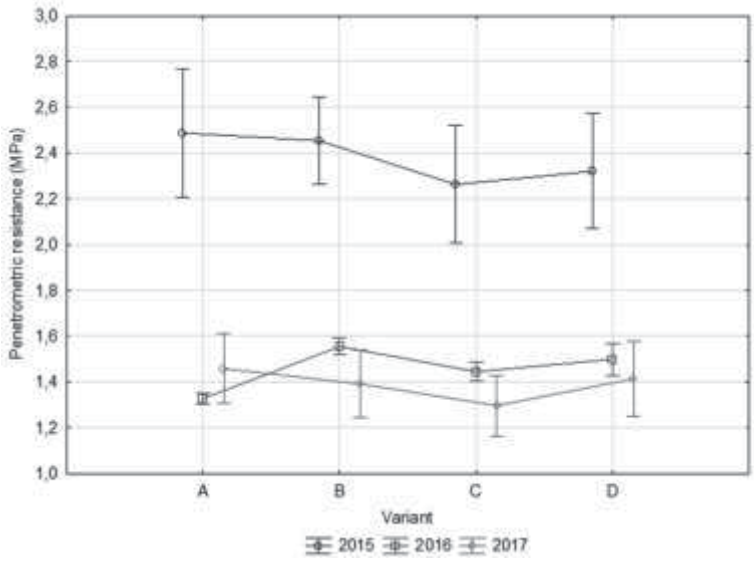
Table 2 Average values of penetrometric resistance of soil

The depth of the soil horizon (mm)	Year	Variant of mixture			
		A	B	C	D
		Average values of penetrometric resistance of soil			
100–200	2015	1.56 ± 0.50^{a}	1.84 ± 0.27^{a}	1.51 ± 0.25^{a}	1.56 ± 0.33^{a}
	2016	1.35 ± 0.06^{ab}	1.46 ± 0.03^{c}	1.42 ± 0.03^{bc}	1.29 ± 0.10^{a}
	2017	1.17 ± 0.29^{a}	1.02 ± 0.37^{a}	1.02 ± 0.31^{a}	0.96 ± 0.36^{a}
200–300	2015	2.90 ± 0.29^{c}	2.65 ± 0.32^{bc}	2.23 ± 0.33^{a}	2.43 ± 0.39^{ab}
	2016	1.29 ± 0.03^{a}	1.55 ± 0.07^{b}	1.35 ± 0.04^{a}	1.50 ± 0.07^{b}
	2017	1.53 ± 0.35^{a}	1.54 ± 0.19^{a}	1.39 ± 0.24^{a}	1.58 ± 0.28^{a}
300–400	2015	3.00 ± 0.22^{a}	2.87 ± 0.16^{a}	3.06 ± 0.12^{a}	2.98 ± 0.26^{a}
	2016	1.34 ± 0.09^{c}	1.66 ± 0.07^{ab}	1.56 ± 0.10^{a}	1.70 ± 0.07^{b}
	2017	1.68 ± 0.42^{a}	1.61 ± 0.33^{a}	1.49 ± 0.34^{a}	1.71 ± 0.26^{a}

Legend: Mean ± SD of three determinations, means in rows, not followed by a common letter are significantly different according to Tukey's multiple range test ($P < 0.05$).

As it can be seen from the values of penetrometric resistance and statistical analyses, there are statistically significant differences between the depths of the soil profile and the evaluated variants of plant mixtures in many cases. In overall, the highest values of soil penetrometric resistance were measured in 2015, shortly after the experiment was established. By comparing these average values with critical values, which are stated for example by Dijck (2002) or Arshad et al. (1996), the soil is most compacted at a depth of 200–400 mm where it reaches a level corresponding to the high compaction. It can be assumed that the reduction of soil compaction over three-year period may be affected by the roots of the plant community, while these roots mostly interfere the soil at a depth of 300–400 mm. Because of these facts, in the recent years, the attention has been focused primarily on the use of various herbal mixtures, which are deliberately introduced in between rows of vines (Escalona et al. 2003).

Figure 1 Comparison of the influence of greenery on the penetrometric resistance (2015–2017)



Legend: Vertical columns indicate 0,95 confidence intervals

Zanathy (2006) describes the high level of soil compaction at those vineyards at which the mechanical soil management is being done. Ramos and Martinez-Casanovas (2006) state, that the alternative soil management techniques which use grassing of the soil surface, such as applying different cover materials on the soil surface, can help to sustain a favourable water balance, soil structure and to lower soil consolidation. Other scientists like Fischer et al. (2014), Peacock (1999), Schuch and Jordan (1981), Gradwell (1968) as well describe the positive effect of vegetation cover on maintaining favourable soil structure, which helps to reduce the extent of soil compaction.

The overall evaluation of the average values of penetrometric soil resistance presented in Figure 1 clearly prove that each variant of plant mixtures has the positive effect of on soil resistance reduction.

The most significant decrease was observed in the variant A (Yearling Mix) and variant B (Perennial mixture - diverse). The penetrometric evaluation of soil compaction in cultivated and grassed rows between the vines in California was studied by Smith et al. (2008) as well. The results of their work confirm (in the longer term) the positive influence of vegetation covered soil on the reduction of soil compaction.

CONCLUSION

In the years 2015–2017, there were carried out experimental measurements in the area of South Moravia focused on the issue of influence of vegetation cover in between rows of vines on reduction of soil compaction. The measurements were carried out at site called Popice/Gotberg. 4 variants of vegetation cover with varied species composition were evaluated with the working designation yearling mixture, perennial mixture – diverse, perennial mixture – dry and yearling mixture – pollinators. The measured values of penetrometric soil resistance show, that the biggest reduction of soil density happened in the area between the vineyards by variant A – yearling mixture (58.8%) as well as the variant B – perennial mixture – diverse (56.7%). Both variants of greening have a major influence on reduction of penetrometric resistance of soil and their sowing in between rows of vines can be especially/particularly recommended as a corrective measure restricting soil compaction.

ACKNOWLEDGEMENTS

Results are based on the solution of the research project TA CR No. TA04020464 Different methods of greening and maintaining of vineyards and their impact on limitation of erosion and quality of production.

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RESEARCH OF BIODEGRADABLE FLUID DURING OPERATING TEST

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Abstract: This paper deals with effect of the biodegradable hydraulic and transmission fluid (Universal Tractor Transmission Oil (UTTO) on operating of the tractor hydraulic and transmission system. This fluid was used in the hydraulic and transmission circuit of a tractor Zetor Proxima 6321. Fluid samples were taken from a Zetor Proxima 6321 tractor at intervals of 250 engine hours. These samples were subjected to an IR spectroscopy analysis and differential scanning calorimetry (DSC). The biodegradable fluid meets the requirements for the operation of agricultural tractors in terms of a low impact on the wear of hydraulic components.

Key Words: biodegradable fluid, IR spectroscopy analysis, pour point

INTRODUCTION

Hydraulic equipment is widely used in powerful mechanisms of agricultural and forest machines as well as in many other areas. The development of modern hydraulic components is aimed at increasing the transmitted power, reducing the energy intensity (smaller reservoirs of hydraulic fluid), minimizing the environmental pollution and increasing the technical life and machine reliability (Tkáč et al. 2017, Hoffmann et al. 2013). Hydraulic and transmission fluid requires monitoring of quality parameters (concentration of metal elements content and concentration of chemical elements representing the additives). Fluid cleanliness is one of the most important features (Majdan et al. 2013, Máchal et al. 2013). Often, the cleanliness and technical condition of the fluid are frequent causes of failures of the transmission and mainly hydraulic system of the tractor. A contaminated fluid creates a risk to the machine in terms of wear and failure (Tkáč et al. 2014, Tulik et al. 2013). Pollution (concentration of metal elements content) is dangerous because it accelerates the degradation and oxidation processes in the fluid. If the fluid is contaminated with dirt above the permitted level, it must be replaced (Angelovič et al. 2013, Majdan et al. 2014). Universal Tractor Transmission Oils (UTTO) are designed for hydraulic and transmission systems in agricultural tractors. These fluids provide lubrication functions for the gear box and the transmission of energy in the tractor's hydraulic system (Hujo et al. 2013). The friction points in the hydraulic and transmission circuit are made from several metals (mostly iron, aluminium, and copper components) (Kumbár et al. 2014). For this reason, there is a need to check for other metals, such as aluminium, copper, chromium, lead, tin, nickel, silver, etc. (Kumbár and Dostál 2013). The aim of this paper is application biodegradable fluid in tractor gear and hydraulic circuit. A biodegradable fluid was used in the gear and hydraulic circuit of a Zetor Proxima 6321 tractor (Zetor Tractors, Czech Republic). The fluid was assessed in terms of the lubrication properties and their effect on the wear during application. Experimental results bring important information from the point of view of oil degradation. The majority of tractors are subjected to the conditions which can cause undesirable phase transition of oil in hydraulic systems. It is necessary to develop the flow of oil due to correct operation of hydraulic equipment (Kosiba et al. 2013).

MATERIALS AND METHODS

An operational test of a biodegradable hydraulic and transmission fluid was set at 500 engine hours (EH). Subsequently, fluid samples were collected for analysis and detection of contamination. As regards biodegradable hydraulic and transmission fluid, the most important is to know the running properties of the fluid, i.e. to know the effect of the fluid on the technical condition of hydraulic

and transmission system parts. Table 1 shows the basic technical parameters of biodegradable fluid type UTTO.

Table 1 Technical parameters of biodegradable synthetic fluid (www.shell.com)

PROPERTIES	UNIT	AMOUNT
KINEMATIC VISCOSITY AT 40°C	mm^2/s	67.52
DENSITY AT 15°C	kg/m^3	931
FLASH POINT	°C	212
POUR POINT	°C	-48

Determining the chemical composition of hydraulic and transmission fluid has been measured using Spectroil Q100 (Spectro Scientific, USA), which is a completely solid state spectrometer. With this spectrometer it can be measured trace levels of elements dissolved or deposited as fine particles in mineral or synthetic oil-based fluids using long established and reliable technique with rotating disk electrode. This spectrometer meets the requirements of ASTM D6595 standard method for the determination of wear metals and contaminants in used lubricating oils and hydraulic mixtures (Kosiba et al. 2016, Kumbár et al. 2014). The following parameters were evaluated:

- concentration of metallic elements (Ag, Al, Cu, Cr, Fe, Mg, Mo, Mn, Ni, Ti, Si)
- concentration of chemical elements representing the additives (B, Ca, Zn)
- pour point by method of differential scanning calorimetry.

A decrease in the content of these chemical elements (concentration of chemical elements representing the additives) is calculated by using the following formula (Kosiba et al. 2016):

$$\Delta ED = \frac{ED_0 - ED_{500}}{ED_0}.100, (\%) \quad (1)$$

ΔED – decrease in concentration of chemical elements

ED_0 – concentration of chemical elements 0 EH

ED_{500} – concentration of chemical elements 500 EH

and an increase of metallic elements is calculated by using the following formula:

$$\Delta EI = \frac{EI_{500} - EI_0}{EI_0}.100, (\%) \quad (2)$$

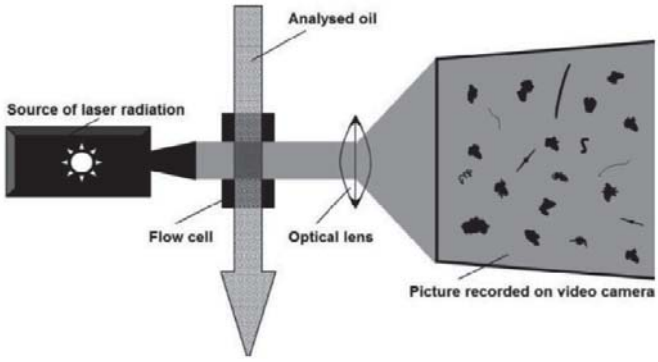
ΔEI – increases in concentration of chemical elements

EI_{500} –concentration of chemical elements 500 EH

EI_0 – concentration of chemical elements 0 EH

LNF is an automated optical oil debris device, which combines the functions of a highly accurate particle counter as well as a particle shape classifier. The basic operating principle of the LNF is illustrated in Fig. 1 (Kučera et al. 2016).

Figure 1 The basic principle of the measuring device LNF (Kučera et al. 2016)



Differential scanning calorimetry (DSC) is a technique in which difference in heat flow (power) to a sample and to a reference is monitored against time or temperature while the temperature of the

sample, in a specified atmosphere, is programmed (Haines 1995). Differential method compares thermal behaviour of reference material with sample. This method provides information on thermal effects which are characterized by an enthalpy change and by temperature range, such as phase transitions (melting, crystallization etc.) (Tulík et al. 2013, Kosiba et al. 2016).

Differential scanning calorimetry (DSC) was performed on a Mettler Toledo DSC (Mettler Toledo, United Kingdom) unit. Samples with weight (8–3) mg were hermetically sealed in aluminium crucibles and thermally treated at a speed of heating 2 K/min in the temperature range from 20 °C to the temperature of -60 °C. The measurement was carried out in an air atmosphere. As a result, we got a DSC thermogram, which was evaluated in STAR^e software (Mettler Toledo, United Kingdom).

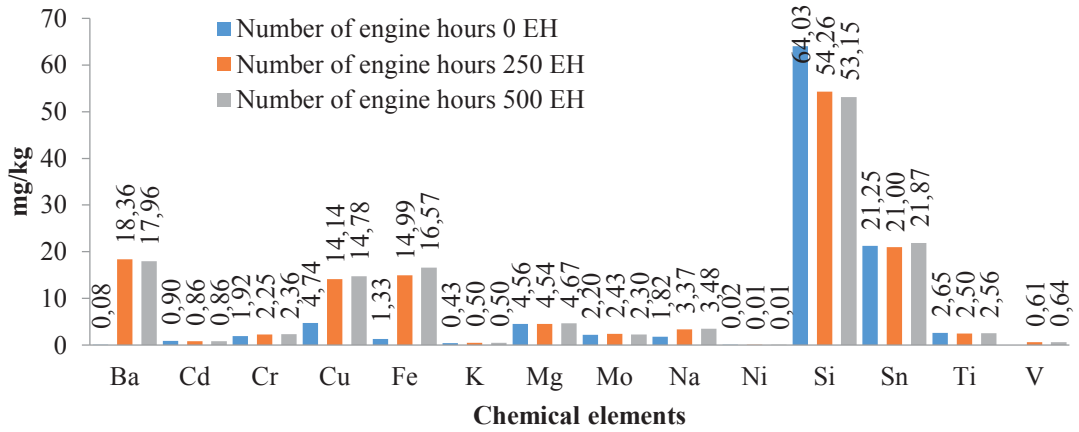
RESULTS AND DISCUSSION

Table 2 and Figure 2 shows an increase in the concentration of chemical elements in hydraulic and transmission fluid during tractor operation

Table 2 Concentration of chemical elements (%)

Chem. content	Ba	Cd	Cr	Cu	Fe	K	Mg
ΔEI (%)	22350		22.92	211.8	1146	16.28	2.41
ΔED (%)		4.44					
Chem. content	Mo	Na	Ni	Si	Sn	Ti	V
ΔEI (%)	4.55	91.21			2.92		45.54
ΔED (%)			50	16.99		3.40	

Figure 2 Concentration of chemical elements (mg/kg)



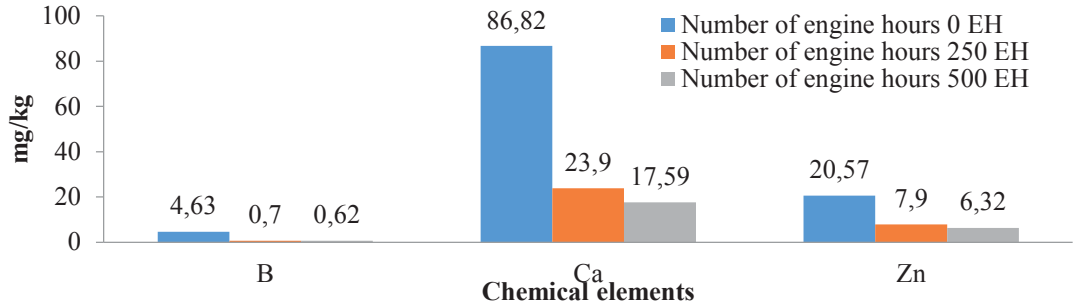
The biggest increase of deposited metals in the oil was observed in relation to barium (Ba), iron (Fe), and copper (Cu). Iron (Fe) and barium (Ba) are used as construction material in the transmission, and copper (Cu) is used as construction material in the oil cooling system. Any concentration of Ba, Fe, and Si are standard values of content according publications by authors (Tarasov et al. 2002) and (Asaff et al. 2014). Other changes in the chemical content of hydraulic and transmission oil are almost negligible.

Table 3 and Figure 3 shows the base elements that characterise set of additive packages. The chemical properties of the hydraulic fluid, being used as the quality evaluation parameters, were monitored in publications by authors (Kučera and Rousek 2008) and (Phillips and Staniewski 2016).

Table 3 Concentration of chemical elements representing the additives (%)

Chemical content	B	Ca	Zn
ΔED (%)	86.61	79.74	69.28

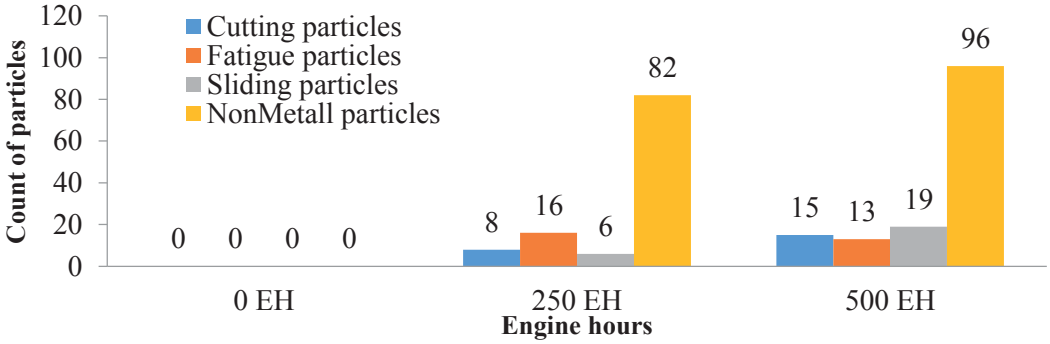
Figure 3 Concentration of chemical elements representing the additives (mg/kg)



The largest decrease was observed in the measuring of boron (B) at 86.61%. Zinc (Zn) is used as an anti-wear agent or as an antioxidant. Hydraulic and transmission oils with zinc additives that are too high have of leading to the corrosion of metals as they chemically attack the metal surfaces (Nicholls et al. 2005).

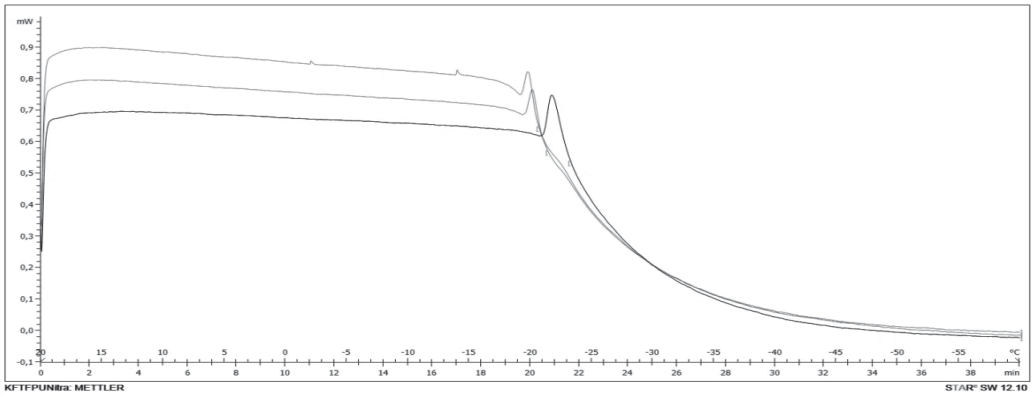
There are several methods how to assess the technical condition of biodegradable fluid. Kučera et al. (2016) used the LaserNet Fines (LNF) tests for the carried out long-term stability of biodegradable fluid. Figure 4 show the LNF tests of biodegradable fluid.

Figure 4 LNF tests of biodegradable fluid



Results of LNF tests of biodegradable fluid correspond by authors by Stachowiak et al. (2008) and Perić et al. (2013). Important consideration of wear particle contamination of gear oil is also focused on trend of cleanliness code according to ISO 4406: 1999 (Kučera et al. 2016). Cleanliness code changed during the experiment from value 19/17/16 to 21/18/15.

Figure 5 Comparison of oil samples by DSC



Legend: SHELL naturelle hf-e46 0MH, 23.06.2017 09:53:06 SHELL naturelle hf-e46 0MH, 12.7700 mg DSC SHELL naturelle hf-e46 250MH, 23.06.2017 10:39:25 DSC SHELL naturelle hf-e46 250MH, 13.2700 mg DSC SHELL naturelle hf-e46 500MH, 23.06.2017 13:31:54 DSC SHELL naturelle hf-e46 500MH, 17.0500 mg

DSC curves which correspond to change of enthalpy due to thermal effects in the samples are on the Figure 5. In the process of oil freezing and in the case of a new oil sample, we observed exothermal peak at the temperature -22.0 °C, which corresponds to the beginning of the change in the crystalline structure of the material. Generally, temperature of phase transition depends

on chemical composition and on crystalline structure of material. In the case of oil with 250 engine hours the temperature of peak was -20.35 °C. In the last sample, representing used oil with 500 engine hours, the temperature of exothermal peak was almost the same as previous -19.8 °C.

CONCLUSION

Tribotechnical diagnostics use oils as media that help obtain information about processes and changes in the systems that they lubricate. If tribodiagnostics are applied properly and thoroughly, they result in significant savings in many areas; for example, they contribute to an increase of the lifetime of machines and devices, to a decrease of consumption of energy, to limiting the idle time (Kučera et al. 2013, Haas et al. 2016). After completing 500 engine hours the operating test for hydraulic oil was completed. In Table 3 the decreasing trends for oil additives can be seen. The biggest decrease in oil additives was observed with boron (B) and cadmium (Ca). Boron (B) content decreases from 4.63 mg/kg to 0.62 mg/kg, and cadmium (Ca) content decreases from 86.82 mg/kg to 17.59 mg/kg. Boron is used as corrosion inhibiter and cadmium is used as a detergent additive.

The graph of DSC indicates that peaks corresponding to the pour point for worn out samples are almost identical, so we can infer that the difference between 250 engine hours and 500 engine hours is not very significant. But we can see the difference between new and worn out sample. Pour point (or temperature of freezing) of both worn out biodegradable hydraulic and transmission fluid samples increased more than 2 °C. The pour point introduced in the specifications of the producer is -48 °C, which corresponds to the measured values.

We can say that the biodegradable hydraulic and transmission fluid does not affect the construction or operation of the Zetor Proxima 6321 tractor. Biodegradable fluid has no negative influence on the rubber components in the hydraulic and transmission system of the Zetor Proxima 6321 tractor.

ACKNOWLEDGEMENTS

Supported by the Ministry of Education of the Slovak Republic, Project VEGA 1/0337/15: ‘Research aimed at influencing the impact of agricultural, forest, and transport machinery on the environment and elimination of any detrimental impact on the basis of the application of ecological measures’.

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CHANGE OF WATER PERMEABILITY OF NONWOVEN GEOTEXTILE EXPLOITED IN EARTHFILL DAM

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Abstract: Geotextiles have been extensively used as filters in drainage systems in geo-environmental and geotechnical engineering for over five decades. The main functions of the filter are to prevent the movement of the base soil fine particles allowing the liquid to flow as freely as possible. The design of a geosynthetics filter is a very complex process because of the large number of parameters involved. However, among the various properties of the nonwoven geotextiles, the hydraulic properties are of major importance. This paper reports the results obtained from the laboratory tests of water permeability characteristics normal to the plane without and under loads of 2, 20 and 200 kPa of nonwoven geotextile samples after 23 years of exploitation in the earthfill dam Białobrzegi in Poland. The results show a marked influence of clogging and loading on flow velocity and permeability coefficient of tested geosynthetic samples.

Key Words: nonwoven geotextile, hydraulic properties, clogging, earthfill dam

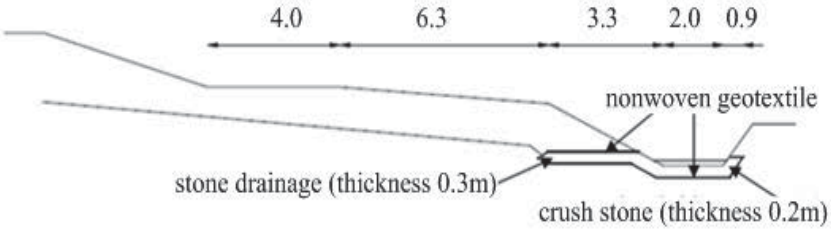
INTRODUCTION

Geotextiles have been used as substitute for mineral materials to provide separation and filtration functions. Nonwoven geotextiles placed horizontally between subgrade fine soils and subbase aggregates can perform the separation function because prevent them from mixing together. In filtration applications the geotextile is used as a filter to prevent the migration of fine particles and allow adequate seepage to flow through the geotextile plane. These geosynthetics are cost-effective in comparison with traditional granular drainage layers, easy to install and reducing the exploitation and use of natural filter materials (Iryo and Rowe 2003, Hong and Wu 2011, Wetzel et al. 2011, Palmeira and Trejos Galvis 2017).

However, the nonwoven geotextiles are required to meet important objectives: permeability, retention and anti-clogging capabilities. The filter clogging is the decrease of the permeability of the filter because of accumulation of materials in the geotextile filter pores. It is a time-dependent process. Three types of mechanism afford accumulation of materials in filter pores: accumulation of soil particles, accumulation of biological and of chemical materials. For that reason, geotextile clogging can be affected by a large number of factors such as the soil composition, the loading state, the sludge content in the water, the composition of the sludge or the fine material and finally type of geotextile. As physical, chemical and biological clogging results in a decrease of drainage capacity of the filtering system and the increase of pore pressure may be the cause of stability problems (Koda et al. 1993, Van Santvoort 1994, Koda and Paprocki 2000, Palmeira and Gardoni 2000, Maheshwari and Gunjagi 2008, Moraci 2010, Wu et al. 2008, Adamcová and Vaverková 2016, Koda et al. 2016), knowledge about this phenomenon is essential to properly design of drainage systems.

The objective of this paper is to examine water permeability in normal direction to the plane of geotextile samples taken from the earthfill dam Białobrzegi. These earthfill dam is one of eight side dams of Zalew Zegrzyński water reservoir. Initially, the dam was drained by a drainaged pipe and discharge into a ditch. Difficult hydro-geological conditions of foundation structures resulted in suffusion and hydraulic break in a sand layer. After the renovation, made in the mid-90s, polyethylene and polypropylene nonwoven geotextiles were used in the drainage system (Figure 1) (Miszkowska et al. 2016).

Figure 1 Nonwoven geotextile in drainage system in Białobrzegi earthill dam (Miszkowska et al. 2016)



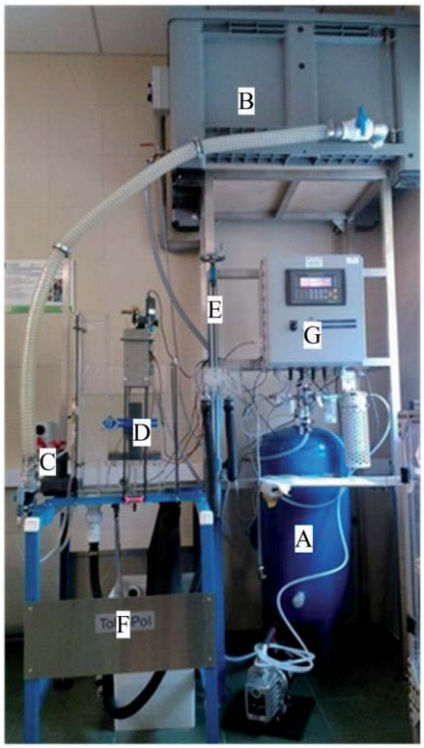
MATERIAL AND METHODS

The PP, PET nonwoven geotextile with mass per unit area of 500 g/m^2 after 23 years of exploitation was tested in laboratory (Figure 2). The geotextile were selected from the ones used in drainage applications in Białobrzegi dam.

Figure 2 The tested nonwoven geotextile after exploitation in the Białobrzegi earthfill dam

The water permeability characteristics normal to the plane without and under load were determined according to PN – EN ISO 11058:2011 and EN ISO 10776:2012 in the Laboratory Water Center at Warsaw University of Life Sciences. Figure 3 presents the test laboratory equipment.

Figure 3 Laboratory equipment for determination of hydraulic properties



Legend: A – water deaeration tank, B – water tank, C – throttle valve, D – sample holder, E – adjustment of head loss, F – water collection tank, G– recording equipment

Testing the flow velocity of flow without the load involved measuring the water flow velocity normal to the plane of a geotextiles sample, in a specified time and hydraulic gradient (14, 28, 42, 56, 70 and additional 3 and 5 mm to calculate water permeability coefficient only). The specimens were placed under water containing a wetting agent and were left to saturate for 24 hours. Then the specimen was placed in the cylinder. A supporting mesh was used in the cylinder to avoid deformation of material by the pressure of water flowing through the holder installed in the device measuring water permeability (Figure 4A). The actual volume of water was determined based on the average from three measurements.

Testing the velocity of flow for specimens under load was carried out with the values of hydraulic gradient being subsequently: 3, 5 and 50 mm. Levels of glass aggregate and supporting steel meshes were used in the cylinder to avoid deformation of geotextile and reproduce the soil conditions similar to the site (Figure 4B). To the prepared specimen a piston was applied, with the loads of subsequently: 2, 20 and 200 kPa. The surface of each of specimen was 0.001963 m^2.

Figure 4 Sample holder

A) Without load *B) Under load*

RESULTS AND DISCUSSION

After the tests without load, the flow velocity v_{20} for each specimen was determined (PN – EN ISO 11058:2011) according to formula:

$$v_{20} = \frac{V \times R_t}{A \times t} \quad [m/s]$$

$$R_t = \frac{\eta_T}{\eta_{20}} = \frac{1{,}762}{1 + 0{,}0337T + 0{,}00022T^2} \quad [-]$$

$$\eta_T = \frac{1{,}78}{1 + 0{,}0337T + 0{,}00022T^2} \quad [mPa \cdot s]$$

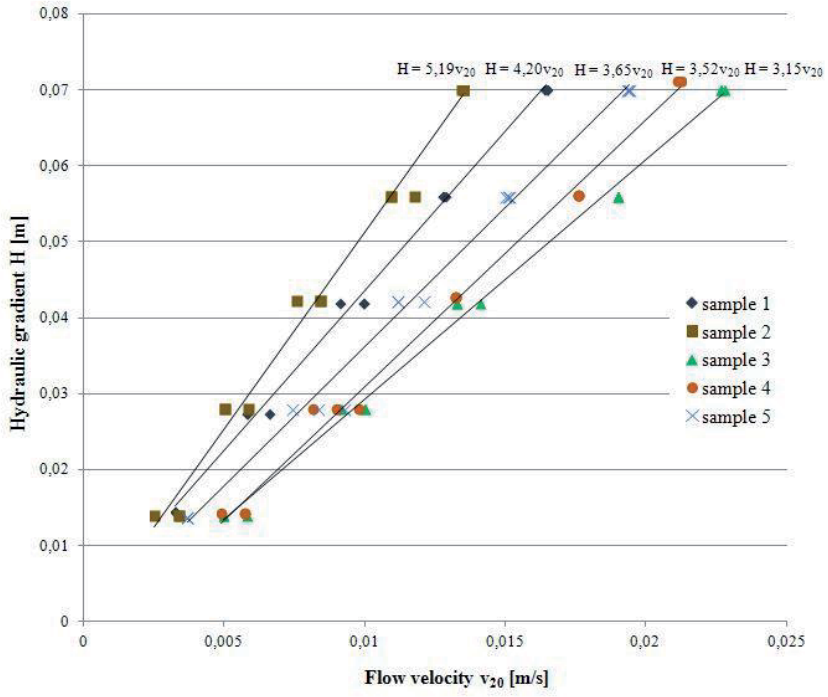
where:

V – water volume measured [m^3], R_t – correction coefficient for water of temperature of 20ºC, A – exposed specimen area [m^2], t – time measured to achieve the volume V [s], ηT – dynamic viscosity at TºC [mPa·s] test temperature, η_{20} - dynamic viscosity at 20 [ºC] test temperature [mPa·s].

Figure 5 presents the relationship between the velocity of water flow and the hydraulic gradient for tested nonwoven geotextile with mass per unit area of 500 g/m^2. Very similar results were obtained by Miszkowska et al. (2016).

Based on the curve equation, the flow velocity index V_{H50} was calculated for hydraulic gradient equal to 50 mm. The range of flow velocity index from the test without load for the nonwoven geotextile is 0.0096–0.0159 m/s. The results depend on the exposure of the specimen to physical, chemical and biological factors and clogging (Palmeira and Gardoni 2000, Wu et al. 2006, Fannin 2010, Veylon et al. 2016). The integrity of the contact at soil-geotextile interface and the stress level also exert a significant influence (Moraci 2010).

Figure 5 Flow velocity characteristics of tested nonwoven geotextile samples

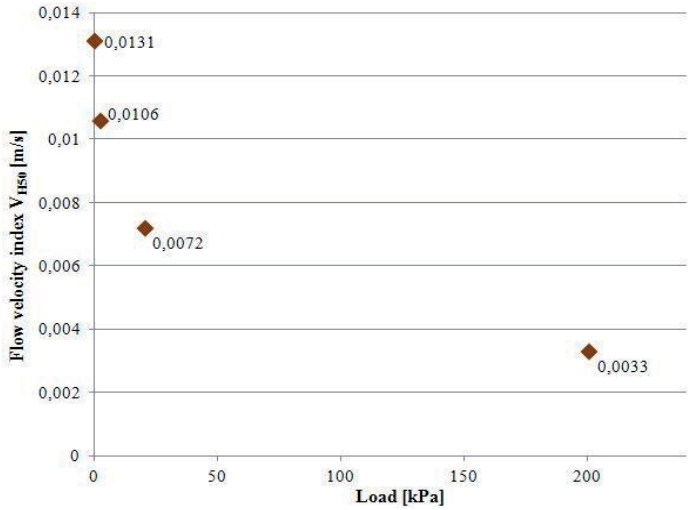


In case of tests under the load of 2, 20 and 200 kPa, the flow velocity index was calculated using the following formula:

$$V_{H50} = \frac{V}{A \times t} \quad [m/s]$$

Figure 6 shows the change of the mean values of flow velocity index without and under load of tested samples of nonwoven geotextile.

Figure 6 Relationship between load and flow velocity index for tested materials



The obtained results show noticeable reduction of flow velocity index under load but it is not linear relationship. The flow velocity index decreased approximately 1.2, 2 and 4 times for the geotextiles subjected to respective loads of 2, 20 and 200 kPa.

Additional, the water permeability coefficient k_g was determined according to formula:

$$k_g = \frac{V \times g}{A \times t \times \Delta h} \quad [m/s]$$

where: g – average thickness of 10 samples of tested material under given load [m] (Figure 7), Δh – pressure differential under and over the specimen, expressed as the height of water column [m].

Water permeability coefficients of tested nonwoven geotextile samples are presented in Table 1.

Figure 7 Statistical thickness characteristics of tested nonwoven geotextile

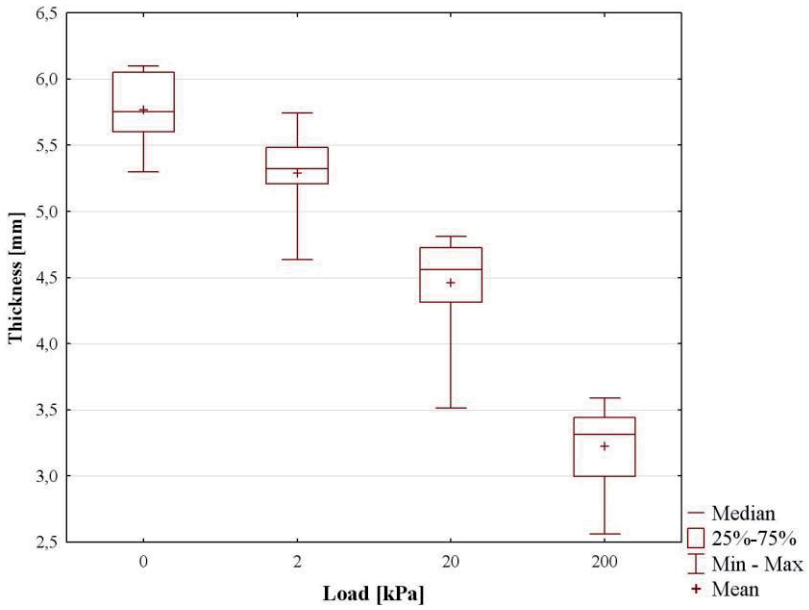


Table 1 Permeability coefficient of nonwoven geotextile under load

Load (kPa)	Water permeability coefficient k_g (m/s)
without load	0.0019
2	0.0017
20	0.0013
200	0.0008

The permeability coefficient decreased approximately 1.2, 2 and 4 times for the geotextiles subjected to respective loads of 2, 20 and 200 kPa. Work presented by Giroud (1982) suggests that the permeability of the geotextile installed in a soil must be only 10 times greater than the soil permeability at all times. At the Białobrzegi earthfill dam, the permeability coefficient of the soil adherent to the geotextile is $k_{soil} = 7.65 \cdot 10^{-5}$ m/s, hence the condition is met even for the most unfavourable case, i.e., a most clogged geotextile under the maximum load of 200 kPa. The water permeability coefficient of tested unworn nonwoven geotextile was 0.00268 m/s so after 23 years of exploitation, this parameter decreased approximately only 1.5 times.

CONCLUSION

This paper presented and discussed the results of water permeability normal to the plane without and under load of of 2, 20 and 200 kPa of nonwoven geotextile after 23 years of exploitation in Białobrzegi earthfill dam. The main conclusions from this investigation are presented below.

After 23 years of exploitation water permeability coefficient of tested materials decreased 1.5 times due to negative phenomena which is clogging but the tested geotextile still meets the requirements of the filters. However, despite of many research and literature data, clogging remains a very complex subject and further research is necessary for the development of more accurate and reliable filter criteria for geosynthetics.

Generally, the hydraulic properties of nonwoven geotextile decreased under load, even 4 times for tested materials. For that reason, it is very important to take this relationship into account in design process to avoid serious consequences in a geo-environmental and geotechnical work.

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SOIL WATER RETENTION BEHAVIOUR OF GRANULAR SOIL – MODIFIED PORE PRESSURE TRANSDUCER TESTS

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Abstract: Unsaturated soil mechanics play a significant role when designing earth structures like embankments, dams, dykes and their foundations. They are commonly designed in shallow subsoil depth. Their mechanical properties are susceptible to the hydraulic status and history which is controlled by the climate events. Soil Water Retention Curves (SWRC) determination is crucial when analysing geotechnical parameters of the soil, especially when analysing rainfall triggered slope failures. The present paper focuses on analysing SWRC for reconstituted sand samples, prepared at different moisture contents and subjected to climatic cycles. To imitate changing in situ environmental conditions the analysed soil samples of sand a research stage employing modified pore pressure transducer was developed. As the soil suction has an impact on mechanical strength of the filling material for earth structures, thus it is so important to firstly determine hydraulic behaviour to be more accurate when analysing mechanical parameters of tested material. The method used for obtaining the suction curves, used in the present study was the tensiometer, attached at the bottom of the sample. The present study was a part of wider research aiming at determination of geotechnical parameters of granular soil influenced by changing hydraulic conditions.

Key Words: Leighton buzzard sand, suction, tensiometer, water content

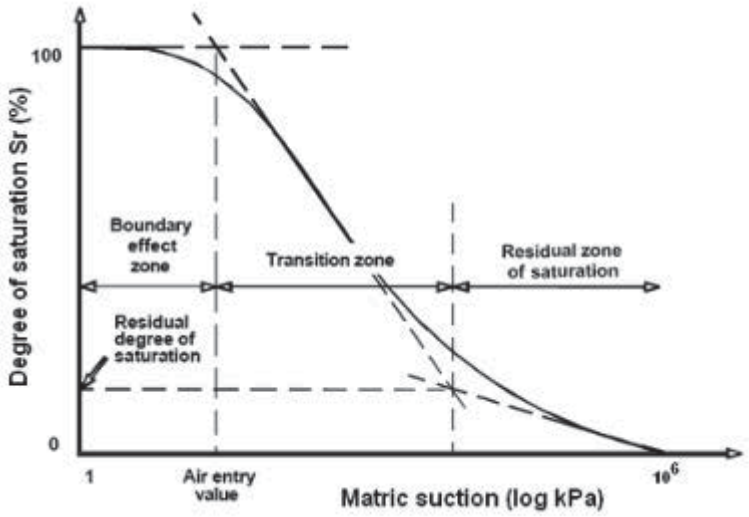
INTRODUCTION

The ability of a soil to attract and retain water is an important property for each soil. A soil water retention curve (SWRC) is defined from the relationship between water content and suction. The soil suction can be defined as the attraction that the soil exerts on free water if the two are placed in contact. This attraction can be stronger if the water inside the voids starts to evaporate. The soil suction, or total suction, has two components: matric suction and osmotic suction. The matric suction is generated by the capillarity phenomenon associated with the existence of surface tension between water and air phases within the soil pores. Matric suction is thus dependent on the soil structure as it is affected by the pore size distribution of the soil. The osmotic suction is associated with salt concentration in the pore water. Changes in osmotic suction are generally less significant than changes in matric suction for most engineering problems although volume change behaviour (shrinkage and swelling) can be strongly influenced by osmotic suction (Fredlund and Rahardjo 1993). The SWRC is dependent on various factors such as soil type, structure and mineralogy. Investigating the hydraulic character of engineering material is crucial not only for civil engineering but is equally important in environmental sciences (Adamcova et al. 2016, Lech et al. 2016, Zabielska-Adamska 2008).

The typical behaviour of a SWRC following a drying path is presented in Figure 1. Initially the suction increases while still maintaining a degree of saturation close to 100% (the boundary effect zone). When the value of suction is sufficient to start draining the pores the air entry value (AEV) of the soil is reached. After the AEV is reached bulk water starts to be pulled from the largest pores and air starts to fill the pores. After this point is reached the soil is considered as being desaturated and

the magnitude of suction necessary to pull water out of the pores may not need to increase significantly until a residual degree of saturation is reached (transition zone). The soil suction can be determined using various techniques. An overview of the various methods can be found in: Fredlund and Rahardjo (1993), Bulut and Leong (2008), Toll et al. (2015). A wide range of systems and methods to measure suction are available in the market, namely: filter paper, psychrometer transistor and high capacity tensiometer (also known as suction probe) and the pressure plate technique, and also less common dew point method.

Figure 1 Typical shape of Soil Water retention Curve with characteristics



MATERIAL AND METHODS

For this particular study samples of granular soil were analysed. For the reconstituted specimen preparation Leighton Buzzard (LB) – Fraction E sand was used. The soil material is classified as fine and uniform uncrushed silica sand. It is deposited around Leighton Buzzard, Bedfordshire in the east of England. LB particles are subrounded and contain mainly quartz. The grain size ranges from 90 to 150 μm. The specific gravity of the sand is 2.65. The maximum and minimum void ratios are found to be 0.99 and 0.65, respectively. The coefficient of permeability is $1.44*10^{-4}$ m/s. All the main physical parameters are presented in table 1.

Table 1 Leighton Buzzard of Fraction E– soil characteristics

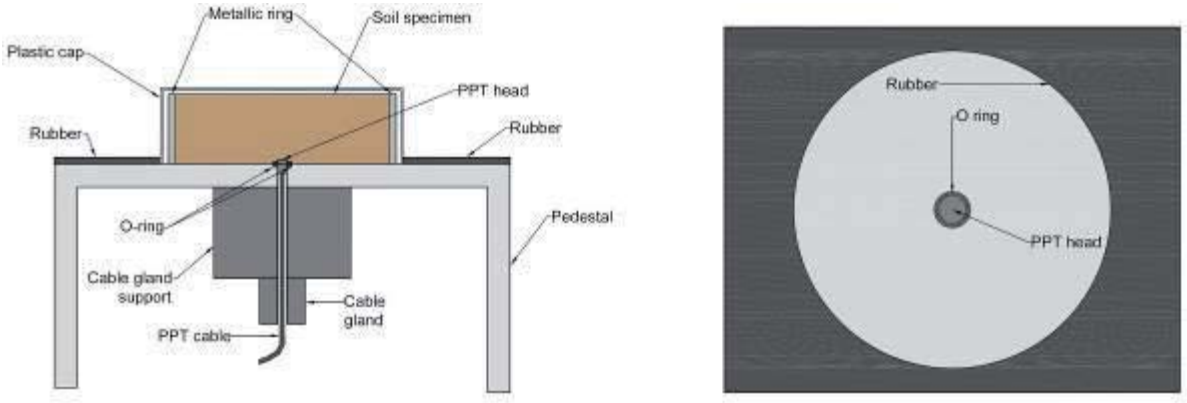
Parameter	Unit	Value
Permeability, k	(m/s)	$1.44*10^{-4}$
Dry density maximum, ρ_{dmax}	(Mg/m^3)	1.61
Dry density minimum, ρ_{dmin}	(Mg/m^3)	1.33
Optimum moisture content, W_{opt}	(%)	11
e_{max}	–	0.99
e_{min}	–	0.65
G	–	2.65
D_{10}	(mm)	0.095
D_{50}	(mm)	0.12
E	–	0.735

The experimental setup used at this study is shown in Figure 2. It included an aluminium pedestal with a fitted miniature Pore Pressure Transducer (PPT) in the middle of its base for direct measurement of matric suction. PPT was supported on the pedestal using a cable gland at the bottom. Soil samples were prepared onto the pedestal due to the non–cohesive nature of the fine sand which

makes challenging their transport from the preparation bench to the testing point. The soil specimens were prepared within a consolidation cutting ring with 75 mm diameter and 20 mm height which was placed on the pedestal. The desired density was achieved by compacting the soil using a tamper. The weight of the sample was determined by weighing the base pedestal and the ring before and after the soil compaction. Surplus material was dried in the oven at 105 °C to determine the initial gravimetric moisture content. Finally, the entire system was placed on an electronic scale with a resolution of 0.001 g allowing continuous measurement of changes in the specimen's weight, due to evaporation.

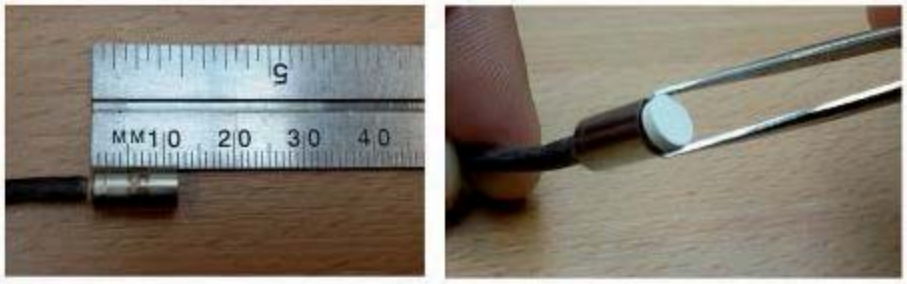
The PPT was used for the direct measurement of matric suction in the specimen during drying and wetting processes. To make it working in desired manner the PPT had to be transformed into a tensiometer (Toll et al. 2013). This is a miniature size transducer (Druck PDCR81 Pore Pressure Transducer, König et al. 1994) with 12 mm length and 6 mm diameter (Figure 3). It consists of a diaphragm which deforms under the pressure of the pore water and produces an electrical signal. An extremely small water reservoir is located between the filter and the diaphragm. The calibration took place in order to correlate the electrical signal produced by the sensor when subjected to pressure changes and the actual pressure. In order to accurately measure also negative pore pressure (i.e. suction), PPT was modified by fitting a high air entry porous disc in front of the diaphragm. The porous disc (filter) was saturated adequately, before testing, using the procedure described by Matziaris et al. (2015). The elimination of air bubbles from the filter and the water reservoir ensured that pore water was in very good contact with the diaphragm and the suction readings were reliable.

Figure 2 Experimental setup for tensiometer (modified PPT) method: (a) side view, (b) plan view.



Several samples were formed by compaction of the material within the soil using tamping method. Same drop height, same mass of a hammer, same volume of soil was used in every test. To change the density of the sample the number of blows was limited to reach required value of the void ratio. When the sample was prepared the tensiometer (being fully saturated at all times) was carefully attached to the bottom of the pedestal on which the specimen was prepared. The tensiometer had to be placed being aware of potential cavitation process taking place in the meantime.

Figure 3 Pore Pressure Transducer (Druck PDCR81), fitted with a high air entry value porous disc.



After the preparation stage, the entire set up was connected to a data acquisition system which was controlled by LabView software. This enabled measurements of suction and current specimen mass to be recorded at regular intervals.

RESULTS AND DISCUSSION

Pre–testing of modified pore pressure transducer (tensiometer)

As mentioned before the tensiometer used in the study was modified to increase the suction measurement ability. To verify the accuracy and the response of the device the material used for pre-tests was clayey silty sand, which was much easier to prepare the sample from and gave more time for the tensiometer to respond to water content changes.

For the pre–testing of tensiometer there were two approaches used in the study: stage drying and continues drying. For the first approach the suction was measured by drying tested simples to required water content value, outside the pedestal. When excepted gravimetric water content of each sample was achieved, the sample was placed in a sealed container and left to allow an even distribution of moisture within the specimen. After 24h the samples were placed on the pedestal and the suction was measured. There were 4 samples prepared at 14%, 15%, 18% and 20% (S1, S2, S3, S4 respectively). For the second approach there was sample prepared at 20% of gravimetric water content and left to dry on the pedestal left on the balance. The suction was measured simultaneously with a mass loss. The results obtained for both approaches are presented on Figure 4 and 5. They present measured suction over time, at different gravimetric water contents.

Figure 4 Suction measurements in stages for pre–test samples at certain water contents.

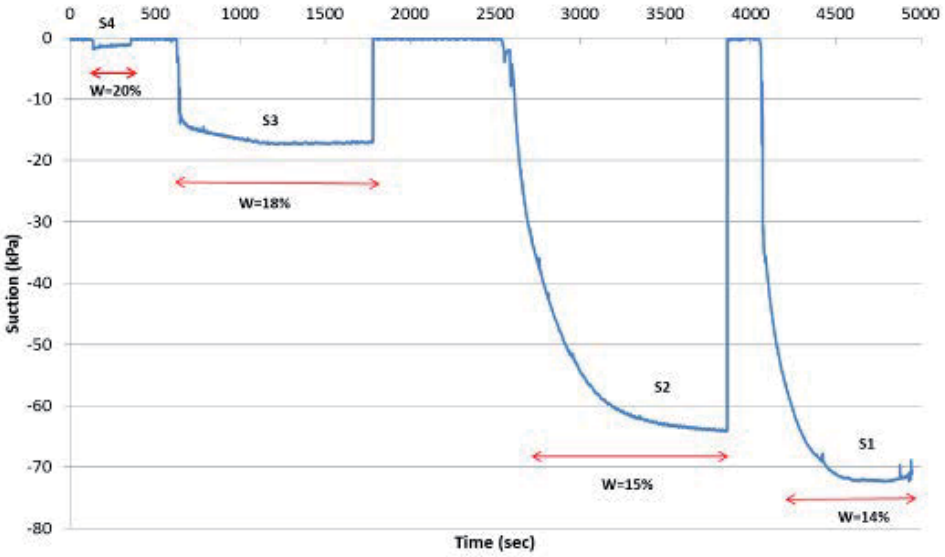


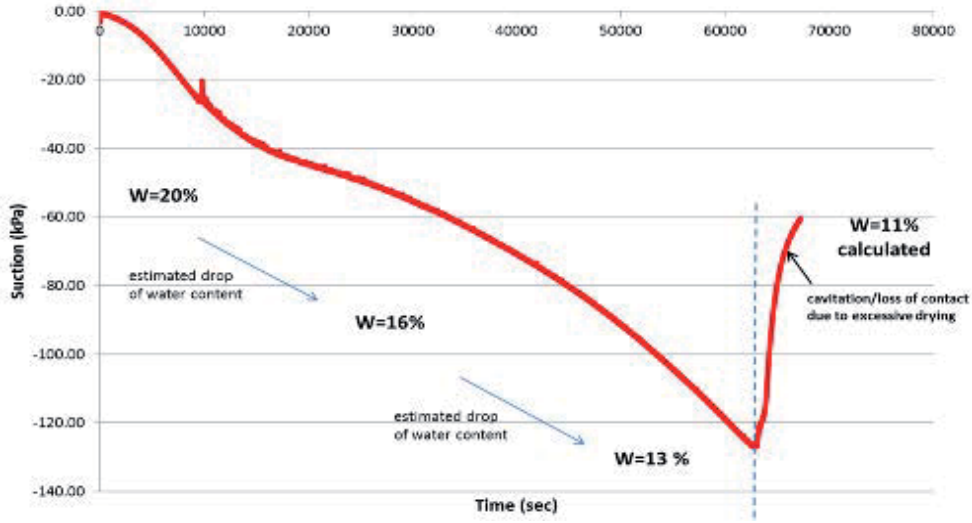
Figure 4 present rapid respond for the changing suction and different water contents. The equilibrium of recorded value of suction stabilizes after short time of about 400–700 sec. After the sample gets in contact with the tensiometer the reading of suction is very clear. However, more importantly when the sample is taken of the pedestal the value of suction is immediately reaching back the initial value of 0–1 kPa. With lower water content the suction reading is increasing what was expected to happen, and proved the tensiometer to be working properly.

The next approach of continuous drying allowed obtaining almost full SWRC using single sample. The sample was saturated up to 20% of water content and left for free evaporation. The whole set up was placed on the balance so the weight loss due to evaporating water could be precisely measured. The sample could be left over night as the all data (suction and weight) was collected using data acquisition system controlled by LabView.

From Figure 5 it is quite clear how the suction is changing over time with water content decrease, caused by evaporation. However, more useful information from this plot is that cavitation point was captured at 125 kPa, what gave the real capability of measuring suction for this particular

(modified) device. The differences in measured suction between the approaches are mainly due to different method of preparing the sample, using different compaction efforts.

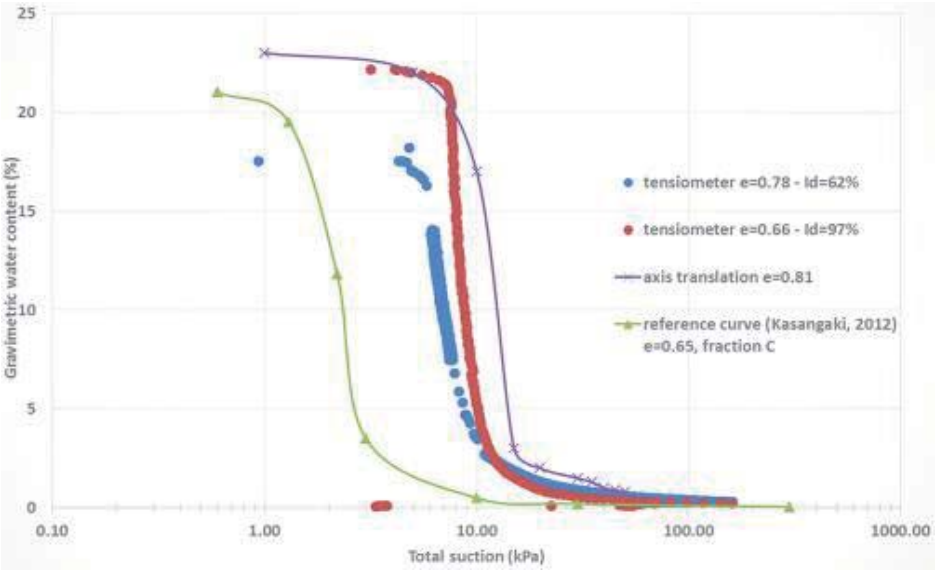
Figure 5 Suction measurement for continues drying path (sample left open to the atmosphere, over two days, starting from 20% of moisture content).



SWRC determination for Leighton Buzzard of Fraction E sand

After confirming that the modified pore pressure transducer (tensiometer) was working properly the samples of Leighton Buzzard of Fraction E sand were prepared and tested. The results are plotted on Figure 6. There were two samples tested on the research set up. The samples differ void ratio of e = 0.66 and 0.78. Due to difficulties when preparing the samples for similar water contents (looser the sample lower the water content) for the same void ratio the initial moisture was 22% for denser specimen and 17% for the loose one. For verification of SWRC shape correctness, there were two more curves added to the plot. One was the referencing SWRC obtained by Kasangaki (2012), for Leighton Buzzard of coarser fraction (C), and second one was SWRC for the same material used in the study but obtained by using axis translation technique (Osinski et al. 2016; Toll et al. 2016)

Figure 6 SWRCs for Leighton Buzzard of Fraction E sand obtained by using modified pore pressure transducer (plus additional curves to compare the results).



As seen from the graph the reference curve for fraction C sand, the suction is lower for the same values of water content for fraction E. The behaviour is as expected, and is mainly caused by differences in fraction (the reference material was coarser). The other curve obtained by using axis translation method plots slightly above the curves of the fraction E sand, which is typical when

applying these two different techniques. Plots of SWRC for fraction E sand (for tensiometer technique) allows clear identification of air entry value zone, transition and residual zone of saturation.

CONCLUSIONS

The aim of the study was to obtain and SWRC of Leighton Buzzard of Fraction E sand and analyse the hydraulic behaviour by using modified pore pressure transducer. This was a part of the wider research aiming at determining changes of mechanical parameters of soil in different water content condition, at known values of suction. For such purpose the SWRCs had to be determined before starting triaxial shearing tests. Using tensiometer technique is an efficient, very accurate and less time consuming method for measuring the suction, comparing to axis translation methods commonly used in triaxial cells. The most important conclusion is that the suction of tested material did not significantly respond to significant moisture content changes. The transitional zone fits within the range from 8 to 10 kPa, when the water content has changed from 20 to 3%. Such findings were very useful when preparing the samples at certain suction, for particular water content, for further tests on mechanical behaviour of unsaturated samples.

ACKNOWLEDGEMENTS

The Authors would like to acknowledge the European Un–ion's Seventh Framework Programme FP7/2007–2013/ under REA grant agreement no 289911. The authors also would like to acknowledge EU COST Action TU1202 “Impact of climate change on engineered slopes for infrastructure”.

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EFFECT OF HEAT TREATMENT OF CMT WELD ON ITS MECHANICAL PROPERTIES

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Abstract: The CMT (Cold Metal Transfer) welding process allows to achieve optimal results when joining different kind of metals. The article deals with the mechanical properties of CMT welded joints, specifically verification of mechanical properties changes before and after heat treatment. CMT welding is a new approach that allows joining of different types of metals, creating a weldment with specific mechanical and chemical properties. A heat treatment is widely used to modify the mechanical properties of the material. In case of different kind of metals weldment, the heat treatment is difficult because of different properties of the basic welded metals. The heat treatment has to be optimized to improve the properties of all the materials used in the welded joint. A tensile strength test will be used to verify changes in the mechanical properties of the weldments.

Key Words: welding, heat treatment, mechanical properties, tensile strength, galvanizing

INTRODUCTION

CMT is a modified GMAW (gas metal arc welding) process that uses a new method of droplet detachment based on short circuit welding. The moment the power source detects a short circuit, the welding current drops and the filler wire starts to retract. Exactly one droplet is detached into the molten weld puddle. The filler wire then moves forwards again and the cycle is repeated. The filler wire is constantly retracted at very short intervals. The precisely defined retraction of the wire facilitates controlled droplet detachment to give a clean, virtually spatter-free material transfer. For mechanized pipeline girth welding, the CMT process is applied with a conventional bug and band system. The typical welding procedure incorporates an Argon/CO_2 shielding gas with a standard J-Bevel joint design. Travel speeds range from 350–406 mm/min, with typical heat inputs ranging from 0.47 to 0.75 kJ/mm. Thanks to this method, qualitatively different materials can be combined. The weld is more resistant to galvanic corrosion, which is a significant factor today (Pickin and Young 2006, Dostal 2014).

The CMT process is characterized by three main features compared to the conventional MIG/MAG process: a rearward motion of the wire that is integrated into the overall digital control of the process, a significantly lower energy input into the weldment and a spatter-free material transfer. The process brings about 20 to 30 percent less heat into the material than the MIG/MAG welding. This makes the material noticeably less distorted and substantially less time will be required for costly corrections for distorted weldments. The CMT process is more reliable than alternative methods and is therefore suitable for automated serial production (Cao 2014).

Weldability of zinc coatings is an important property of the coating, since most galvanized product is joined in this manner. Arc welding of galvanized steel sheet produces defects such as gas cavities (blowholes) and spatters (Matsui 1998).

It is well known that, when a welding operation has been performed, the weld and heat-affected zone (i.e. the weld area) may tend to cool in a stressed condition, sometimes with unwanted phases present or with other undesirable properties which might cause a subsequent failure of the weld. In order to minimise such undesirable characteristics, a post-weld heat treatment of the weld area may be effected. The success of such heat treating operations depends very largely on the ability to heat the weld area to a definite temperature and then to maintain the temperature within specified limits for a specified period of time and/ or to control its cooling rate thereafter (Ptacek 2002).

This paper is focused on preparation and evaluation of CMT weld joints. The advantage of this method consists mostly in welding thin metal sheets up to 2 mm thick, anticorrosion steels and in joining heterogeneous materials. The article deals with the mechanical properties of CMT welded joints, specifically verification of mechanical properties changes before and after heat treatment.

MATERIAL AND METHODS

The S235JRG1 steel material with a carbon content of 0.17% was selected for the experiment. The content of P, S and N is 0.045, 0.045, and 0.007% respectively. It is a low-grade structural steel of the usual quality which is suitable for welding. Fusion-welded parts of structures and machines, statically and slightly dynamically stressed, less stressed welded pipes and branches, and weir structures, and forge-welded parts. The tensile strength [Rm] for thermally unprocessed version is min 370 MPa and after normalizing annealing is min 350 MPa as specified in CSN 41 1373 standard. Zinc coated steel sheet: It is a classical construction material used in a usual technical engineering practice. The base is formed by a ferrite-pearlite steel class 11, whose surface treatment consists of a combination of passivating elements according to the chemical composition of a melting bath. They are zinc and aluminium intermetallic compounds. The thickness of passivating coating is up to 30 μm and is guaranteed by zinc dipping method and removing the excessive material by nitrogen (Votava et al. 2014).

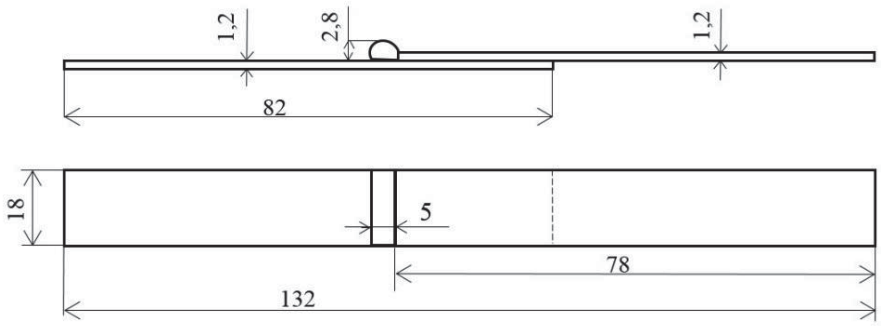
Prior to welding the selected material, an anti-corrosion layer was applied. The first part was hot-dip galvanized, while the other part was hot-dip Al-Zn (50/50) plated. To verify the quality of the applied anti-corrosion layer, the size of the two coating types was measured with a CM-8825 digital ultrasonic thickness gauge (manufactured by TQC, Nederland).

The CMT welding method was used to weld these two differently galvanized steels. Additive material used for this experiment was a solid wire $CuSi_3$. Chemical composition of the wire itself is 97% Cu and 3% Si. Basic welding parameters: Weld beads were produced on a welding robot using the cold metal transfer method. Protective atmosphere of welding bath consists of 98% of Ar and 2% CO_2. The speed of welding jet was 10 m/min. Welding current for materials up to 2 mm was set at 59 A. Flow of the protective gas depending on the speed of welding jet was 10 to 16 l/min. Electric voltage of the welding aggregate is 11.5 V (Yang et al. 2013).

Four groups of five samples each were created. In total, 20 samples were generated. One group of welded samples was not heat treated. Three groups welded samples were subjected to heating at a temperature of 150 °C, 300 °C and 450 °C in the school 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 (Polakova 2016).

For tensile testing of model sample the universal testing machine ZDM 5/51 was employed. ZMD 5/51 is ripper for testing of the samples under tensile stress (manufactured by Labortech, Czech Republic). Samples were fixed in self-locking jaws and loaded with jaws dilation at 5 mm/min (Zacal 2015). A diagram of the welded sample is in Figure 1.

Figure 1 Diagram of the welded sample



To modify the mechanical properties of the weld, the mentioned thermal exploitation of samples was proposed. Thermal resistance tests up to 450 °C were chosen with respect to the information in literature (Ptacek 2002) stating that $CuSi_3$, which was used as an auxiliary material for the CMT welding method, is resistant to 200 °C. It was therefore desirable to ascertain what influence the temperatures up to 450 °C have on the welded joint strength during at least short-term exploitation. Heating temperatures were chosen with respect to the material used. The zinc coating can be used at operating temperatures up to 300 °C, since the melting temperature of zinc is 419 °C (Marder 2000). To compare the differences, we also used lower temperatures of 150 °C and 300 °C. The heating time of 1 hour was chosen as optimal for this experiment. This is the lower time limit used for recrystallization annealing (Ptacek 2002).

RESULTS AND DISCUSSION

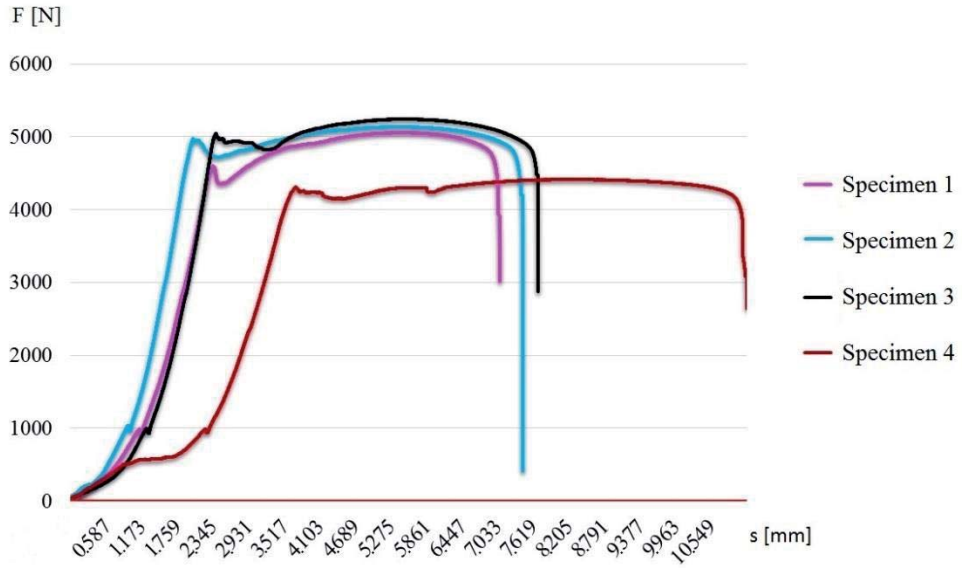
On randomly selected sample locations, the thickness of surface layers was measured using a CM-8825 digital ultrasonic thickness gauge. The measured values are given in Table 1.

Table 1 Surface coating

S235JRG1									
Zn					50/50 AlZn				
Num.	Measured values [µm]	Average [µm]	Standard deviation [µm]	Variance [%]	Num.	Measured values [µm]	Average [µm]	Standard deviation [µm]	Variance [%]
1	14				1	20			
2	14				2	18			
3	17				3	17			
4	15				4	20			
5	17	15.30	1.35	0.09	5	19	18.40	1.02	0.06
6	15				6	18			
7	13				7	18			
8	15				8	19			
9	17				9	18			
10	16				10	17			

The measured values correspond to the size of the surface layer deposited by hot dip galvanizing according to the literature (Reimann 2017). The tensile diagram after the tensile strength testing is shown in Figure 2. Table 2 shows the tensile test results. The maximum tensile force was recalculated to the strength limit according to the formula Rm $[MPa] = \frac{F_{max}[N]}{S_0[mm^2]}$.

Figure 2 Tensile diagram of samples



Legend: Specimen 1 without heat treatment, specimen 2 tempering 150 °C for one hour, specimen 3 tempering 300 °C for one hour, specimen 4 tempering 450 °C for one hour

Table 2 The result of the tensile test

Heat treatment	Tensile strength [MPa]	Total average [MPa]	Variance [%]	Standard deviation [MPa]
Without heat tr. 1	350.74			
Without heat tr. 2	350.35			
Without heat tr. 3	350.81			
Without heat tr. 4	350.29			
Without heat tr. 5	350.69	350.58	0.00	0.21
Tempering 150 °C 1	356.74			
Tempering 150 °C 2	354.65			
Tempering 150 °C 3	348.71			
Tempering 150 °C 4	350.63			
Tempering 150 °C 5	351.64	352.47	0.01	2.87
Tempering 300 °C 1	364.38			
Tempering 300 °C 2	362.43			
Tempering 300 °C 3	359.36			
Tempering 300 °C 4	361.32			
Tempering 300 °C 5	359.51	361.40	0.01	1.88
Tempering 450 °C 1	306.64			
Tempering 450 °C 2	309.38			
Tempering 450 °C 3	307.57			
Tempering 450 °C 4	306.45			
Tempering 450 °C 5	304.89	306.99	0.00	1.47

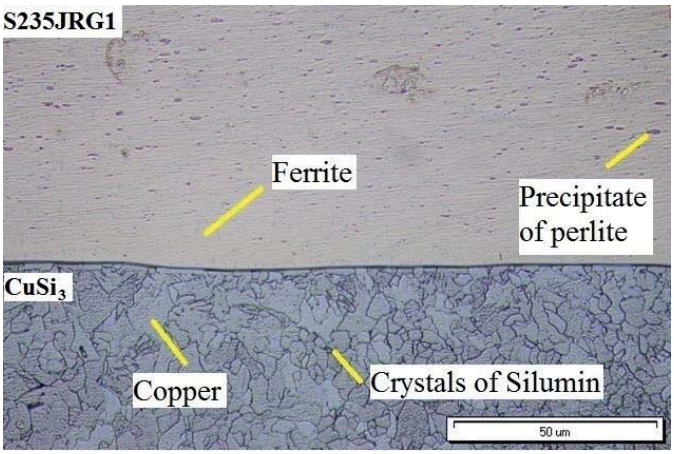
The not heated samples have reached an average tensile strength of 350.58 MPa and the samples after thermal exploitation at 150 °C showed an average tensile strength of 352.47 MPa. Nearly identical values can be explained by the low carbon content of the steel (the insignificant content of the perlite, i.e. carbides). Slightly higher tensile strengths were measured in samples heated at 300 °C. The average tensile strength measured was 361.40 MPa. These values can be explained by variance of tensile tests (Zhang et al. 2016). The lowest tensile strength reached the samples after heating at 450 °C. The average Rm value was 306.99 MPa. This is due to the recrystallization annealing of the material under this temperature (reduction of the deformation reinforcement after cold

forming), thereby reducing hardness and strength (Weigl et al. 2011). An important result of the experiment was the finding that in all cases of tensile tests, the sample failure was outside the welded joint. The rupture occurred in the base material of the steel galvanized strip. The sample image before rupture and after rupture can be seen in Figure 3.

Figure 3 Specimen before and after tensile strength test

Another result includes image of a metallographic abrasive cut. The structure of the CMT weld zone steel is shown in Figure 4.

Figure 4 The structure of the welded zone



The base material S235JRG1 (the upper part of the figure) consists of a ferrite (light part) and partially precipitates of perlite at the grain boundaries (dark grains) (Reimann et al. 2017). The $CuSi_3$ additive (bottom of the figure) is made of copper and silumin crystals. There is a sharp transition edge between the base material and the additive material of the CMT welded joint (Di Cocco et al. 2014, Marder et al. 2000).

CONCLUSION

Galvanized steel sheets with a low carbon content (up to 0.2%) with Zn or AlZn 50/50 surface finish can be metallurgically joined by the CMT welding method with an additional $CuSi_3$ electrode under a protective atmosphere composed of 98% of Ar and 2% of CO_2 (Matsui 1998). The hypothesis was that the welded joint would be broken by the tensile strength test.

Based on the results of the tensile tests at temperatures up to 450 °C, it has been proven that there has been no failure of the welded joint. For all samples, the galvanized steel strip ruptured outside the weld. The hot-dip Al-Zn (50/50) plated steel strip withstands higher temperatures; therefore no rupture occurred in this part of the weldment. All tensile strength measurements show that the CMT joint is stronger than the base material. Therefore, the joint welded by this method cannot be a source of failure, for example, in machinery. Average tensile strengths ranged from 350.58 to 361.40 MPa in the thermal exploitation range of 0 to 300 °C. Lower average tensile strengths of 306.99 MPa were measured in samples thermally processed at 450 °C. The reason is that at this temperature, the recrystallization annealing of the material already starts and thus the hardness and strength decrease (Ptacek 2002, Weigl et al. 2011).

The second important finding of the experiment is that the hypothesis that the $CuSi_3$ electrode can be used up to operating temperatures of 200 °C as reported in literature (Ptacek 2002) has been

refuted. The experiment has shown that the mentioned type of joint can be used for components operating at temperatures up to 300 °C.

Images from metallographic analysis prove the quality of the welded joint, since the chemical composition corresponds to the composition of the CMT welded joint according to literature (Reimann et al. 2017, Di Cocco et al. 2014, Marder et al. 2000).

ACKNOWLEDGEMENTS

The research has been supported by the project TP 6/2017: Defectoscopic quality assessment of technical and organic materials; financed by IGA AF MENDELU.

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TECHNICAL-ECONOMIC ASPECTS OF THE ERADICATION OF ENERGY WILLOW PLANTATIONS

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Abstract: The study contains the evaluation of technical aspects of the eradication of energy willow plantations taking into account the working widths and depth of the machines used. Also determined were the costs of the eradication procedure with the use of various machine units. The studies were performed in the plantation of energy willow in Kaniów locality situated in southern Poland. The machinery used permitted the theoretical removal of 23–100% of horizontal roots, and 43–60% of vertical roots. The costs of the eradication of plantation ranged from 3381 PLN/hectare to 15824 PLN/hectare.

Key Words: energy willow, work expenditure, eradication costs

INTRODUCTION

As indicated in relevant references (Czeczko 2012, Rahman et al. 2014, Isebrands et al. 2014), willow plantations provide satisfactory yield for about 25 years. Later, the yield begins to drop, and the eradication of willow plantation becomes inevitable. As the energy willow is cultivated within farmlands, it is necessary to know what to do in order to be able to grow other plants on the areas left by willow plantations (Larsson 2006).

The priority in the studies on willow plantations most often concerns the determination of financial profits from their operation whereas little attention is paid to the issues pertaining to their eradication. At present, the most frequent analyses deal with the cost of establishing the plantation as well as what income can be achieved (Ericsson et al. 2009).

As pointed out by (Ericsson et al. 2009, Adamczyk et al. 2016) in the process of biomass production from energy willow, and in economic analyses, the costs of establishing and running plantations are considered before any other costs whereas the work required and the cost of eradication of the plantation are often neglected.

In the body of literature on this subject, there is only little information pertaining to the eradication of multi-year plantations of energy crops. The published data on this topic involves theoretical analyses associated with the eradication of the multi-year plantations of energy crops. The information published on this subject are theoretical analyses not supported by relevant field studies (Kwaśniewski et al. 2010). Among the published theoretical treatises, one can find the estimates (Larsson 2006) according to which the cost of liquidating 1 hectare of plantation amounts to 1500 SEK, or the analyses (Stolarski et al. 2008) indicating the costs of eradication to be approximately 2075 PLN per hectare.

The objective of this study was to evaluate the technical aspects of the eradication of plantation, taking into account the working width and depth of the machinery used, as well as determining the cost of eradication of the energy willow plantation when the various types of machine units are used.

MATERIAL AND METHODS

The scope of work consisted of the field studies conducted within the plantation of energy willow in Kaniów (Bielsko poviat, Silesian voievodship), involving the determination of effective,

operating efficiency as well as work expenditures and cost of the eradication of the plantation. The following machine units were used during the studies:

- variant V1 – CRYSTAL ORION 170 tractor and FAO FAR model FV 4088 cutter for strip preparation of soil (working width 0.4 m, working speed 0.8 km/h),
- variant V2 – CRYSTAL ORION 170 tractor and FAO FAR model FV 4088 cutter for strip preparation of soil (working width 0.4 m, working speed 1.2 km/h),
- variant V3 – CRYSTAL ORION 161 tractor and Meri Crusher model MJS-2.0 DT (working width 2 m, working speed 0.8 km/h),
- variant V4 – CRYSTAL ORION 170 tractor and FAO FAR model FV 4088 cutter for strip preparation of soil (working width 0.4 m, working speed 0.5 km/h),
- variant V5 – CRYSTAL ORION 170 tractor and FAE mulcher model SFM 225 (working width 2.3 m, working speed 0.5 km/h).

The studies were implemented in two stages: in May and in December, and constituted a part of research project No. PBS2/A8/26/2014 "Developing the new technology and functional model of machine used for the reclamation of fields left after the cultivation of energy willow".

In order to establish the technical aspects of plantation eradication, the results of studies by (Juliszewski et al. 2015) of 30 energy willow rootstocks obtained from the plantation maintained at the Faculty of Production and Power Engineering of the University of Agriculture in Krakow were used. These studies pertained to the allocation of willow root biomass. The proportion of roots eradicated was calculated as a quotient of the relevant parameter of a given machine (the working width – in the case of horizontal roots, and the working depth – in the case of vertical roots) and the reach of roots (horizontal or vertical).

The following parameters were determined for the above variants in field studies over the distance of 100 m:

- T_1 effective time of the runs of studied machine units,
- time for turnings on headlands,
- operating time T_{07} (effective time and time for turnings),
- fuel (diesel oil) consumption measured by the full-tank method.

The times were measured with a stop watch. The T_1 and T_{07} times were necessary to calculate the coefficient of operating time usage k_{07} which was used to determine operating efficiency.

The efficiency of the operation of machine units was determined with their working widths and speeds. In the study, the effective efficiency (in the effective operating time) and next – the operating efficiency (in operating time – considering the time for backing) were determined.

The machine operating costs for given machine units were calculated following the universally applied methodology of cost calculations (Michałek et al. 1998, Muzalewski 2009).

The following component costs were taken into account in the calculations of operating costs for machine units:

- maintenance costs (fixed costs): depreciation, tractor insurance costs, storage costs,
- operating costs (variable costs): costs of fuel and lubricants, costs of technical service and repairs, labour costs.

The assumptions used for the calculations included, among other things:

- average annual use rate of tractors – 600 h/year,
- average annual use rate of machinery – 70 h/year,
- prices of tractors and machines as of the first quarter of 2017 based on telephone call to the commercial representatives of the FAO FAR and UTECH companies:
  - CRYSTAL ORION 170 tractor – 335000 PLN,
  - CRYSTAL ORION 161 tractor – 308000 PLN,
  - FAO FAR model FV 4088 cutter for strip preparation of soil – 52000 PLN,

- Meri Crusher model MJS-2.0 DT – 120000 PLN,
- FAE mulcher model SFM 225 – 217800 PLN,
- price of diesel oil – 4.5 PLN/l,
- storage cost – 11 PLN/m^2/year,
- annual insurance for tractors – 300 PLN/year,
- labour costs – 13 PLN/mhr.

RESULTS AND DISCUSSION

The results of studies carried out by (Juliszewski et al. 2015) are compiled in (Table 1). The expression "thick roots" means the roots with diameter exceeding 30 mm. The mean values of particular parameters were adopted in further considerations.

Table 1 Parameters of the energy willow root distribution

Serial No.	Parameter	Mean	Min.	Max.	SD
		(cm)			
1.	Rootstock diameter of root neck at soil level	20.9	11.7	36.7	5.8
2.	Diameter of main root at the thickest place	11.2	6.3	16.5	2.6
3.	Maximum vertical reach of roots	58.3	27.5	87.5	12.8
4.	Measured vertical reach of thick roots	42.3	18.5	69.5	17.1
5.	Maximum horizontal reach of roots	75.2	53.5	96.0	11.6
6.	Measured horizontal reach of thick roots	51.6	31.0	80.5	11.7

The percentage proportions of eradicated roots obtained with the use of studied machine units are presented in (Table 2). The machine units applied in variants V3 and V4 will theoretically allow the complete eradication of horizontal roots. It results from the great working widths of machines used. In variants V1, V2, and V4 it will be possible to eradicate 100% of rootstock at root neck. Nevertheless, more than 20% of thick horizontal roots as well as nearly half of the roots of greatest horizontal reach will be left.

Table 2 Percentage proportions of the roots removed by the machine units used

Parameter	Variant		
	V1, V2, V4	V3	V5
Rootstock diameter of root neck at soil level	100%	100%	100%
Maximum vertical reach of roots	60%	43%	51%
Measured vertical reach of thick roots	83%	59%	71%
Measured horizontal reach of roots	53%	100%	100%
Measured horizontal reach of thick roots	78%	100%	100%

In the case of vertical roots, better results will be obtained by using variants V1–V4 than by variant V3 which will allow the eradication of only 59% of thick roots and 43% of roots with the greatest vertical reach. The reason for it is the low working depth of this machine which amounts to 0.25 m. The machine units used in the remaining variants will allow eradication of between 71% and 83% of vertical thick roots.

In order to find the cost of eradication of plantation, first the work input for eradication by particular machine units in the operating working time must be found. The obtained results as well as fuel consumption for particular variants are presented in (Table 3).

In order to determine the total cost of eradicating a plantation it is necessary to find earlier the unit costs of operation for the studied machine units (denominated in PLN/h). These unit costs are compiled in (Table 4).

Table 3 Fuel consumption and work input during the eradication of plantation

Detailed list	Unit	Spring studies (May)			Autumn studies (December)	
		Variants				
		V1	V2	V3	V4	V5
Working width	(m)	0.4	0.4	2.0	0.4	2.3
Working speed	(km/h)	0.8	1.2	0.8	0.5	0.5
Fuel consumption	(l/h)	26.2	25.5	17.6	28.0	48.3
Fuel consumption	(l/ha)	771.5	555.0	143.2	1400.0	555.2
Effective efficiency	(ha/h)	0.04	0.06	0.16	0.025	0.115
Operating efficiency	(ha/h)	0.034	0.046	0.123	0.02	0.087
Work input (during operating time)	(mhr/ha)	29.6	21.5	8.1	50.4	11.4

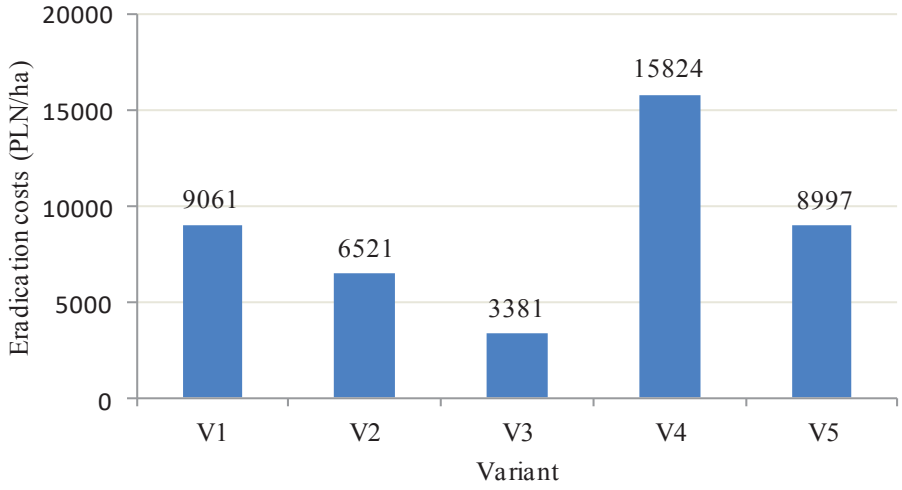
Table 4 Operating costs per machine unit (PLN/h)

Detailed list	Spring studies (May)			Autumn studies (December)	
	Variants				
	V1	V2	V3	V4	V5
Depreciation	89.8	89.8	168.5	89.8	287.2
Insurance costs	0.5	0.5	0.5	0.5	0.5
Storage costs	1.4	1.4	2.1	1.4	2.4
Maintenance costs	91.8	91.8	171.1	91.8	290.1
Costs of technical services and repairs	80.8	80.8	151.7	80.8	258.5
Costs of fuel and lubricants	120.4	117.2	80.8	128.5	224.8
Labour costs	13.0	13.0	13.0	13.0	13.0
Costs of use	214.2	211.0	245.5	222.3	496.2
OPERATING COSTS	306.0	302.8	416.5	314.1	786.3

As the variants V1, V2, and V4 involved the use of the same tractor and the same machine, the maintenance costs remain fixed. The only variable component is the cost of fuel and lubricants. They stay between 117.2 and 128.5 PLN/h and constitute from 39 to 41% of the operating costs. In the case of variants V3 and V5, the machines applied were much more costly. For these reasons, the amounts of depreciation, and the costs of technical services and repairs for variant V3 were almost two times higher, and for variant V5 – more than three times higher than those for the remaining variants. The lowest operating costs amounted to 302.8 PLN/h (variant V2) whereas the highest - 786.3 PLN/h (variant V5). The costs generated by the last of the considered variants (variant V5) are in sharp contrast with the values obtained in other variants. They are caused by the great financial outlays involved in the purchase of a new machine. The costs directly connected with its price (depreciation and the costs of technical services and repairs) constitute almost 70% of the total operating costs.

After he operating costs have been established, and with the operating efficiency taken into account, it is possible to calculate the total cost of the eradication of energy willow plantation, expressed in PLN/ha. The costs of eradication are presented in (Figure 1).

Figure 1 Costs of plantation eradication in particular variants



Major disproportions are noticeable between the results obtained for particular variants. The eradication costs amounted from 3381 PLN/ha for variant V3 (tractor CRYSTAL ORION 161, Meri Crusher model MJS-2.0 DT, working speed 0.8 km/h) to 15824 PLN/ha for variant V4 (tractor CRYSTAL ORION 170, FAO FAR model FV 4088 cutter for strip preparation of soil, working speed 0.5 km/h). The reasons behind such high costs for variant V4 was the small working width amounting to 0.4 m, but also the low speed of the movement of the machine unit (0.5 km/h). The comparisons between variants V1, V2, and V4 where the same machine was used reveals the significant effect of the working speed upon the value of final costs. The relationship occurring between these values is inversely proportional, is a fact which might encourage an attempt to reduce the costs of eradication by increasing the working speed of the machine unit. It should nevertheless be mentioned that in the view of great forces exerted on the working element of the machine during willow rootstocks cutting, the working speed may not be too high. The price of the machine used seems to be an equally essential element affecting the cost of eradication, as can be suggested by variant V5. The FAE mulcher applied in this variant shows the greatest working width among all studied machines, but the low speed and high price, as well as the low annual utilization rate of the machine resulted in generating the cost of this variant amounting to as much as 8997 PLN/ha.

As pointed out by (Mańkowski et al. 2014) few studies on the topic pertain to the use of simple time- or energy-intensive methods. (Larsson 2006) estimated that cost of the plantation eradication amounts to 1500 SEK/ha. In conformity to the analyses (Stolarski et al. 2008) the costs be approximately 2075 PLN/ha. In this study for eradication procedure used advanced technical methods and the calculated cost hesitated between from 3381 PLN/ha to 15824 PLN/ha.That range largely hung on environments conditions and time when the studies was done (2015 and 2016). In future, the results obtained in this study will be able to provide the foundation for the determination of the costs of *Salix viminalis* willows biomass production.

CONCLUSION

Considered from the viewpoint of the technical aspects of plantation eradication, the machines used in variants V1–V5 will theoretically allow to remove 23–100% of horizontal roots, and 43–60% of vertical roots.

The work inputs for eradication of the studied energy willow plantation fell into the range from 8.1 mhr/ha to as much as 50.4 mhr/ha. The levels of work inputs were affected significantly by the working speeds of machine units (from 0.5 km/h to 1.2 km/h) and by their working widths (from 0.4 to 2.3 m).

The operating costs of particular machine units amounted from 302.8 PLN/h to 786.3 PLN/h. The greatest proportion of costs was that of the costs of depreciation (high costs of tractors and machines), the costs of technical services and repairs, as well as the costs of fuel and lubricants.

The highest cost of plantation eradication amounting to as much as 15824 PLN/ha were noted for variant V4 where CRYSTAL ORION 170 tractor and FAO FAR model FV 4088 cutter for strip preparation of soil, moving with the speed of 0.5 km/h, whereas the lowest (3381 PLN/ha), for variant V3, i.e. the machine unit composed of CRYSTAL ORION 161 tractor, Meri Crusher model MJS-2.0 DT, moving with the speed of 0.8 km/h.

All calculations followed the assumed methodology therefore the conclusions should be treated as correct, however, because of the specific nature of the procedure of eradicating energy willow plantations, there is a number of factors which can reduce or increase the calculated costs incurred during the eradication of an energy willow plantation.

ACKNOWLEDGEMENTS

The project financing was supplemented by the National Center for Research and Development under the framework of the Applied Research Program in path A.

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COMPARISON OF DIFFERENTIAL HYDROMECHANICAL AND MECHANICAL TRANSMISSIONS IN TERMS OF IMPACT ON THE DRAWBAR PULL PROPERTIES OF A TRACTOR

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Abstract: The aim of this paper is to compare drawbar pull properties of tractors with mechanical (powershift transmission) and differential hydromechanical transmissions (continuously variable hydromechanical transmission). The tire inflation pressure was 100 kPa. The weight of tractor with the differential hydromechanical transmissions was 120 kg higher than that of tractor with mechanical transmissions. The drawbar pull properties were measured on a dry, straight section of an asphalt road. The results show a considerable difference in the highest drawbar pulling power achieved by individual tractors. Significantly lower performance was achieved by the tractor with continuously variable transmission in all measured travel speeds.

Key Words: continuously variable transmission (CVT), transmission engaged under load, drawbar pull, drawbar pull performance, slippage, potential drawbar pulling power

INTRODUCTION

Working conditions not only of tractors but also of other motor vehicles are very diverse. In some situations, there is a need for high drawbar pull at a lower working speed or, on the contrary, for a high working speed, for example, when transporting material. If the combustion engine had the so-called ideal rotational characteristic, or the dependence of torque on rotations is hyperbolic, then the above-mentioned requirement could be met without any problem. However, as the course of the curve is different, it is necessary to include a transmission between the combustion engine and the drive wheels. The transmission will make it possible to change the gear ratio to make better use of the engine's performance and therefore better features of the tractor as a whole. In addition, it transmits torque for front axle and PTO shaft (Semetko 1985).

Until recently, tractors used only mechanical transmissions, which were gradually improved from the basic directly manually operated individual gears to today's mechanical powershift transmissions. Mechanical transmissions have one disadvantage, namely a gradual change in the gear ratio, which makes it impossible to achieve the hyperbolic dependence of the driving force on the working speed, or the ideal course. This disadvantage is eliminated by using transmissions with a higher number of gears (Figure 1).

As we can see in the figure, there is a loss area between the two gears, or an unusable range of engine power. The loss area expresses the difference between the ideal and actual gradation. The size of the loss area decreases, particularly with the increasing number of gears and the torque increase or with the flatness of the engine power curve. However, a higher number of gears also results in the increase of size and weight of the entire transmission. Another developmental direction how to get closer to achieving the ideal distribution is to use a continuously variable transmission (CVT) as it offers a theoretically infinite number of gear ratios between the basic and maximum gear. In a tractor, this transmission consists of a hydrostatic transducer and a mechanical clutch planetary gear as seen in Figure 2 (Bauer et al. 2013).

Figure 1 Gradation in transmission with 10 gears

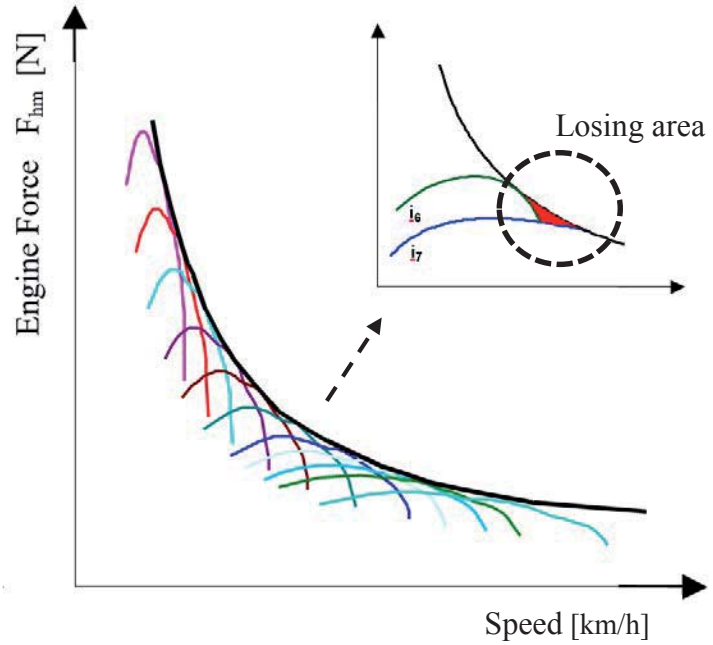
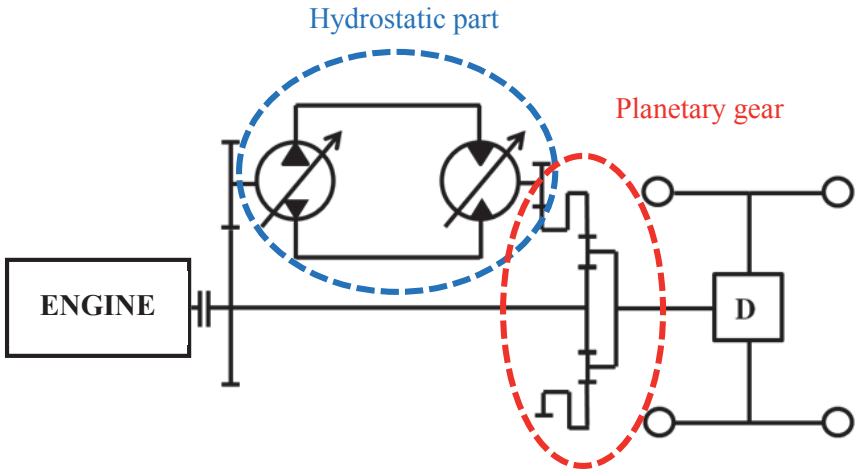


Figure 2 Design of differential hydromechanical transmission, Bauer et al. 2013



However, the disadvantage of the differential hydromechanical transmission is a low efficiency of the hydrostatic part, which is approximately 17% lower against the mechanical transfer (Grečenko 1994). This disadvantage is a limiting factor for the use of these types of transmissions in higher performance tractors.

The aim of this paper is to compare the drawbar pull properties of tractors with mechanical and differential hydromechanical (CVT) transmissions.

MATERIAL AND METHODS

To determine the effect of the transmission type on drawbar pull properties of a tractor, drawbar pull tests were carried out. The measurements were carried out on tractor fitted with the mechanical powershift transmission, and in the second case, with the continuously variable hydromechanical transmission. The outputs parameters of tractor engines were the same. The weight of tractor with the differential hydromechanical transmissions was 120 kg higher than that of tractor with mechanical transmissions. The tire sizes were the same for both tractors. The tire inflation pressure was 100 kPa.

The drawbar pull properties were measured on a dry, straight section of an asphalt road. On a straight section of the road, a 50 m stretch was defined, on which the drawbar pull properties of both tested tractors were measured. For the tractor with mechanical transmission, drawbar pull tests were performed on four gears. For the tractor with continuously variable transmission, four travel speeds corresponding to the gears of the previous tractor were set. The measurements were performed at set speeds of 5 km/h, 8 km/h, 11 km/h, and 12 km/h. At individual speeds, the load was gradually increased until the maximum drawbar pulling power was reached. In the manually operated transmission, the control electronics set the default measurement speed as the maximum speed. At each indicated speed, six to nine measurements were carried out. Each measurement proceeded with a constant braking force (drawbar pull), which was gradually increased with subsequent measurements. The braking force was kept constant in the measuring section with the another tractor in tow. The measured and braking tractors were connected by a steel rope with an inserted Hottinger U2A type strain gauge with a force range of 0–200 kN. Measurements were conducted in one travel direction. Prior to the beginning of the measuring section, there was a sufficiently long path to achieve the desired speed and stabilization of measured parameters. Measured data was continuously transmitted using a WIFI set to a stationary workstation situated near the measuring track. During drawbar pull tests, in addition to drawbar pull, additional data from internal and external sensors added to the tractor for drawbar pull testing, was measured simultaneously. Data from internal sensors was obtained by connecting to a Can-Bus data bus, from which it was sent to the memory of the measuring computer at the same 20 Hz frequency as other data. The data included for example the engine speed, as well as theoretical and actual speeds. A GPS module was used to determine the actual speed of the tractor. The actual and theoretical speed information was used to calculate the slippage of wheels.

The slippage calculation was according to equation 1:

$$\delta = 1 - \frac{v_s}{v_t}.100 \quad [\%] \tag{1}$$

where: v_t - theoretical speed *[m/s]*

v_s - actual speed from GPS [m/s]

For evaluation purposes also the drawbar pulling power Pt was calculated according to equation 2:

$$P_t = F_t.v_s \quad [W] \tag{2}$$

where: Ft - drawbar pull [N]

RESULTS AND DISCUSSION

The drawbar pull characteristics of both tractors were measured under the same conditions. This allows for their accurate mutual comparison. For easier comparison of the achieved results, the drawbar pull characteristics of both tractors at the same inflation pressure were plotted on a graph. To determine the impact of the transmission on drawbar pull properties, a graph of dependence of drawbar pulling power and slippage on drawbar pull (Figure 3) and a graph of dependence of potential drawbar pulling power on drawbar pull (Figure 4) were generated.

As shown in Figure 3, the tractor with mechanical transmission achieved a maximum drawbar pulling power of 112.8 kW on the asphalt measuring track. The maximum drawbar pull of 70 kN was achieved in first tested gear. At this drawbar pull, the drawbar pulling power was 100 kW and the asphalt track wheel slippage was 8.8%.

As we already mentioned in the Material and Methods chapter, the travel speeds of the tractor with differential hydromechanical transmission were chosen so as to match the measured speeds of the tractor with mechanical transmission. The set speeds were 5 km/h (equivalent to first gear for mechanical transmission), 8 km/h (equivalent to second gear), 11 km/h (equivalent to third gear) and 12 km/h (equivalent to fourth gear). In this case the same tire pressure was used as for the previous tractor. The tracking results of the tractor with differential hydromechanical transmissions indicate that for the initial speeds of 8 km/h, 11 km/h, and 12 km/h, almost the same maximum drawbar pulling power was measured. The difference is up to 1 kW. The highest

performance was measured for the initial speed of 8 km/h, namely 86 kW at the drawbar pull of 48.5 kN. The maximum drawbar pull of 68 kN was measured for 5 km/h with a wheel slippage of 5.8%.

Figure 3 Dependence of drawbar pulling power and slip on drawbar pull

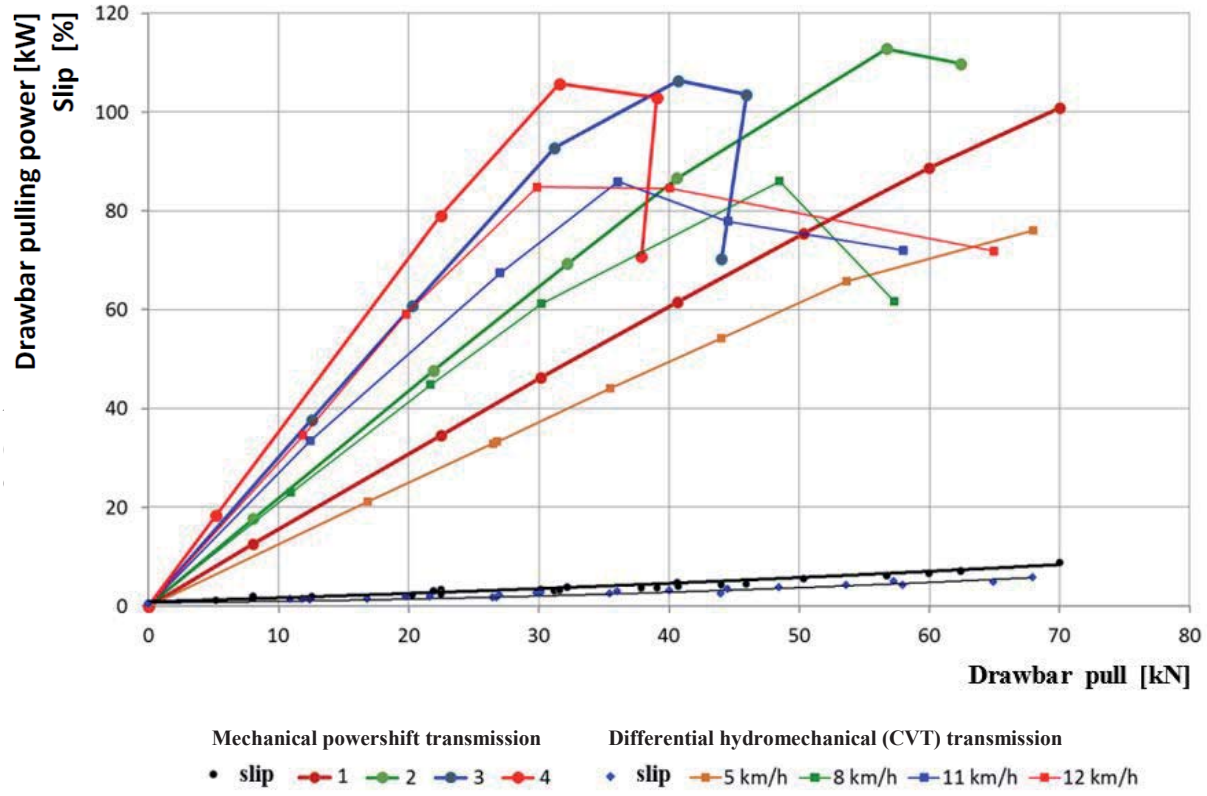
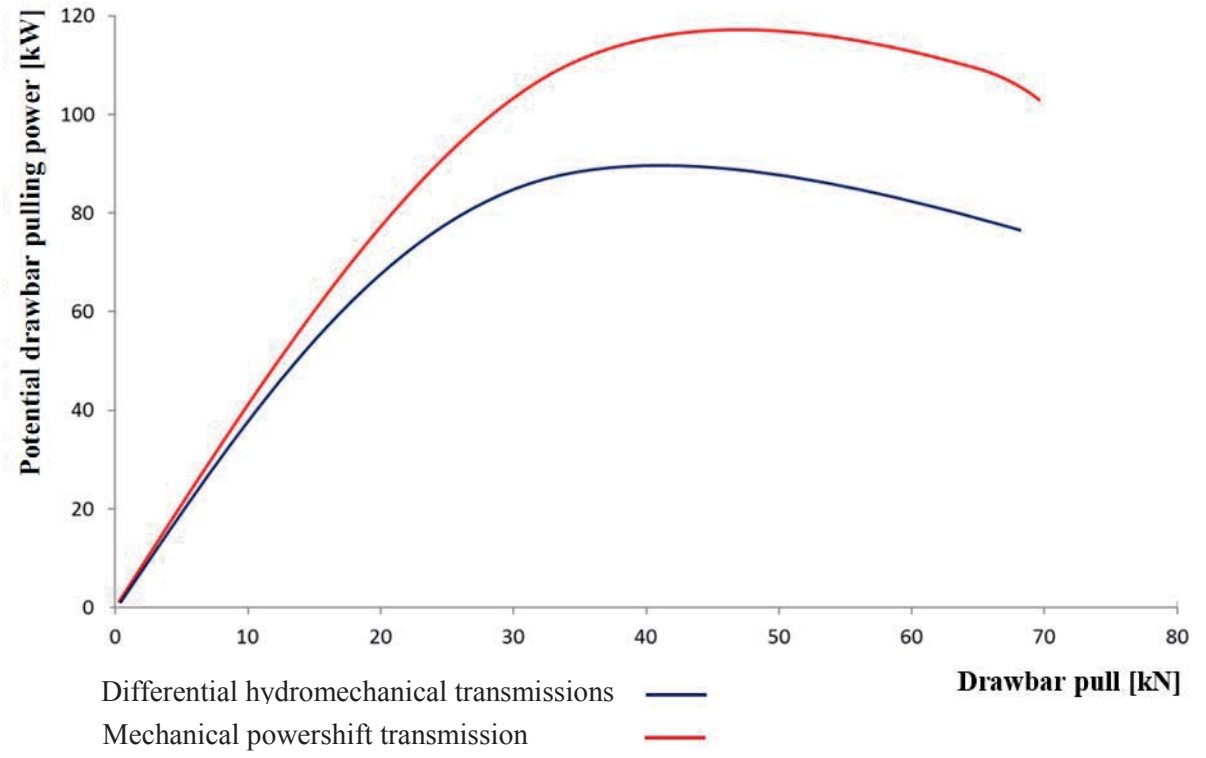


Figure 4 Dependence of potential drawbar pulling power on drawbar pull



As we can see from the graph in Figure 4, the tractor with the differential hydromechanical transmissions achieved significantly lower power at all measured travel speeds. At the lowest travel speed of 5 km/h in compare to first gear for mechanical transmission gear, the measured difference in the maximum drawbar pulling power of both tractors was 24.8 kW. At a travel speed of 8 km/h (in compare to second gear), the difference in the highest drawbar pull performances was 26.8 kW. At the initial travel speed of 11 km/h (in compare to third gear), a difference of 20.4 kW was measured and at the speed of 12 km/h (in compare to fourth gear) the drawbar pulling power difference was 20.4 kW. For both tractors, nearly the same wheel slippage was measured in the drawbar pull tests, yet the tractor with differential hydromechanical transmission showed a slightly higher value. At the maximum drawbar pull it was about 3%.

The results of drawbar pull tests of the tractor with differential hydromechanical transmission tractor also clearly show that drawbar pull performances produced unusual patterns compared to the tractor with the mechanical transmission. For this reason, a graph of engine rotations depending on the theoretical speed for one gear or speed was plotted. With a constant gear or set speed, this dependence should be linear. However, when plotting this dependence for the tractor with a hydromechanical variable transmission, it was found that it is not linear. Thus, from the plotted dependence it is clear that even with the manual control setting the electronics seem to be interfering with the transmission control. In particular, at higher drawbar pulls it adjusts the gear ratio. In the drawbar pull characteristic it will be reflected so that when the maximum drawbar pulling power is reached, with any further load the performance curve reaches significantly higher drawbar pulls (sometimes twice the force at maximum performance). This is due to an increase in gear ratio on the transmission. For the tractor with variable transmission, the drawbar pull varied by more than 40 kN. This is more than 70% from the average drawbar pull measured in the test.

The comparison of the powershift mechanical and variable transmissions in the tractor was also performed by Čupera et al. in 2011. Transmission comparison was performed during ploughing in combination with the plough in two different engine modes (characteristic with a constant speed range). The average fuel consumption in ml per m^3 of overturned soil was chosen as the main assessment criterion. The set with the variable transmission achieved savings of 14.5% compared to the one with powershift mechanical transmission. The higher fuel economy of the tractor with the variable transmission was due in particular to the "better cooperation" of the engine with the transmission, in other words the variable transmission has maintained the set engine speed better. Higher speed fluctuations in a relatively short period of time cause the acceleration and deceleration of individual function groups, which results in higher power consumption for accelerating individual parts of gears. The comparison of these two transmissions (powershift mechanical and variable transmissions) in the operation of tractors in transport was also carried out by Bauer et al. in 2011. Their results also show higher fuel economy for the transmission with a continuously variable gear ratio change due to the "better" adjustment to the desired driving mode or desired constant speed setting.

CONCLUSION

In conclusion, it cannot be clearly determined on the basis of measurements made and the results of studies dealing with similar issues which transmission is generally better. However, it is possible to distinguish which transmission is more suitable for the respective engine mode. For drawbar pull work at constant drawbar pull or speed, the mechanical transmission is more suitable thanks to its higher overall efficiency. For a tractor moving in traffic and during ploughing at set constant speeds, the variable transmission is more preferable.

ACKNOWLEDGEMENTS

This work was supported by the project IGA AF no. IP 2017/039: „Spatial transformation of forces and torques in the three-point hitch depending on the tractor's hydraulic system setting“ financed by Internal Grant Agency of Mendel University in Brno, Faculty of AgriSciences.

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UTILIZATION OF ACOUSTIC EMISSIONS IN THE EVALUATION OF MACHINING PROCESS

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Abstract: The thesis deals with the use of non-destructive testing methods (acoustic emission) for the description and identification of stages during the machining process. For this measurement, advanced machining was selected. Selected cutting speeds-0.0028 m/min, 0.004 m/min and 0.008 m/min, and the rotor speed was 56 rpm with a chip size of 1mm. At each cutting speed, the resulting graphs were recorded. The test material was chosen as the structural steel 12 050-structural plain steel for tilth and hardening ISO C60E4 ISO 683-1-87 (Ferona). In the experimental part the methodology of continuous emission signal recording, continuous processing and evaluation of the measured data and monitoring of the response of the stressed material (during the machining process) was designed in real time. The detected values were then inserted into this methodology, subsequently evaluated, it was the detection of emission signals using the acoustic emission apparatus. Acoustic emission is a fast, short release of energy in the form of elastic waves. The acoustic emission curve has several parameters that can be used to characterize the source defect. Through an appropriately designed methodology for detecting, processing, and evaluating AE signals, it is possible to track the progress of the machining process, the measured data allow you to obtain new information about the processes that accompany the machining. Thus, the emission signals can be used to indicate microcracks in the internal structure of the stressed materials.

Key Words: machining, cutting material, acoustic emission, machining monitoring

INTRODUCTION

Nowadays, most engineering companies are engaged in increasing the productivity of machining processes and also reducing operator demands. Therefore, it is very important to choose a suitable monitoring system to prevent the production of faulty pieces. Machining is defined as the stage at which the machining operation can be performed in the highest productivity with the lowest cost (Seco). The principle of monitoring the machining process is based on the monitoring or measurement of selected quantities or their combination. In the future, sound analysis could be used, for example, in adaptive machine control. Auditory analysis can identify excessive tool wear or vibrations that are undesirable in the machining process.

The aim of the measurement is to monitor the course of the machining process by means of a non-destructive method using the acoustic emission monitoring system AE, which enables us to identify defects such as cracks, corrosion, etc. Delamination creates transient elastic waves resulting from sudden change in stress in the material. These elastic waves are called acoustic emission (AE) phenomena. Acoustic emission measurement allows you to "listen" to the resulting defects in the material using piezoelectric sensors. (Mistras) The machining process is realized in a machining system, which is generally subdivided into subsystems of machine tools, cutting tools, handling devices and machining environment. The machining system consists of a machine tool, a cutting tool, a workpiece, and a tool (Humar 2008). For this measurement, a high-speed milling was chosen. The machining process is a complicated process due to many ambient influences that can affect the machining result. Therefore, it is necessary to identify these influences and identify such influences that positively affect and remove unwanted effects.

MATERIAL AND METHODS

The material for the test specimens was chosen to be 12 050 steel (ISO C60E4 ISO 683-1-87), carbon steel. This steel is suitable, for example, for surface hardening. The cutter speed setting was 56 rpm at a single chip size of 1 mm. For these measurements, these feed rates were 28 mm/min, 40 mm/min and 80 mm/min. The cutting angles of cutting plates were γ=20°, α= 8° (Tumlikovo). The tested samples were of the same shape with dimensions: height-4 cm, width-10 cm, length-20 cm. The following table shows the chemical composition of the test steel.

Tabel 1 Chemical compocition of steel, mechanical properties of steel 12 050

$C_{max.}$	Mn_{max}	Si_{max}	P_{max}	S_{max}	Cr_{max}
0.51	*0.69*	*0.25*	*0.023*	*0.017*	*0.15*
Mechanical properties					
Tensile strength R_m	*min. 370 MPa*	*Hardness*	*225 HB*	*Yield strength R_e*	*min. 250 MPa*

TOS FNK 25 milling brackets with six indexable inserts from Pramet were used for machining. The plates were made of sintered carbides coated with a TiD-based PVD method (Figure 1).

Figure 1 Cutter with indexable inserts – Place the piezoelectric sensor on the sample

For each measurement, the piezoelectric sensor IDK-09 was placed on the sample. The acoustic emission signals were scanned and analyzed by the Dakel XEDO measuring system with one piezoelectric sensor. This sensor was fixed at the bottom of the samples with a second adhesive. The contact surface of the sensor was equipped with an ultrasonic gel. Figure 2. The 2 to 8 MHz sample signal processing with 12-bit resolution was fully digitized. The gain is programmable in the range of 0 to 80 dB. The counts 1 and 2, RMS and emission event parameters (time of arrival, maximum, length, count 1 and 2 and the whole event) are evaluated (Dakel). Configuration of the measuring instrument has been set: Amplifier values 10 dB, set levels (count 1) to 302%, (count 2) 600% range, 4 MHz sampling and 1000 ms period.

Table 2 Technical data of piezoelectric sensor(Dakel)

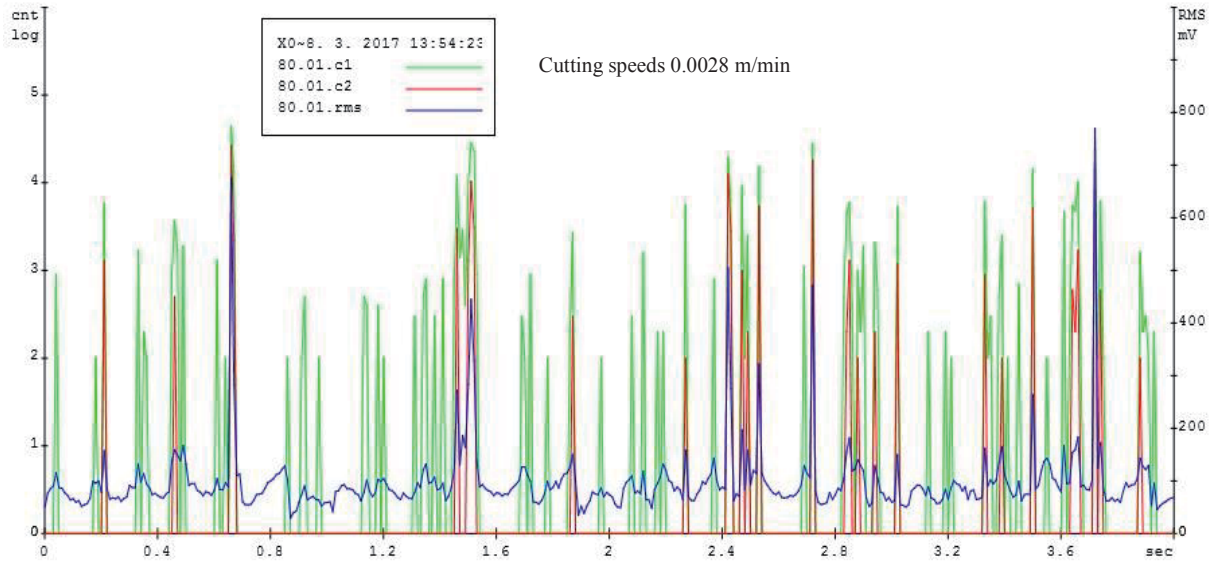
PZT material	*Dakel Class 200*
Size PZT element	*4 x 3 mm*
Capacity	*60 pF*
Output	*cabel with conector*
Cover	*Stainless steel*
Touch screen	*Corundum, 6 mm*

When measuring acoustic emission, the RMS signal of acoustic emission with the number of exceedances of C1 (Counts 1) and C2 (Counts 2) was monitored. This parameter indicates the so-called effective signal value. For alternating voltage, the RMS is equal to the DC voltage which, when applied to the resistive load, would give the same average power. The RMS unit is mV. This value corresponds to the quantitative characteristic of the measured acoustic emission events (amount of energy). The overshoots of the set signal levels C1 and C2 characterize the behaviour of the signal with respect to its time course.

RESULTS AND DISCUSSION

When measured, the acoustic emission sensor was placed directly on the material. The material here serves as a waveguide for an acoustic signal. From which it follows that the composition of the machined material can affect the signal characteristics. From Figure 2 to 4 are apparent when comparing them that increasing the cutting speed increases the number of overshoots in hard steel 12 050, as well as the RMS values and the regularity of the individual acoustic signals. Individual overshoots across the set threshold are relatively accurate, and for this reason we can assume that they are shots of each cutting tool's blades. During the measurement, it is possible to record and identify individual shots of the tool's teeth, according to the total energy of the RMS signal. From the forecast, we found that vibratory machines did not affect our measurement. The acoustic emission depicts the dependence of the RMS signal size on the speed of the feed. Therefore, we can assume that individual acoustic events characterize the cutting of material by the individual blades of the cutter.

Figure 2 Record AE steel 12 050 at cutting speeds 0.0028 m/min



On Figure 2 we can observe a 4 second record, in terms of the appropriate machining process characteristics. As mentioned above, the feed rate affects the size and frequency of the RMS signal. We can see that during the measurement, the signal did not exceed 800 mV. The RMS signal overlaps here with the blades.

From Figure 3 we can see an increase in the frequency of signal overruns, this phenomenon will be even more noticeable at Figure 4.

Figure 3 Record AE steel 12 050 at cutting speeds 0.004 m/min

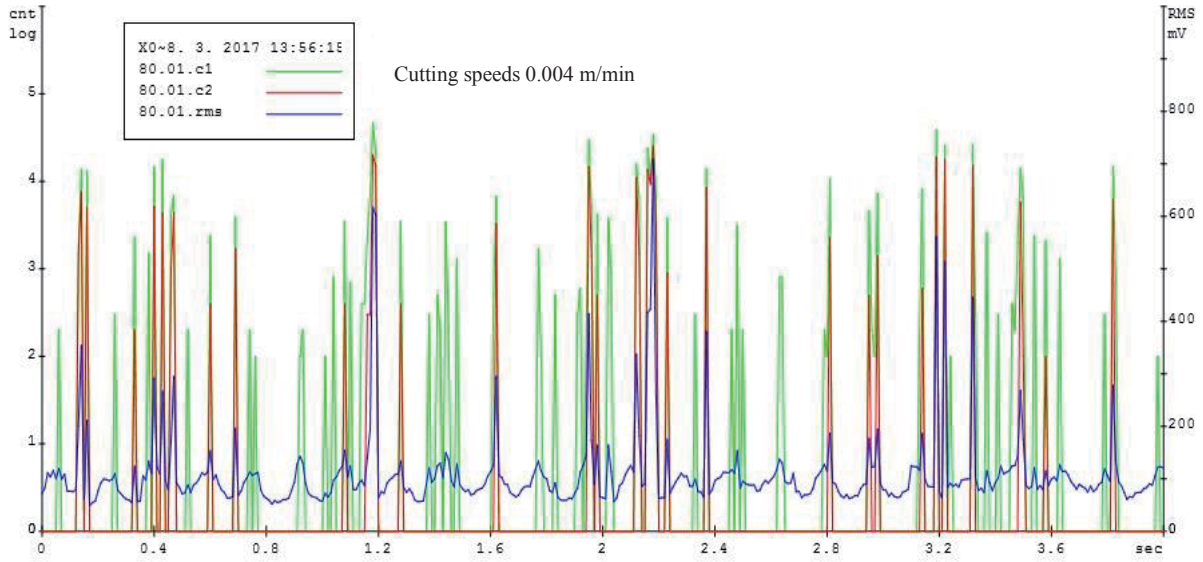
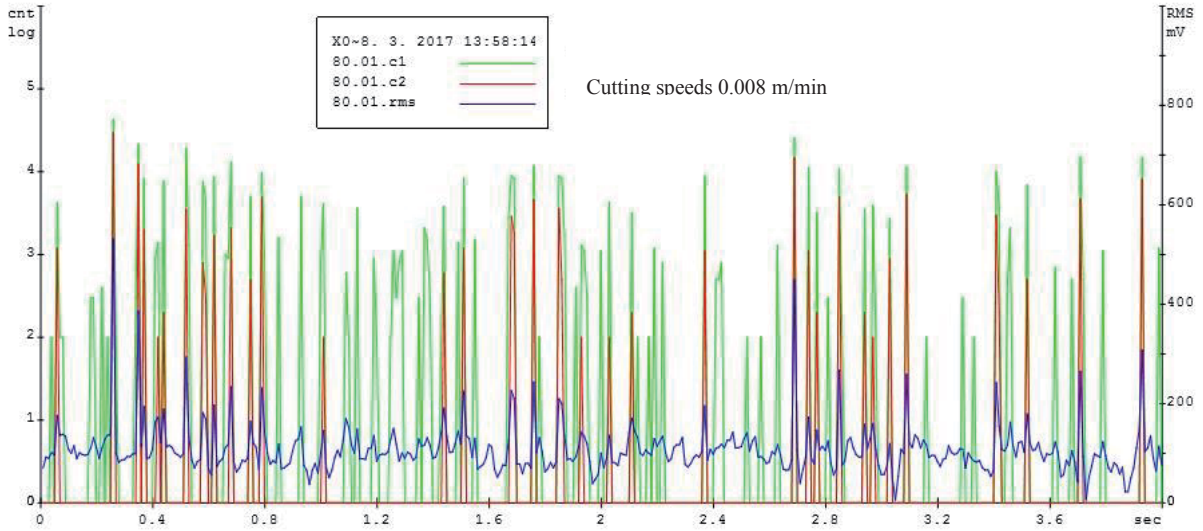


Figure 4 Record AE steel 12 050 at cutting speeds 0.008 m/min



There are sudden RMS anomalies in all records, but at the moment it is not known why this is happening, it would be necessary to subject the sample to a larger number of measurements to produce statistics. It can be assumed that acoustic emission statistics could be used here, but this is a matter of more extensive research.

The acoustic emission system AE is usable as a monitoring process, but we can not directly evaluate the machining conditions, Cutting speed, removal, etc.

Part of her record we can evaluate the hardness of the material. The hardness or toughness of the machined material is one of the factors that influences the machining process, and the acoustic emission is noticeable.

CONCLUSIONS

The work deals with the use of acoustic emission during machining. For the machining method, the steep milling of the steel 12 050 was chosen, with a shift of 0.0028 m/min, 0.004 m/min and 0.008 m/min. Chip removal of 1 mm for all feeds remained the same. The main tool used to describe the internal characteristics during the measurement was the non-destructive acoustic emission method. The paper demonstrates the real-time visualization capability of AE.

The machining process is constantly influenced by a number of defect variables, such as workpiece shape, material machining, cutting depth, cutting tool etc. The wear of the cutting edge increases the number of pulses of acoustic emission when the cutting edge is worn. As a result, the wear and tear of the cutting edge can also be observed. In this work, turning is monitored, turning in comparison with milling is a major difference in the location of the acoustic emission sensor. (Novotný 2013) After evaluating the measured data, it can be seen that the acoustic emission system appears to be a suitable monitoring system for machining, the acoustic signal is very dependent on the material to be machined and the feed rate. It can be assumed that in the emission records, acoustic events represent shots of the individual blade of the milling cutter. The acoustic emission sensor thus provides inputs corresponding to the reality of the machining. For this reason, AE can be used for continuous monitoring, especially for serial production, where thousands of dimensionally identical workpieces are produced. In this way it would be possible to react in a timely and correct manner to changes in the machining process, thus preventing the production of a non-conforming workpiece from the point of view of dimensional or surface tolerances.

ACKNOWLEDGEMENT
The research has been supported by the project TP 6/2017: Defectoscopic quality assessment of technical and organic materials; financed by IGA FA MENDELU.

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APPLIED CHEMISTRY AND BIOCHEMISTRY

ANTIOXIADANT ACTIVITY OF YOGHURT SUPPLEMENTED WITH NATURAL ADDITIVES

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Abstract: The aims of this study were the estimation of difference in physiochemical properties and antioxidant activity of yoghurts supplemented by different natural additives. In addition, the changes in physiochemical properties and antioxidant activity during five weeks storage were performed. The change in antioxidant activities was evaluated using the ABTS radical cations decolorizing assay. The statistical analysis showed that the yoghurt samples with the natural supplement have higher antioxidant activity than the plain yoghurt. In addition, the storage period affect the yoghurt properties by increasing its acidity and antioxidant activities by increasing the storage time. Furthermore, the plain and supplemented yoghurts are still consumable until the fifth week of storage but the favourable time to consume it with its higher antioxidant capacity is the 3rd week of storage.

Key Words: yoghurt, natural additives, antioxidant activity, Trolox equivalent

INTRODUCTION

Various reactive oxygen species such as hydrogen peroxide, superoxide and hydroxyl radical are known to cause oxidative damage not only to the food systems but also to living systems (Liu, Chen et al. 2005). The free radicals are the causative agent of many serious diseases in human such as cancer (Kinnula and Crapo 2004), cardiovascular diseases (Singh and Jialal 2006), neural disorder (Sas et al. 2007), Alzheimer's disease (Smith et al. 2000), Parkinson's disease (Bolton et al. 2000), aging (Hyun et al. 2006), and atherosclerosis (Upston et al. 2003). The natural antioxidants from food can protect the human body from the harmful effects of the free radicals, and they able to delay the progress of many chronic diseases (Liu et al. 2005). The natural antioxidants are preferable than the synthetic ones because some synthetic antioxidants have been reported to be carcinogenic (Liu et al. 2005).

Milk proteins considered the main source of the bioactive peptides that played a vital role in maintenance of antioxidant defence system (Gupta et al. 2009), the highest number of these peptides have been in the hydrolysates milk protein and fermented dairy products (Korhonen and Pihlanto 2006, Nagpal et al. 2011). Yoghurts are one of the most common fermented milk products and it is a worldwide food. It is traditionally consumed as a healthy food, because of their high nutritional values, health benefits, and its sensory properties (Gilliland 1989). Yoghurt is a coagulated milk produced by fermentation using the lactic acid bacteria, *Streptococcus thermophilus*, *and Lactobacillus Bulgaricus* (Gahruieet al. 2015). The fermentation process evolves the yoghurt antioxidant activity by releasing various bioactive peptides and free amino acids during lactic acid fermentation (Kudoh et al. 2001, Korhonen 2009). Yoghurts supplemented by different fruits,

vegetables, and other natural products to increase their antioxidant activity by providing more bioactive peptides and amino acids that act as a scavenger for the free radicals.

The aims of this study were to estimate the antioxidant activity and the pH of yoghurt supplemented with a different weight percentage (1, 3, and 5 wt%) of different natural additives that already known with their high antioxidant activity. Another aim of this study was to estimate the effect of storage time on the antioxidant activity and pH of the different yoghurt samples for five weeks storage in the fridge at 4 °C.

MATERIALS AND METHODS

pH measurement

The pH of the different yoghurt samples was measured according to *Zainoldin and Baba 2009* (Zainoldin and Baba 2009) using the pH meter (WTW 720 pH meter, Germany), by mixing 1 ml of yoghurt and 3 ml of distilled water . The measurements were performed weekly in triplicate during five weeks storage period to estimate the change in pH during the storage time.

Yoghurt samples preparation

The yoghurt samples were prepared at the Department of Food Technology laboratories at Mendel University, different types of natural additives with different weight percentage (1, 3, and 5 wt%) were added to yoghurt after fermentation process completion. The natural additives that used includes the Quinoa (Qu), Nopal (No), Apple fibres (Af), and Bamboo fibres (Bf), in addition to the plain yoghurt sample without any additives. All samples were stored at 4 °C and the pH and antioxidant activity were measured weekly to detect the changes.

Preparation of water-soluble extracts of the yoghurt samples (WSE)

The water soluble extract of the yoghurt samples was prepared according to *Perna et al. 2014* (Perna et al. 2014) with some modifications. Briefly, 50 ml of yoghurt samples were centrifuged at 9000 rpm (Hettich Universal 32 R centrifuge, Germany) at 4 °C for 15 min. The supernatant was filtrated separately through 0.45 µm membrane filter and the filtrates were stored at 4 °C for the analysis.

Antioxidant activity of ABTS radical scavenging system assay

The antioxidant activity of the different yoghurt samples was assayed according the method described by *Re et al. 1999* (Re et al. 1999). The ABTS (2, 2 – Azino- bis (3- ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical solution was created by oxidation of 7 mM ABTS solution with 140 mM potassium persulfate (K_2SO_4). The stock solutions of both reactants were prepared in ACS water and the ABTS radical solution was prepared by mixing 10 ml of 7 mM ABTS stock with 175 µl of the K_2SO_4 solution, and the mixture kept in the dark at room temperature for 12–16 hours before use. For estimation of the antioxidant activity, the ABTS radical solution was diluted with (96%) ethanol to get the absorbance of 0.700 ± 0.020 at 734 nm. Two millilitres of the diluted ABTS radical solution were mix with 100 µl of the yoghurt WSE and incubated at room temperature and the absorbance was measure after 30 min at 734 nm. The Trolox standard curve (0–2 mM) was created to calculate the Trolox equivalent antioxidant capacity (TEAC) for each sample and the results were expressed as µg TEAC/ml of yoghurt extract. The 96% ethanol was used as a blank in each assay, all the measurement was carried in triplicate and the percent of inhibition (%) was calculated using the following equation: Inhibition (%) = $[(A_{control} — A_{sample})/ (A_{control})] \times 100$

Statistical analysis

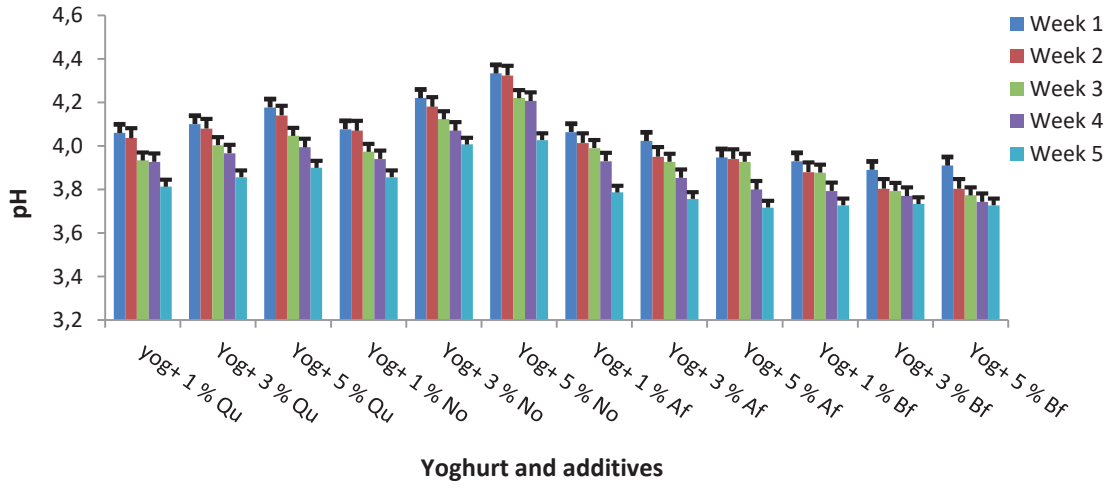
The result analysis performed by IBM SPSS Statistics 21 software. The one-way ANOVA was performed to detect the significance variations in pH, antioxidant activities, and TEAC between and within the yoghurt samples during the storage period at the significant level ($P < 0.05$). The correlation between the antioxidant activities and the additives percentage was estimated by Pearson correlation at the significant level ($P < 0.01$).

RESULTS AND DISCUSSION

Changes in pH during storage periods

The pH measurement considered a very sensitive tool to detect the acidity change of yoghurt during a refrigerated storage (Salij and Ismail 1983). In our study, the initial pH of all yoghurt samples during the first week was acidic and then its start to decline and be more acidic by increasing the storage time. The changes in pH values for the different yoghurt samples during the storage periods are shown in figure.1. The pH values were significantly ($P < 0.05$) decrease during the different storage periods in all yoghurt samples including the control. In addition, there was a significant difference ($P < 0.05$) in pH values between the different samples and within the same samples. The change in pH values for the yoghurt samples with the additives compared to the control related to the percentage and additives type, which affect the yoghurt taste and pH (Laye et al. 1993, Tarakçi and Kucukoner 2003). The continuous decline in pH of the yoghurt samples by increasing the storage time is related to the amount of lactic acid production because of acidification process, which is the main process during yoghurt fermentation (Aloğlu and Öner 2011, Nguyen and Hwang 2016). Furthermore, the continuous decrease in pH is considered an indicator for the high metabolic activities of the lactic acid bacteria which is the starter culture in almost all yoghurt types (Zainoldin and Baba 2009).

Figure 1 Change of pH of the yoghurts during different storage periods



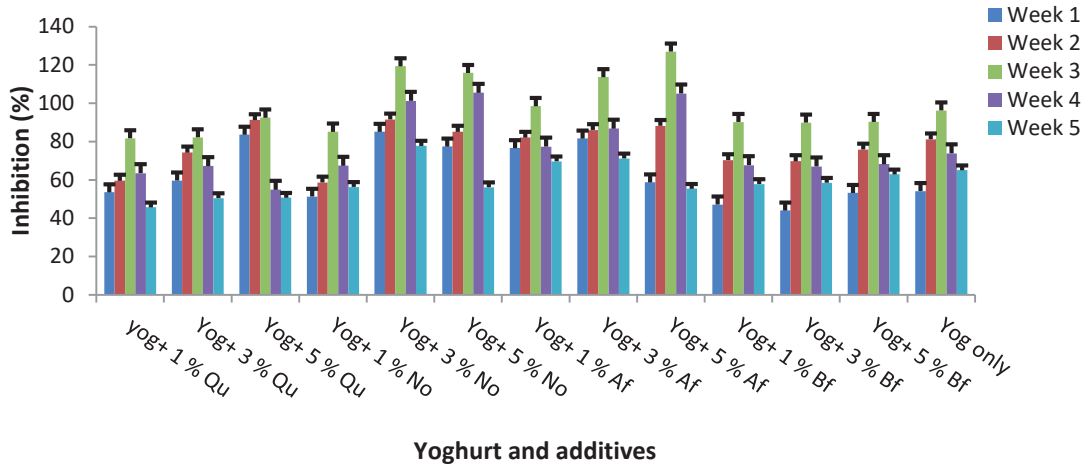
Legend: Yog– yoghurt, Qu – Quinoa, No – Nopal, Af – Apple fibre, Bf – Bamboo fibres

Change in Antioxidant activity of ABTS radical scavenger assay and Trolox equivalent antioxidant capacity (TEAC)

The most important feature of the antioxidants is their capability to trap the free radicals which are exist in the biological system from different sources (Aloğlu and Öner 2011). The free radicals may oxidize the macromolecules (nucleic acids, proteins and lipids) and can initiate a serious diseases (Zainoldin and Baba 2009, Srivastava et al. 2016). In our experiment, the yoghurt samples showed a significant variation ($P < 0.05$) in their antioxidant activities and the TEAC (Figure 2 and Figure 3) respectively. These variations between the different samples and within the sample with different percentage of the supplement are strongly related to the percentage and additives types especially there are many studies reported the antioxidant activities of our additives like Alvarez-Jubete et al. 2010, Wu et al. 2012, El-Mostafa et al. 2014. Generally, it has been reported that the natural additives enhance the antioxidant activity of the yoghurt and other foodstuff to evolve the consumer's health against the pathological process that are related to the free radicals (Pereira et al. 2013). Furthermore, the results showed that the antioxidant activity significantly ($P < 0.05$) increase with the storage time, it has reached the maximum value in the third week of storage and then start to decrease gradually for all samples including the control. The increase in antioxidant activity and TEAC equivalent are strongly related to the fermentation and post acidification during storage that determine the production of organic acids, and the decline in the antioxidant activity after the third week is related

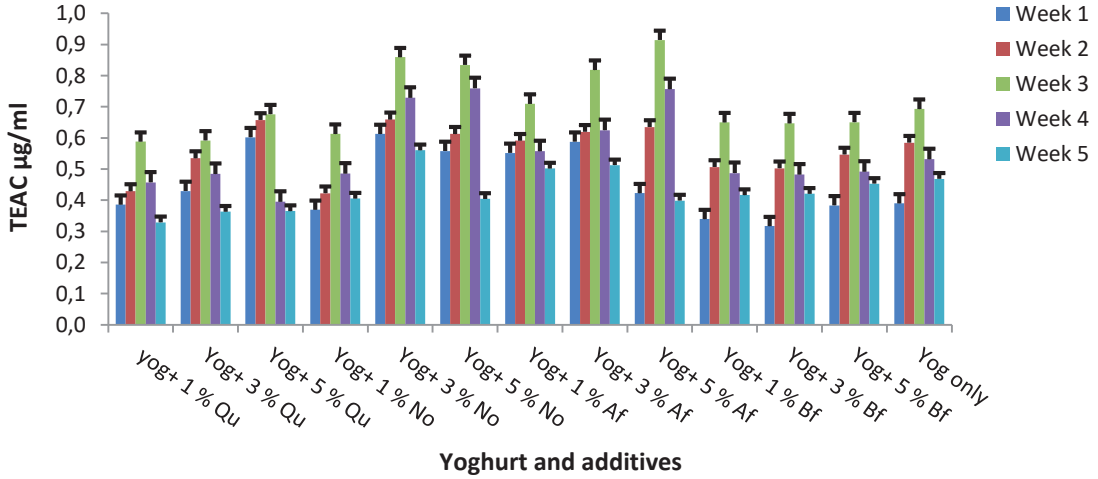
to the extensive proteolysis that produce smaller and less bioactive peptides (Correiaet al. 2005, Perna et al. 2014). Our results are in agreement with (Shori and Baba 2013, Perna et al. 2014, Shori and Baba 2014) who reported the increase in antioxidant activity of the yoghurt during the storage period and then start to decrease after the 21 days (3 weeks). Moreover, the results showed that the plain yoghurt without additives have also an antioxidant activity and its activity and the amount of TEAC improved by the additives. Our results are in agreement with different studies that reported the antioxidant activity of plain yoghurt like (Aloğlu and Öner 2011, Gjorgievskiet al. 2014, Yilmaz-Ersan et al. 2016). Furthermore, there was no significant ($P > 0.01$) correlation between the antioxidant activity and the additives amount in spite of different studies like (Prior et al. 1998, El Samh et al. 2013) reported the increase of antioxidant activity of yoghurts or foods by increasing the amount of additives.

Figure 2 Percentage change of antioxidant activity of yoghurts during storage periods



Legend: Yog– yoghurt, Qu – Quinoa, No – Nopal, Af – Apple fibre, Bf – Bamboo fibres

Figure 3 Change in Trolox equivalent capacity (TEAC) of yoghurts during storage periods



Legend: Yog– yoghurt, Qu – Quinoa, No – Nopal, Af – Apple fibre, Bf – Bamboo fibres

CONCLUSION

This study shows the effect of storage on the physiochemical properties and antioxidant activity of plain yoghurt supplemented with different natural additives. The results showed that the plain yoghurt has antioxidant activity but this activity can be improved by these supplements which are known for their antioxidant activities. Furthermore, the study showed that the storage conditions have an effect on the physiochemical properties of yoghurt like increasing the acidity, probably due to the increase of lactic acid production. Moreover, increasing the storage period of yoghurts improve

their antioxidant activity regardless if they have additives or not, the progress fermentation during the storage results in production of peptides and free amino acids that enhance the yoghurt antioxidant properties. In addition, based on our results we can say that the proper storage time of the yoghurt is 3 weeks (21 days), after this period the yoghurt is still acceptable to use by the consumers but it will be less quality considering our results that related to pH and antioxidant activity changes during the storage period.

AKNOWLEDGEMENT

The research was supported by the project TP 2/2017 "Effect of additives on the rheological behaviour of foodstuffs and raw materials for their production" financed by the Internal Grant Agency FA MENDELU and by the Ministry of Education, Youth and Sports of the Czech Republic under the project CEITEC 2020 (LQ1601).

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LIMITED DRYING AND ITS EFFECT ON PEPTIDE RECOVERY RATES

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Abstract: Protein and/or peptide loss is an undesired but inevitable side effect of purification procedures. We compared three different containers, three different peptide standards and a representative complex proteome digest, and show that a partial drying could improve sample recovery from a glass surface and standard polypropylene tubes. Further, we show that the partial drying minimizes differences between the low-binding and standard polypropylene tubes.

Key Words: proteomics, digest, sample preparation

INTRODUCTION

Peptide-based analyses often employ techniques that concentrate small-volume samples to achieve the desired concentration or to remove volatiles such as methanol or acetonitrile. Lyophilization, vacuum evaporation or evaporation under a stream of gaseous nitrogen are the most common methods to reduce the sample volume. However, each of these methods presents a different obstacle. The evaporation in a centrifugal vacuum concentrator which is often the method of choice in proteomics does not require a sample freezing and vacuum effectively limits oxidations. However, the dried sample has a high potential for adsorption to the wall of a container containing the sample. Here, we compare the peptide recovery rates from three different containers and show that limited drying may significantly improve the yield.

MATERIAL AND METHODS

Plant material

Leaf blades of barley (cv. Sebastian) were homogenized (Retsch Mill MM400), aliquoted and stored at -80 °C.

Protein standards

Lyophilized ovalbumin and albumin (>97% purity) were purchased from Sigma Aldrich and dissolved (8 M urea, 50 mM ammonium bicarbonate) to the final concentration of 1 mg/ml. Aliquots corresponding to 500 µg were diluted and digested overnight with an immobilized trypsin (Promega) as described previously (e.g. Skalák et al. 2016).

Protein extraction

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.

Protein desalting

Samples were desalted on C18 SPE (Agilent) and dried (Speed-vac system, Thermo).

Peptide content determination

Peptide concentration was determined with a modified BCA colorimetric assay (Pierce Quantitative Colorimetric Peptide Assay kit; Tecan Spectra Rainbow microplate reader) and a commercial peptide standard included within this kit.

LC-MS analysis

Protein analysis was performed as described previously (e.g. Baldrianová et al. 2015). Briefly, tryptic digests 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 a 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

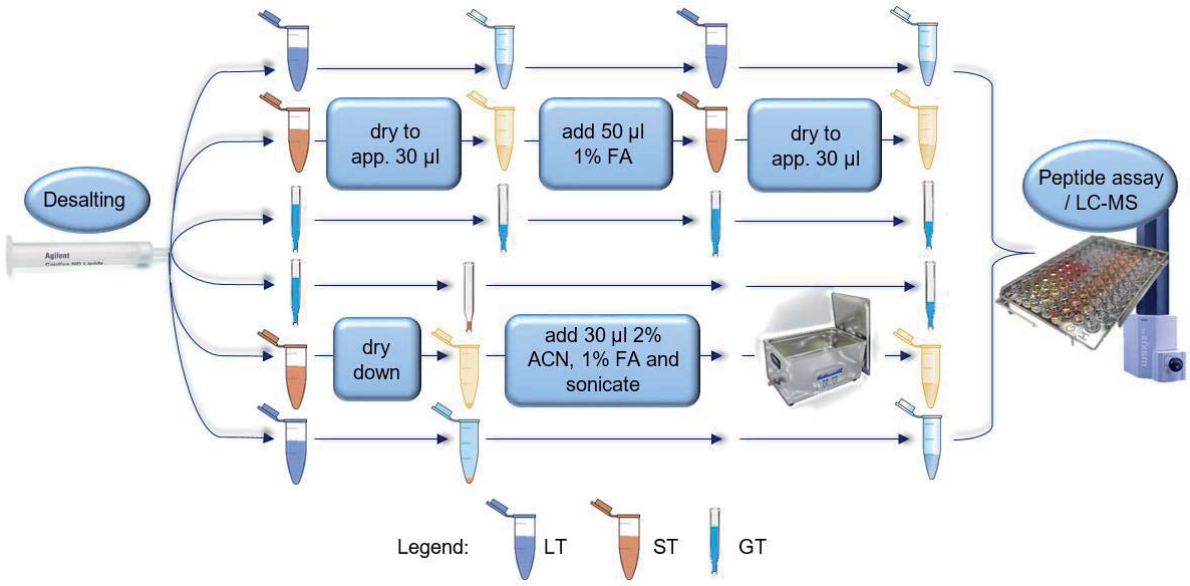
The measured spectra were extracted by Bruker's Data Analysis 4.1 and processed as described previously (e.g. Cerna et al. 2016) with minor modifications. In brief, spectra were recalibrated by Preview (www.proteinmetrics.com) and were searched against barley (6/2016) protein sequence database by Sequest HT 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. Data were processed and visualized by ProteomeDiscoverer 2.2 (Thermo).

RESULTS AND DISCUSSION

In silico trypsin digestion

Routine protein digestion protocols employ an in-gel digestion (e.g. Dobrá et al. 2014) or an in-sol digestion (e.g. Černý et al. 2013). Prior the MS analysis, the resulting peptides have to be extracted, desalted and concentrated. These steps are prone to an extensive sample loss. Hydrophilic peptides are lost during the washing steps, shorter and more volatile peptides during the drying, and large and hydrophobic peptides precipitate or may stick to the surface of the container.

Figure 1 Analysis of peptide loss in three different containers.



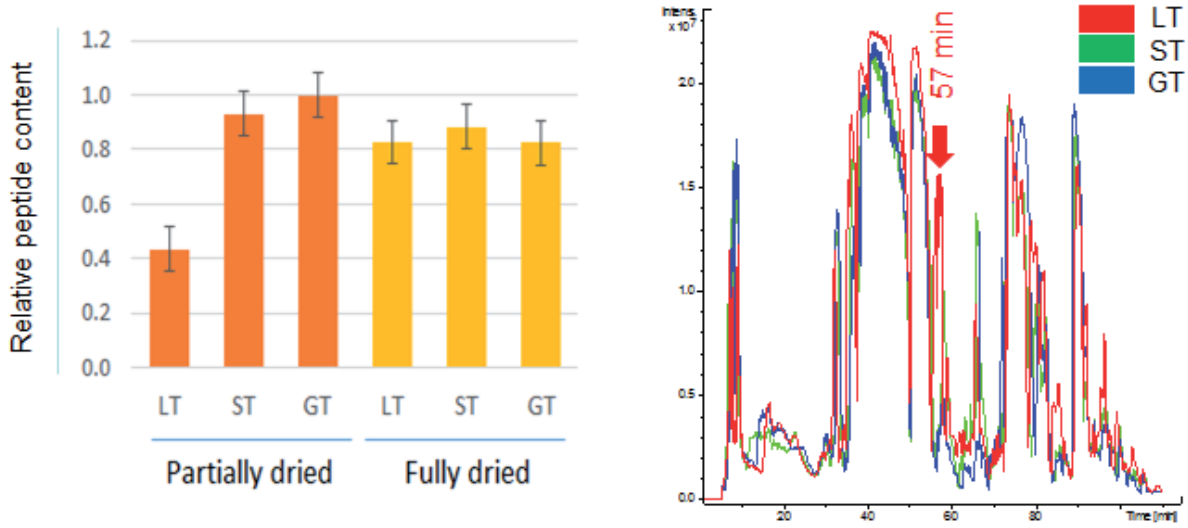
Here, we followed the last step in the procedure and compared sample loss in three different containers: a low-binding tube designed to decrease protein/peptide interactions with the tube surface (LT; LoBind Tube, Eppendorf), standard polypropylene 0.5 ml lab tube (ST) and glass insert (GT). Samples of protein standards and barley leaf total protein extracts were desalted on C18 SPE columns (Agilent) and eluted with a three-step polarity gradient (150 µl methanol, 150 µl acetonitrile, 150 µl acetone). Each sample was divided into six equal fractions and processed as indicated (Figure 1). For each experiment, three samples were dried completely, reconstituted in app. 30 µl solution of 2% acetonitrile in 1% formic acid and sonicated for 10 min. In parallel, three samples were dried to app. 30 µl, mixed with 50 µl 1% formic acid in ultrapure water (LC-MS grade, Sigma-Aldrich) and dried again to app. 30 µl.

Partial drying improves peptide yield

Previous studies have found that the peptide-surface interaction depends on the peptide concentration and its primary sequence, and the materials and solutions used in the sample preparation (Hoofnagle et al. 2016). The surface adsorption is not easy to compensate because the additives that could improve the peptide recovery (e.g. surfactants dimethyl sulfoxide or Triton X-100) are not compatible with an LC-MS analysis (Suelter and Deluca 1983, Midwoud et al. 2007). To compare the peptide recovery rates from three different containers in fully- and partially-dried samples, we employed a colorimetric assay and a label-free LC-MS analysis (Figure 2). We did not find any significant difference in the absolute peptide content of fully dried samples of albumin (BSA), ovalbumin (OVA) or a commercial peptide standard. However, we note that the qualitative results from the LT tubes were slightly better which was reflected in the LC-MS base peak chromatograms (e.g. a peak at 57.0 min; Figure 2B). Amines tend to adsorb to glassware via its positively charged chain (Broek et al. 2008) but our results indicate that this binding is either reversible or that the glass insert surface capacity is lower compared to that of polypropylene tubes. The partial sample drying improved the peptide yields in ST and GT but not in the LT samples. The LT polypropylene tube features a protein-repelling surface that prevents a protein adsorption and thus should have the highest peptide recovery rate. However, it seems that this surface is increasing the sample loss during the repeated evaporation steps.

Figure 2 Peptide recovery rates in a high concentration low-complexity peptide sample. Effects of drying and container material; (A) The means and standard deviations of total PSMs count in BSA and OVA samples; (B) Representative base peak chromatograms of fully dried peptide samples.

A) *B)*



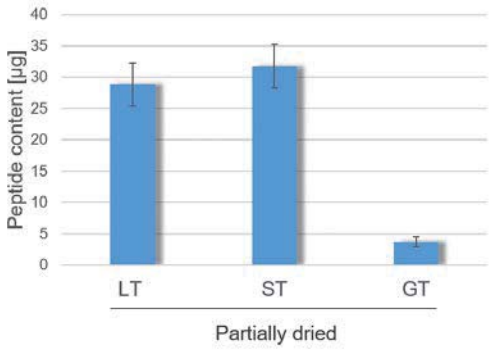
Complications with a complex sample

Our results indicated that it would be beneficial to modify the sample preparation technique and employ a method of a partial drying. To further support these results, we analyzed a complex proteome sample obtained from barley leaf blades. In these experiments, the total protein input for the digestion step was decreased by app. 90% to obtain samples of a high complexity and a low concentration. We have analyzed three complete experimental replicates (Figure 3). In contrast to the previous assays with standard digests, the testing with a complex barley proteome digest showed a much lower recovery rate from the glass surface, indicating more than 80% higher sample loss than in polypropylene tubes. The difference between the total yields in the LT and ST sets was not statistically significant ($p<0.05$). We believe that the extensive sample loss in GT is related to peptide-surface interactions and that the benefits of a glass surface observed earlier (Figure 2) are revealed only once the surface is saturated. This seems to be well in line with the reported lower binding capacity of the wetted solid surface area (John et al. 2004).

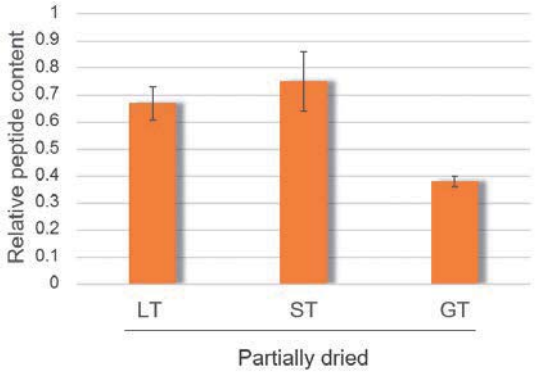
We have also found a surprising difference between quantitative data from the peptide assay (Figure 3A) and the PSMs-based total protein content approximation (Figure 3B). We did not see this difference with the high-abundant standard digests and we believe that this overestimation in the LC-MS quantitation originates in a disproportional sample loss and a depletion of some of the high abundant peptides. In accordance, we have identified 5% more proteins in the GT samples but we did not detect HORVU0Hr1G036870.1 and HORVU6Hr1G016850.1 that in polypropylene tubes represent over 2% and 1% of the total PSMs, respectively.

Figure 3 Peptide recovery in a complex barley proteome digest in partially dried samples. (A) Peptide content determined by the peptide assay (B) Relative peptide content from the total PSMs count; The means and standard deviations of three independent replicates.

A)



B)



CONCLUSION

Our results showed that a limited drying could improve the peptide sample yield. We demonstrated that the benefits of a modified low-binding surface are minimized during the drying and that inexpensive polypropylene tubes have similar or higher peptide recovery rates. Our analyses also imply that a glass surface could improve the protein identification by a limited depletion of abundant proteins. However, the extensive sample loss and a certain level of unpredictability will limit the applicability of this depletion technique.

ACKNOWLEDGEMENTS

The research was financially supported by grant TE02000177 (TACR) and IGA grant no. IP 15/2017.

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RUTHENIUM-BASED CORE-SHELL NANOPARTICLES WITH EXCEPTIONAL *IN VITRO* BIOCOMPATIBILITY

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Abstract: The current study demonstrates design preparation and characterization of biocompatible hybrid ruthenium core-shell nanoparticles (RuNPs) coated with polyvinylpyrrolidone (PVP) and polyoxyethylene stearate (POES). The resulting RuNPs were loaded with doxorubicin, as model anticancer drug. Resulting complex has an exceptional stability in physiological conditions. The cytotoxic effects of the complex were tested using cell lines representing breast and ovarian cancers and neuroblastoma. Although bare RuNPs had only negligible cytotoxicity, RuPDox caused an enhancement of doxorubicin cytotoxicity when compared to free doxorubicin. RuPDox promoted significantly increased stability of doxorubicin in human plasma and pronounced hemocompatibility assayed on human red blood cells. Results demonstrate that biocompatible RuNPs could have a great potential as versatile nanoplatform to enhance efficiency of anticancer therapy.

Key Words: biocompatibility, nanomedicine, polyvinylpyrrolidone, polyoxyethylene stearate

INTRODUCTION

Nanotechnology is a term that defines design and testing of functional units on a nanoscale (Sahoo et al. 2007). Material scientists have performed exceptional accomplishments in the design of various types of materials that can be used in nanomedical (Giner-Casares et al. 2016).

The frequent drawback of these materials is common systemic toxicity. One potent solution is encapsulation of nanoparticles into polymeric shells. (Quarta et al. 2012). Among the most promising belong biodegradable polymers, such as polyethylene glycol (PEG) or polyvinylpyrrolidone (PVP) (Beik et al. 2016, Calvo et al. 2001, Moghimi et al. 2001).

Herein, we present hybrid organic-inorganic core-shell ruthenium nanoparticles (RuNPs) coated with PVP-POES shell for delivery of conventional cytostatic agent doxorubicin (Dox, hereinafter RuPDox for the whole complex). Ruthenium is notable for its pronounced biocompatibility, attributed to ability to mimic the binding of iron to serum transferrins (Kostova 2006). Particularly, biocompatibility and magnetic properties make RuNPs primary candidate of selection for manifold biomedical applications (Gibb et al. 1973).

Overall, we show that our designed RuPDox exhibit exceptional stability in non-target plasma environment with only negligible adsorption of plasma proteins. Moreover, RuPDox possess pH-responsive properties enabling for burst release of Dox in acidic pH present in endosomes and tumor hypoxic tissue. RuPDox cytotoxicity was tested in vitro on three types of malignat cells - ovarian, breast cancer and neuroblastoma. RuNPs are pronouncedly biocompatible, RuPDox

formulation significantly enhanced Dox intranuclear accumulation. Our results imply high potential of the use of RuNPs with PVP-POES as versatile nanoplatform to enhance efficiency of cancer treatment.

MATERIAL AND METHODS

Reductive colloidal synthesis of RuNPs capped with PVP

$RuCl_3 \cdot 2.5\ H_2O$ in water was added to a stirred solution of PVP dissolved in 80 ml of water. Black solution was stirred, $NaBH_4$ was supplemented and release of hydrogen was observed. The product was stirred overnight followed by volume reduction on Amicon 3k to final volume of 50 ml.

RuNPs coating with POES and non-covalent complexation of Dox

Equal volumes of RuNPs and 20% solution of POES were mixed and ultrasonicated. After that, the solution of RuNPs was mixed with Dox and ultrasonicated. Finally, resulting RuPDox was centrifuged to remove unbound Dox and resuspended in MilliQ water. Loading efficiency (LE) of Dox to RuNPs was analysed by UV-Vis spectroscope Infinite 200 PRO (Tecan, Männedorf, Switzerland) at λ 480 nm.

Attenuated total reflectance Fourier transform-infrared spectroscopy (ATR-FT-IR)

FT-IR spectra were collected using a Nicolet iS10 FT-IR spectrometer with diamond ATR attachment (Thermo Electron Inc., San Jose, USA).

Transmission electron microscopy (TEM), Doppler electrophoresis and quasielastic dynamic light scattering (DLS)

TEM analyses were performed using the sample deposited onto 400-mesch copper grids coated with a continuous carbon layer by Tecnai F20 TEM (FEI, Eindhoven, Netherlands). ζ-potential was evaluated using Doppler electrophoresis on Zetasizer Nano ZS90 (Malvern instruments, Malvern, UK) as well as particle size analysis by DLS.

Evaluation of colloidal stability of RuNPs and RuPDox in physiological environments

To demonstrate their colloidal stability nanoparticles dispersed in the Ringer´s solution were placed in the stationary rack and kept at 25 °C.

Cell lines and culture conditions

Three human cell lines were used: *i*) the A2780 human ovarian cancer cell line, *ii*) the MDA-MB-231 - human breast cancer cell line, and *iii*) the UKF-NB-4 neuroblastoma cell line. All cell lines were purchased from Health Protection Agency Culture Collections (Salisbury, UK).

A2780 and MDA-MB-231 were cultured in RPMI-1640 with 10% foetal bovine serum (FBS), UKF-NB-4 Iscove's modified Dulbecco medium (IMDM) with 10% FBS. Media were supplemented with penicillin and streptomycin, and the cells were maintained in a humidified incubator Galaxy® 170 R (Eppendorf, Hamburg, Germany). Prior all analyses, cells were counted using Countess II FL (Thermo Fisher Scientific, Waltham, MA, USA).

Estimation of cytotoxicity

The viability was assayed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Cell was incubation for 24 h at 37 °C with 5% CO_2 to ensure cell growth. After treatment, 10 µl of MTT [5 mg/ml in phosphate buffered saline (PBS)] was added to the cells and incubated. After that, MTT-containing medium was replaced by 100 µl of dimethyl sulfoxide (DMSO) and, absorbance was determined at 570 nm using Infinite 200 PRO (Tecan, Männedorf, Switzerland).

Analysis of formation of protein coronas and hemocompatibility

Plasma was isolated from whole blood. RuNPs, RuPDox and Dox were incubated in plasma. The protein coronas were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained by Coomassie Brilliant Blue. Gels were visualized using Azure c600 (Azure Biosystems, Dublin, CA, USA).

Hemocompatibility was assayed using human red blood cells sampled. The degree of hemolysis was determined by measuring the absorbance of the supernatant at λ 540 nm after centrifugation.

Descriptive statistics

For the statistical evaluation of the results using paired *t*-test and ANOVA. Unless noted otherwise, the threshold for significance was $p < 0.05$. For analyses Software Statistica 12 (StatSoft, Tulsa, OK, USA) was employed.

RESULTS AND DISCUSSION

Physico-chemical characterization of RuNPs and complexation with Dox

Dox is one of the most commonly used chemotherapeutic agents (Denel-Bobrowska and Marczak 2017). Several studies have shown Dox loading to different carriers such as liposomes, polymeric nanoparticles and others (Dawidczyk et al. 2017, Ding et al. 2017, Wang et al. 2017). Therefore, we have designed, prepared and tested cytotoxicity and bioavailability of hybrid organic-inorganic RuNPs loaded with Dox in this study. Reductive colloidal synthesis with Na $[BH]_4$ and PVP ends resulted in well dispersed and colloid-stable RuNPs. Further surface functionalization was performed using POES (Figure 1A).

RuPDox were tested for their LEs towards Dox. Table 1 illustrates that the highest LE (63.7%) was achieved for RuNPs coated with 20% POES. Moreover, using 20% POES, RuPDox was found to disperse readily and remained stable in dispersion for more than 24 h (Figure 1B).

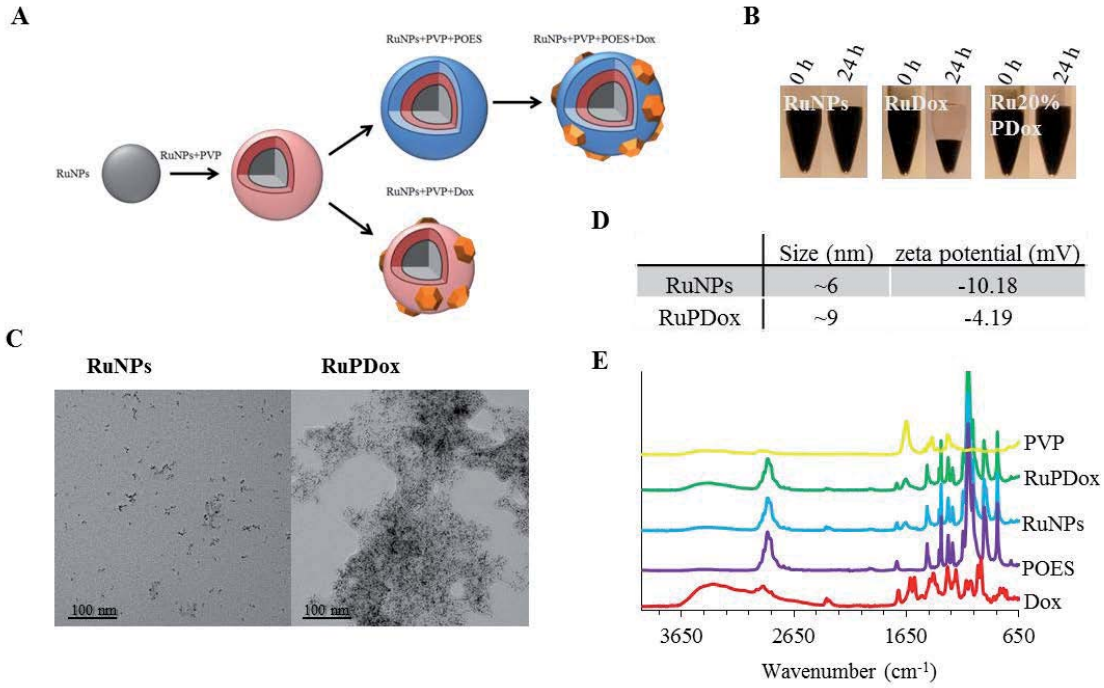
TEM micrographs showed that both RuNPs and RuPDox demonstrated relatively uniform oval-to-spherical morphology and were well dispersed (Figure 1C). The ζ-potential values of RuNPs and RuPDox in physiological conditions were -10.18 mV and -4.19 mV respectively. Although the ζ-potentials demonstrated relatively low values, nanoparticles were stable due to the presence of large molecular weight stabilizers, which shift the plane of shear to a further distance from the particle system, and thus results in a reduction in the value of ζ-potentials (Quaglia et al. 2009). The average maximum distributions of hydrodynamic diameters under physiological conditions were ~6 nm for RuNPs and ~9 nm for RuPDox (Figure 1D). The FT-IR spectra confirmed the formation of RuPDox and represented characteristic fingerprints for individual components forming RuPDox (Figure 1E).

Table 1 Analysis of Dox loading efficiency to RuNPs coated with various amount of POES.

	LE ± SD (%)
RuNPs	58.0 ± 2.6
10% POES	54.1 ± 3.4
20% POES	63.7 ± 4.0

Legend: Data are shown as means ± SD of triplicate in three independent experiments.

Figure 1 (A) Schematic illustration of synthesis of RuPDox from RuNPs by POES coating. (B) Photodocumentation of colloidal stability of synthesized RuNPs, RuDox and RuPDox with 2% POES demonstrating exceptional stability of RuPDox with 20% POES in start-point (0 h), and 24 h. (C) TEM micrographs of RuNPs (left) and RuPDox (right). (D) Hydrodynamic diameters of RuNPs and RuPDox determined by quasielastic DLS and ζ potential values determined by Doppler electrophoresis. (E) FT-IR spectra of RuPDox and individual components used for synthesis



RuNPs potentiate cytotoxicity of Dox in RuPDox formulation

Cytotoxic testing on three different types cells - breast (MDA-MB-231), ovarian cancer (A2780) and neuroblastoma (UKF-NB-4) revealed that RuNPs exhibited only negligible cytotoxic effects, while a complexation with Dox (RuPDox) resulted in a significant ($p < 0.05$) increase in cytotoxic effects in all tested cells (with the IC_{50} values between 1.2 ± 0.2 - 3.2 ± 0.2 μg/ml, all IC_{50} values are summarized in Table 1).

Generally, cytotoxicity of RuNPs is poorly known. Ramasamy and co-workers have shown that hollow mesoporous RuNPs have only slight cytotoxicity at concentrations higher than 100 μg/ml (Ramasamy et al. 2015), which is consistent with our findings and supports the low cytotoxicity of our RuNPs.

Table 2 Summary of IC_{50} values obtained from MTT assay for tested cell lines after 24 h treatments. All values are presented as mean ± SD of six biological replicates

		Mean IC_{50} ± SD (μg/ml)		
Cell line	Time (h)	Dox	RuNPs	RuPDox
A2780	24	8.5 ± 1.2	74.2 ± 2.1	3.2 ± 0.2
MDA-MB-231	24	7.9 ± 0.7	153.5 ± 3.9	1.1 ± 0.1
UKF-NB-4	24	3.4 ± 0.2	148.0 ± 1.8	1.2 ± 0.2

Estimation of RuPDox biocompatibility

In general, high degree of biocompatibility is achieved when a tested nanomaterial interacts with the body without inducing unacceptable toxic responses.

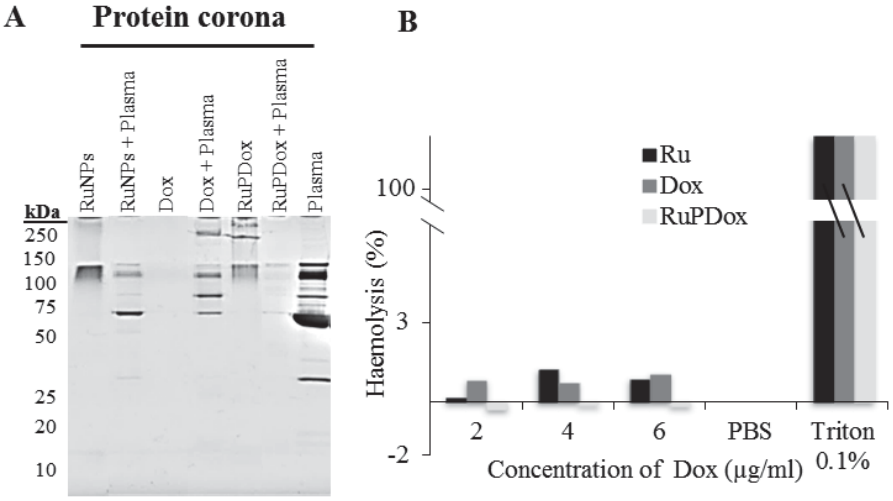
Pivotal aspect of biocompatibility is nanoparticle-blood interactions. Therefore, we firstly studied a rate of protein coronas formation, which are plasma proteins adsorbed on the surface of nanoparticles. Profiles of eluted proteins clearly demonstrate that bare RuNPs are capable to adsorb some amount of plasma proteins (Figure 2A). Moreover, we show that even free Dox is able to cause

plasma protein aggregation. Interestingly, RuPDox was shown to avoid most of unwanted interactions with plasma proteins with only small amount of protein adsorbed on the surface.

Finally, as hemolysis is often toxic effect of nanoparticles, we studied hemolysis on human RBCs. Hemotoxicity is connected with a positive surface charge, which is not present for RuNPs and RuPDox. Figure 2B demonstrates that all tested formulations caused only insignificant (max. 1%) release of hemoglobin from RBCs, which highlights exceptional hemocompatibility of RuPDox.

In general, surface sewing of the polymer produces extremely biocompatible hybrid materials. That is why the development of drug delivery systems is the most important. We have remarkably shown that RuNPs do not cause DNA fragmentation and, moreover, does not contribute to the natural genotoxic potential of free Dox, which in itself causes relatively massive DNA damage due to induction of DNA cleavage (Manjanatha et al. 2014).

Figure 2 (A) Protein corona profiles obtained after incubation of RuNPs, Dox and RuPDox with human plasma and loading onto SDS-PAGE. (B) Hemocompatibility of RuPDox assayed on human RBCs. PBS and 0.1% Triton X-100 were utilized as negative and positive controls, respectively.



CONCLUSION

In conclusion, we designed, prepared and tested cytotoxicity and biocompatibility of novel RuPDox nanoparticles. We show that hybrid organic-inorganic nanoparticles based on RuNPs must be taken into account as exceptional nanomedicine platforms. We also demonstrate that combining PVP with FDA-approved POES, such core-shell nanoparticles can act in multiple ways, which significantly enhances the Dox performance. Despite the validity of our in vitro results is limited and further in vivo experiments might be conducted, it is obvious that ruthenium-based nanomaterials have enormous potential for nanomedicine and our RuNPs with PVP-POES shell can serve as a versatile platform for complexation with distinct antiproliferative agents. Finally, based on available literature, RuNPs or ruthenium complexes are promising MRI contrast agents, hence their use will most likely enable for tracing and imaging of accumulation.

ACKNOWLEDGEMENTS

Financial support from Czech Science Foundation (project GA CR 17-12816S), IGA IP no. 16/2017 and CEITEC 2020 (LQ1601) is highly acknowledged. We also acknowledge Eliska Zakova for support with obtaining scientific data presented in this paper.

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THE EFFECT OF COFFEE SUPPLEMENTATION ON GLUTATHIONE AND TOTAL THIOLS LEVELS

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Abstract: Antioxidants are very important substances that counteract the formation of free radicals. They are divided into exogenous, such as vitamin C which the body receives with food, and endogenous. One of the most important endogenous antioxidants is glutathione (γ-glutamyl-L-cysteinyl-glycine), which plays an important role in cellular defense against oxidative damage. Free glutathione is presented within organisms in both reduced (GSH) and oxidized forms (GSSG). Oxidative stress leads to a decrease in GSH level and therefore a GSH/GSSG ratio that can be used as an indicator of oxidative stress and an indicator of various diseases. The aim of presented study was to develop a sampling method for capillary blood testing, where we have found that this amount (15 µl) is sufficient for our testing. Another aim was to supplement group of volunteers with coffee and to determine GSH levels and levels of total thiols after 0, 48 h and 96 h of supplementation in capillary blood. HPLC with electrochemical detection was used for GSH determination and Ellman's method for determination of total thiols. We could see the GSH slight increase as well as the levels of total thiols.

Key Words: antioxidant, coffee, glutathione, HPLC-ED, total thiols

INTRODUCTION

Antioxidants are extremely important molecules preventing negative effects of free radicals. Oxidative stress results in damage to DNA, proteins, lipids and carbohydrates and is the cause of many human diseases (Carru et al. 2003, Childs et al. 2016, Squellerio et al. 2012). Glutathione is one of the most important intracellular non-enzymatic antioxidant (Giustarini et al. 2011, Wu et al. 2004). GSH contains a thiol group in its molecule that is very reactive. Thiols have been and continue to be of interest because they play an important role in a number of biological processes (Giustarini et al. 2015, Rossi et al. 2006). The primary function of glutathione is to remove reactive oxygen species (ROS) (Kominkova et al. 2015, Minelli and Gogele 2011, Zhang et al. 2014). It also has many other important physiological functions (Childs et al. 2016, Townsend et al. 2003, Wu et al. 2004). The experiment was conducted on a beverage with proven antioxidant effects – coffee. Coffee consists of several biological active compounds, such as caffeine, diterpenes, chlorogenic acids, and melanoidins, which may affect human health (Godos et al. 2014). The most important antioxidants in coffee are polyphenols and the most represented polyphenol with antioxidant effects is chlorogenic acid. Studies in recent years have generated new information regarding the effects of coffee consumption on health, disproving the common belief that coffee is mostly harmful. A number of recent experimental and epidemiological studies reported a substantial positive effect of coffee consumption on human health, especially in relation with cardio-metabolic risk factors (Abrahao et al. 2013, Bakuradze et al. 2011, Ludwig et al. 2014, Salomone et al. 2014). Data published by Jung et al. suggest that coffee has a physiological antioxidative and anti-inflammatory effect and these effects are negatively correlated with roasting levels where antioxidant activity decreases with roasting time (Jung et al. 2017, Kotyczka et al. 2011).

For such experiments venous blood is commonly used (Wink et al. 2016), but for the detection of glutathione in the blood by HPLC-ED and for spectrophotometric assay we can use a small amount of capillary blood. This study is focused on supplementing a selected sample of coffee according to the highest amount of polyphenols and determining GSH and total thiols in capillary blood.

MATERIAL AND METHODS

Chemicals

GSH and GSSG, 5,5'-dithiobis-(2-nitrobenzoic acid), cysteine, sodium acetate, Coomassie Brilliant Blue G-250, Folin-Ciocalteu reagent (FCR), phosphoric acid, ferulic acid and trifluoroacetic acid (TFA) were obtained from Sigma-Aldrich (St. Louis, MA, USA). Methanol in HPLC grade was obtained from Chromservis (Prague, Czech Republic).

Biological material

The capillary blood was collected using the capillary from the heated (60 °C) lateral part of the finger. A single lancet pen was used for injection. In total, 10-15 µl of blood was collected and divided into three aliquots. All volunteers provided informed agreement and the experiment was approved by the ethics committee of Mendel University in Brno.

Determination of total polyphenols

Folin-Ciocalteu method was used to determine total polyphenol compounds. Initial calibration was performed on ferulic acid. 250 µl of FCR and 10 µl of sample were pipetted into the tubes, then all the tubes were mixed. Then 200 µl of 7.5% sodium carbonate solution was added. Samples were incubated at room temperature for 30 min. The colored product of reaction was determined using Infinite M200Pro (Tecan, Männedorf, Switzerland) at 580 nm.

HPLC-ED

Analysis of GSH and GSSG was performed using HPLC-ED comprising two chromatographic pumps, twelve-channel CoulArray electrochemical detector column containing reverse phase Zorbax eclipse AAA C18. Detector consisted of three flow analytical chambers (Zitka et al. 2011). Mobile phase consisted of A: TFA-water (3:97, w/w) and B: 100% methanol. The most optimal preparation procedure found from optimization: an aliquot of 5 µl was taken from the collected blood (15 µl) and diluted with 45 µl of 10% TFA, followed by freezing in liquid nitrogen for approximately one minute. Each sample was then sonicated with an ultrasonic needle for 30 seconds, vortexed and centrifuged for 20 minutes at 25,000 rpm and 4 °C. Approximately 40 µl were taken from each sample.

Total thiols analysis

Blood samples (5 µl, five times diluted with water) were mixed with 138 µl of Ellman's reagent (2 mM 5,5'-dithiobis-(2-nitrobenzoic acid) in 50 mM sodium acetate). The reaction was started using addition of 16.5 µl Tris base buffer (1 M, pH 8 was adjusted using acetic acid). The colored product of reaction (159.5 µl) was determined using Infinite M200Pro at 436 nm within 96-well plate with flat bottom.

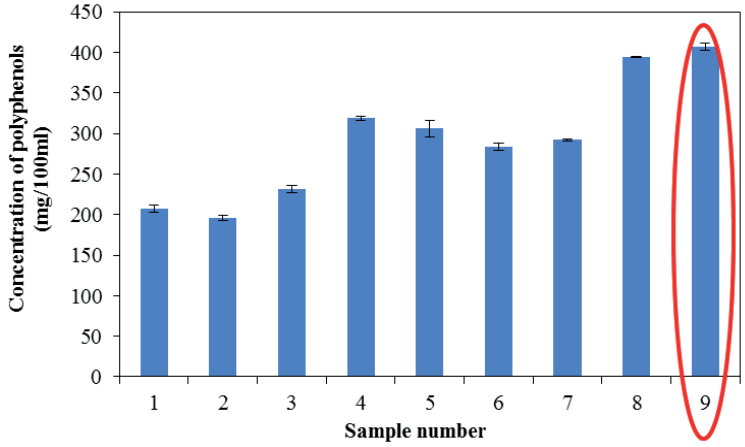
Bradford's assay

Bovine serum albumin was used as a standard. Bradford's reagent was prepared as follows: 10 mg of Coomasie Brilliang Blue G-250 was dissolved in 5 ml of 100% ethanol, subsequently 10 ml of 85% phosphoric acid was added and solution was filled to 100 ml with distilled water. 90 µl of Bradford's reagent was mixed with blood samples. The coloured product of reaction was determined using Infinite M200Pro at 595 nm. The scheme is shown in Figure 1.

RESULTS AND DISCUSION

The measured data is an average of three determinations. First, the total amount of polyphenols was determined in 9 samples of coffee using Folin-Ciocalteu method. 2.5 g of coffee was poured into 100 ml of hot water (80 °C) and filtered after 5 minutes.

Figure 1 Concentration of polyphenols in 9 samples of roasted coffee.

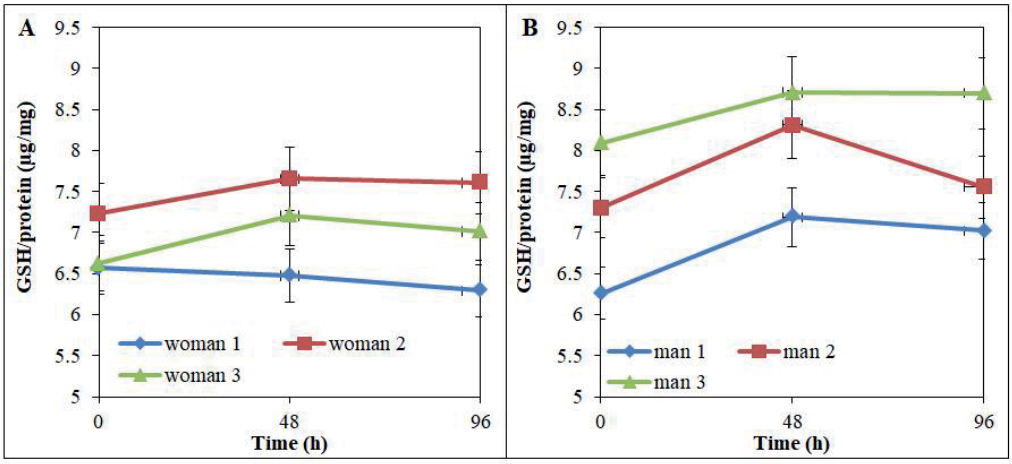


For the experiment with the supplementation with roasted coffee, a sample 9, which contained the highest concentration of polyphenols (namely 407.1 mg/100 ml of coffee ± 4.81), was selected (Figure 1). The lowest concentration of polyphenols was detected in sample 2.

Content of GSH

Six volunteers took part in the experiment (23–30 years). Optimized method for GSH/GSSG analysis was used for investigation of GSH levels in capillary blood samples of group of six volunteers supplemented with coffee. Their GSH levels were determined before the start of supplementation, 48 and 96 h after supplementation. The dose of coffee was calculated according to volunteer weight (2.5 g of coffee per 50 kg of weight for 1 cup) and 4 cups of coffee per day. Limited intake of food and beverages with high antioxidant concentrations like green tea, wine, fruit, vegetable and dietary supplements was recommended to volunteers. 15 µl of capillary blood were taken and GSH levels and total protein concentrations were determined.

*Figure 2 GSH levels during experiment in case of women (**A**) and men (**B**).*

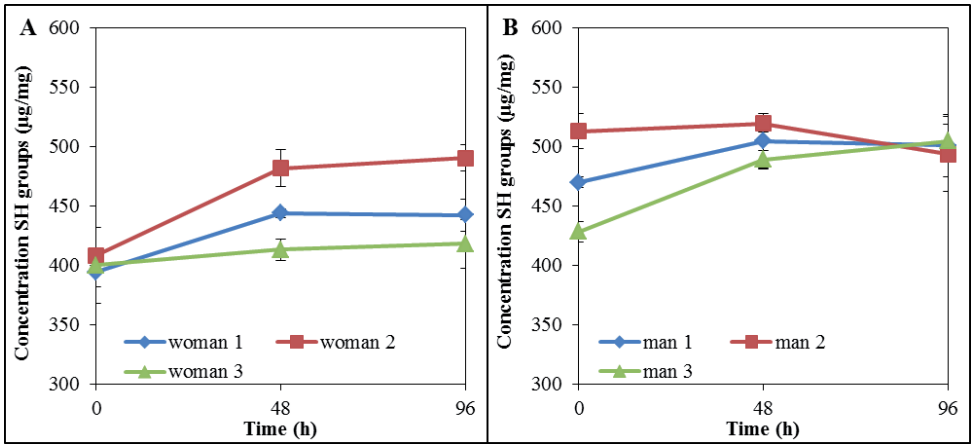


From the results, we can conclude that consumption of coffee induced a slight increase of GSH concentration in the blood (Figure 2). No increase was observed in case of women, but some changes were observed in case of men. Increases occurred mainly between first and second sampling. At the third sampling, the concentration was mostly stagnant or slightly declining. This may be due to the fact that high antioxidant intake resulted in GSH pool saturation. GSH levels elevation after coffee consumption also reporte (Bakuradze et al. 2011).

Another results suggested that coffee didn´t induced statistically significant changes of GSH levels during the test period (Teekachunhatean et al. 2012). Another important outcome is that we are able to perform this analysis even from a small amount of capillary blood and which causes only a minimal discomfort for volunteers.

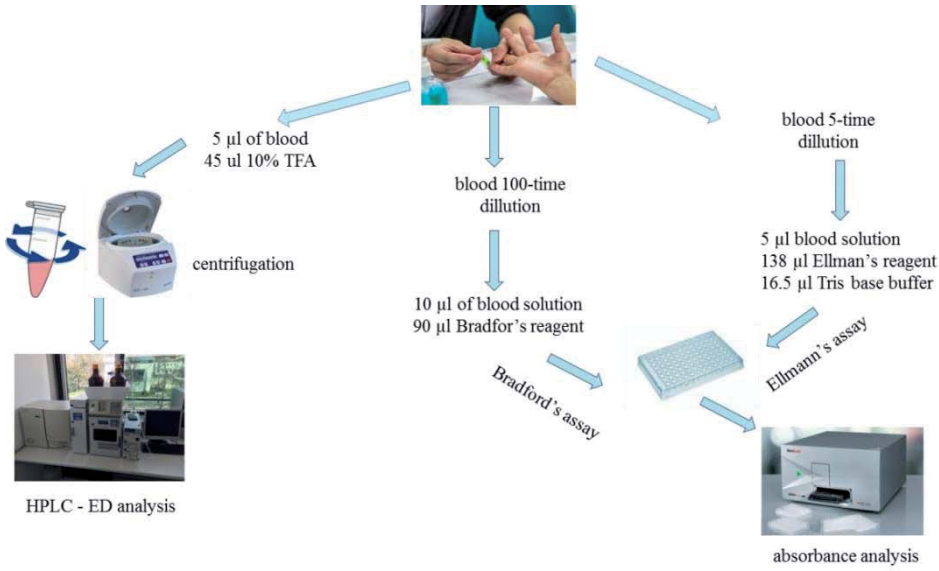
Content of total thiols

*Figure 3 Total thiol levels during experiment in case of women (**A**) and men (**B**).*



Analysis of total thiols was performed on a spectrophotometer using the Ellman‘s method. For this analysis, 2.5 µl of blood was needed, which was taken at the same time as blood for GSH determination and which was diluted with an appropriate amount of phosphate buffer. The results of determination of SH groups mostly correlate with GSH content. This also confirms the accuracy of the experimental data because GSH is one of the most abundant molecules in blood together with the SH group (Giustarini et al. 2016, Wang et al. 2014). Almost all volunteers who consumed coffee exhibited an increase in SH group concentrations in the first 48 hours. In the following days, this growth of total SH group concentrations was stabilized and even decreased (Figure 3). The scheme of sample preparation is shown in Figure 4.

Figure 4 Scheme of sample preparation



CONCLUSION

The aim of the study was to find out if we are able to observe changes in the content of thiol substances in capillary blood affected by supplementation with beverage containing high antioxidant concentration. First, it was important to optimize GSH assay methods on a high-pressure liquid chromatograph with electrochemical detection. The coffee experiment, which was attended by 6 volunteers (three men and three women), showed a slight increase in both GSH and total SH group levels.

The highest increase was observed between the first (the average value is 7.0 ± 0.6 µg GSH/mg protein) sand the second sampling (the average value is 7.6 ± 0.7 µg GSH/mg protein). At the third

sampling, the increase was much smaller or even stagnated (the average value is 7.4 ± 0.8 µg GSH/mg protein). We showed that capillary blood is suitable for GSH/GSSG electrochemical analysis in fast short-term study where due to this method change of GSH levels can be determined prior to the change of the state of organism.

ACKNOWLEDGEMENT
This research was carried out under the project CEITEC 2020 (LQ1601) with financial support from the Ministry of Education, Youth and Sports of the Czech Republic under the National Sustainability Programme II and MENDELU IGA IP 23/2017.

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ANTIBACTERIAL ACTIVITY OF COMPOSITE OF GRAPHENE OXIDE WITH SILVER NANOPARTICLES

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Abstract: Looking for strategies against the development of antibiotic resistance is a major global object of interest for the public health. This work deals with synthesis of antimicrobial composites of graphene oxide (GO) with metal nanoparticles. GO has been prepared by modified Hummers' method and characterized using scanning electron microscopy, Fourier transform infrared spectroscopy (FT-IR) and differential pulse voltammetry. Composites of GO have been synthetized with silve nanoparticles, which have been characterized. Potential antimicrobial activity of the nanocomposites was tested against *Escherichia coli*, *Staphylococcus aureus* and methicilin resistant *Staphylococcus aureus* (MRSA).

Key Words: graphene oxide, nanocomposite antimicrobial, *Staphylococcus aureus, Escherichia coli*

INTRODUCTION

Pathogens, whose resistance profiles present a new challenge for containing their spread and their impact on human health, are appearing among drug-resistant bacteria (Webb et al. 2005). For example, methicillin-resistant *Staphylococcus aureus* (MRSA) is worldwide a major nosocomial pathogen (Wenzel et al. 1991, Cunha 1998, Witte 1999). The occurrence of bacterial resistance phenotypes has been linked to the clinical use of antimicrobial agents to which the bacteria express resistance (Rice 1999). In spite of antimicrobial therapy, morbidity and mortality associated with these bacterial infections remain high partially as a result of the ability of these organisms to develop resistance to practically all antibiotics. Therefore, new strategies are needed to identify and develop the new generation of drugs or agents to control bacterial infections. Hence, the key for prevention of staphylococcal and related bacterial infections are good standards of hygiene, avoidance of skin trauma, and the use of antibacterial ointments or surface-coating agents with potential antibacterial properties. (Jones et al. 2008). Recent advances in the field of nanotechnology, especially the ability to prepare highly ordered nanoparticulates of any size and shape, have led to the development of new biocidal agents. Several studies have shown that nanoparticles formulations can be used as effective bactericidal agents (Tiller et al. 2001, Lin et al. 2002, Stoimenov et al. 2002, Kuhn et al. 2003, Sawai 2003, Sondi and Salopek-Sondi 2004, Lewis and Klibanov 2005, Rosi and Mirkin 2005, Ma et al. 2006). Nanotechnology provides a good platform for development and modification of nanoparticles based on metal, with promising applications in diagnostics, cell labelling, biomarkers, contrast agents for biological imaging, drug delivery systems, antimicrobial agents, and drugs based on nanotechnology for treatment of various diseases (Marcato and Duran 2008, Singh and Nalwa 2011). Therefore, researchers are focusing on nanoparticles in general and silver nanoparticles in particular to solve the problem with emergence of multi-drug resistant bacteria (Gemmell et al. 2006).

It has been presumed that graphene exhibits antibacterial efficiency against pathogenic bacteria strains via lipid peroxidation. The recent studies have shown that antibacterial effect of graphene is caused by inducing oxidative stress and membrane damage (Krishnamoorthy et al. 2012).

The modifications of graphene with metal nanoparticles render this material more effective against pathogenic bacteria.

MATERIAL AND METHODS

Preparation of graphene oxide

The graphene oxide (GO) was prepared by chemical oxidation of 5 g graphite flakes (Sigma-Aldrich, and 100 mesh, ≥75% min) in a mixture of concentrated H_2SO_4 (670 ml, Sigma-Aldrich) and 30 g $KMnO_4$ (Sigma-Aldrich) according to the simplified Hummer's method (Hummers and Offeman 1958). The reaction mixture was stirred vigorously. After 4 days, the oxidation of graphite was terminated by addition of H_2O_2 solution (250 ml, 30 wt% in H_2O, Sigma-Aldrich). Formed graphene oxide was washed 3 times with 1 M HCl (37 wt% in H_2O, Sigma-Aldrich) and several times with Milli-Q water (total volume used 10 l) until constant pH value (3–4) was achieved.

Synthesis of composite of graphene oxide with silver nanoparticles (AgNPs)

A solution of silver nitrate (50 ml, $AgNO_3$, 1 mM, 2 mM, 4 mM, 8 mM resp., Sigma-Aldrich) was added dropwise to the GO solution (1 ml, 5 mg/ml) under vigorous stirring. After that sodium borohydride (40 mg $NaBH_4$, Sigma-Aldrich) was added slowly to the reaction mixture and the resulting mixture was stirred intensively for 24 h at room temperature to allow reduction. The prepared composite was washed with Milli-Q water several times.

Differential Pulse Voltammetry

The electrochemical determination of silver by differential pulse voltammetry was performed using a CH Instruments Electrochemical Workstation (CH Instrument Inc., Austin, TX, USA), using glass measuring cell with three electrodes. The glassy carbon electrode was the working electrode, an Ag/AgCl/3 M KCl was the reference and a platinum wire was the auxiliary one. The parameters of this method were as follows: initial potential −0.2 V, end potential 0.5 V, modulation amplitude 0.05 V, step potential 1 mV. 0.2 M acetate buffer (pH 5) was used as a supporting electrolyte. The volume of injected sample was 20 µl; the volume of electrolyte was 1980 µl. For results evaluation, the software CHI 440A (CH Instrument Inc., Austin, TX, USA) was used.

Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FT-IR) spectra were collected using a Nicolet iS10 FT-IR spectrometer with a diamond ATR attachment (Thermo Electron Inc., San Jose, USA). IR spectra were recorded from 4000 to 650 cm^{-1} at a resolution of 4 cm^{-1}. Each spectrum was acquired by adding together 32 interferograms. Spectra were taken at 22 °C. The OMNIC™ software was used for IR spectra recording, and the JDXview v0.2 software was used for further spectra evaluation.

Scanning Electron Microscopy

The structures of the GO with AgNPs composites were characterized by scanning electron microscopy (SEM). For documentation of the nanoparticles structure, a MIRA3 LMU (Tescan, Brno, Czech Republic) was used. This model is equipped with a high brightness Schottky field emitter for low noise imaging at fast scanning rates. The SEM was fitted with In-Beam SE detector. For automated acquisition of selected areas a TESCAN proprietary software tool called Image Snapper (Tescan, Brno, Czech Republic) was used. The software enabled automatic acquisition of selected areas with defined resolution. An accelerating voltage of 15 kV gave satisfactory results regarding maximum throughput.

Cultivation of bacteria strains

Staphylococcus aureus (NCTC 8511), *Escherichia coli* (NCTC 13216) and MRSA (ST239) were obtained from the Czech Collection of Microorganisms, Faculty of Science, Masaryk University (Brno, Czech Republic). Cultivation media (Mueller Hinton) (Oxoid) were inoculated with bacterial culture and were cultivated for 24 h on a shaker at 600 rpm and 37 °C.

Colony-Forming Capability Test

Bacterial cultures (*E. coli*, *S. aureus*, MRSA) were diluted with MH medium to an absorbance of 0.1 measured using a spectrophotometer Ultrospec 10 (Biochrom, Cambridge, United Kingdom) at a wavelength of 600 nm. After that cultures were diluted by decimal dilution (to 10^{-7} cells per millilitre) and incubated at 37 °C for 2 h. After being exposed to different concentrations of composites of GO with AgNPs at 37 °C for 2 h. 100 µl of the cell suspension was spread onto MH agar plates. The number of the colonies was counted after agar plates were incubated at 37 °C in the dark overnight. The survival percentage was used to evaluate the antimicrobial effect of composites of GO with AgNPs and it was defined as the following formula:

$$\text{Survival\%} = \frac{\text{Colony numbers of treated bacteria}}{\text{Colony numbers of control bacteria}} \times 100\%$$

RESULTS AND DISCUSSION

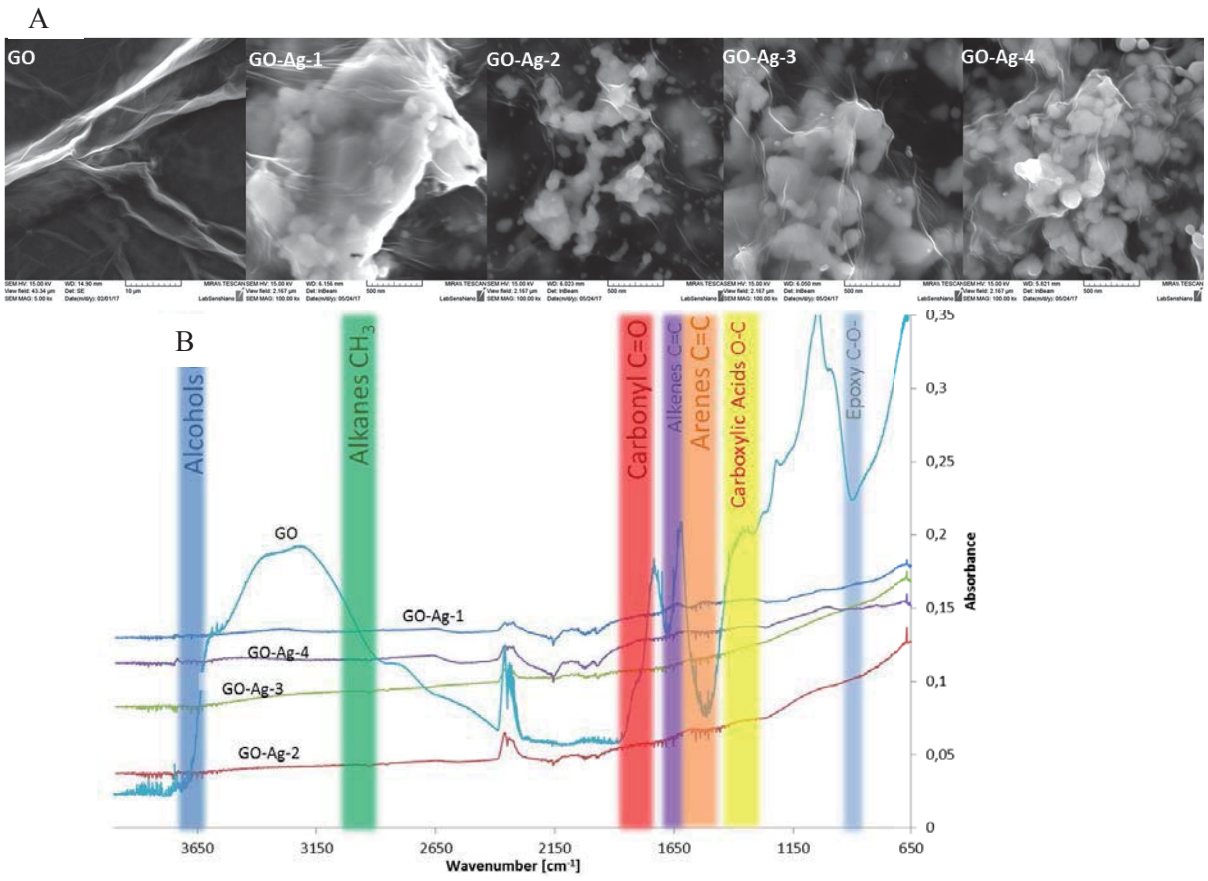
Characterization of composites

Silver was detected as Ag(I) by glassy carbon electrode, and the electrochemical signal of Ag(I) was observed at the potential +0.18 V. Based on the electrochemical determination, it can be confirmed that the silver content was significant. The silver content in individual samples is shown in Table 1.

Table 1 Determination of silver content using Differential Pulse Voltammetry

Name of sample	Concentration of Ag in sample [mg/ml]
GO-Ag-1	1.36
GO-Ag-2	1.46
GO-Ag-3	2.74
GO-Ag-4	4.08

Figure 1 Characterization of GO and composites of GO-AgNPs by using A) SEM and B) FT-IR



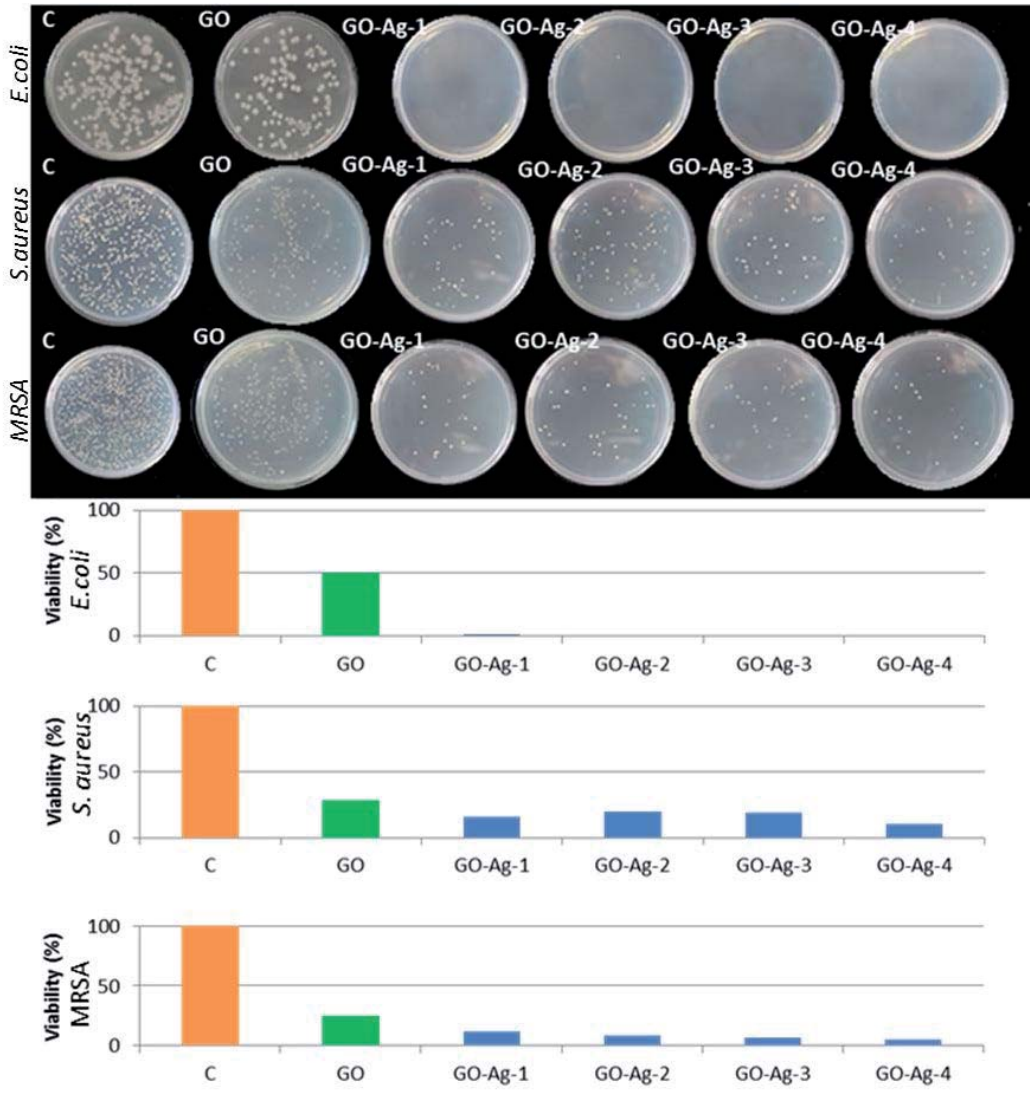
The SEM micrographs (Figure 1A) confirmed the preservation of the original structure of the large area, which remained maintained in comparison with starting material. This method also enabled determining the degree of exfoliation, which is crucial for nanoparticle character. The SEM micrographs also allowed the rating of AgNPs adhesion to GO-based carrier.

The efficiency of GO modifications with AgNPs loading was investigated by FT-IR (Figure 1B). The addition of Ag NPs to the GO resulted in an increase in C=C moiety signals in the 1500–1650 cm^{-1} region. Another increase has occurred in C=O signals in the 1700–1800 cm^{-1} region, O−H signals in the 3580−3650 cm^{-1} region, and O–C signals in the 1320 cm^{-1} region. Increase in overall intensity around 1500 cm^{-1} can be connected with the oxidation. The degree of drying may affect the occurrence and number of O−H groups. Hydrogen bonds in the 3200–3500 cm^{-1} region may be affected by degree of drying. The FT-IR analysis showed the presence of hydroxyl, carbonyl and epoxy groups on the GO sample surface.

The composites influence on pathogenic microorganisms

Antibacterial activity of GO and GO with AgNPs was determined using colony-forming capability test and expressed in terms of the colonies after incubation. GO and composites of GO with AgNPs were tested based on their antimicrobial effects on *S. aureus*, MRSA and *E. coli* strains. Effect of GO and composites of GO with AgNPs on bacterial strains is shown in Figure 2. The highest inhibitory effect after 24 hours of incubation can be seen after the addition of all composites on *E. coli* (Figure 2). All composites of GO with AgNPs had stronger antimicrobial effect against all used bacterial strains than GO itself.

Figure 2 Colony-Forming Capability Test



Legend: C – control, GO – graphene oxide, GO-Ag-1, GO-Ag-2, GO-Ag-3, and GO-Ag-4 are nanocomposites with different concentration of silver nanoparticles

CONCLUSION

In this study, the composites of graphene oxide with silver nanoparticles were synthetized and characterized by different methods and their effect on bacterial strains was tested. The composite showed inhibitory effect on three selected bacterial strains (*S. aureus; E. coli*; MRSA) and the inhibitory effect were stronger than graphene oxide itself. It can be said that combination of GO with metal nanoparticles seems like a promising way to fight against drug-resistant bacteria.

ACKNOWLEDGEMENTS

Research described in this paper was financed by Czech Ministry of Education, Youth and Sports of the Czech Republic in frame of National Sustainability Program under grant LO1401 and by the Internal Grant Agency of Mendel University in Brno (IP 20/2017). For research, infrastructure of the SIX Center was used.

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CLASSIFICATION OF ARCHAEOLOGICAL GLASS SAMPLES USING LA-ICP-MS

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Abstract: Elemental composition of glass differs based on raw material and additives used during manufacturing process, therefore elemental analysis provides substantial information from an archaeological point of view. Major, minor and trace elements in glass samples from Moldava, Kyjov and Visegrad were determined. LA-ICP-MS was used for elemental analysis. Concentrations of Na_2O, MgO, Al_2O_3, SiO_2, K_2O, CaO, TiO_2, MnO, Fe_2O_3, CoO, CuO, SnO_2, Sb_2O_3, PbO and ^{7}Li, ^{11}B, ^{45}Sc, ^{51}V, ^{52}Cr, ^{60}Ni, ^{66}Zn, ^{75}As, ^{85}Rb, ^{88}Sr, ^{89}Y, ^{90}Zr, ^{93}Nb, ^{95}Mo, ^{107}Ag, ^{111}Cd, ^{133}Cs, ^{137}Ba, ^{139}La, ^{140}Ce, ^{141}Pr, ^{146}Nd, ^{147}Sm, ^{153}Eu, ^{157}Gd, ^{159}Tb, ^{163}Dy, ^{165}Ho, ^{166}Er, ^{169}Tm, ^{172}Yb, ^{175}Lu, ^{178}Hf, ^{232}Th, ^{238}U in 44 glass samples were determined. Samples were discriminated according to finding sites using multivariate statistical methods. Elements used for discrimination (Sc, Zr, Ti, Zn, La) were determined by the Random Forrest algorithm. For archaeological sites discrimination, Primary Component Analysis (PCA) was used.

Key Words: medieval glass, LA-ICP-MS, elemental analysis, multivariate statistical analysis

INTRODUCTION

Elemental composition of glass depends on raw materials used during manufacture (Blomme et al. 2016, Kurkjian and Prindle 1998). Manufacturing process differ across time as well as being affected by the availability of materials from the local area. Therefore, elemental analysis is one of the most important techniques used for classification of archaeological glass samples. Elemental composition is also influenced by the addition of small quantities of colorants, opacifiers and clarifiers to the glass melt (Henderson 1985). This work concerns the utilisation of LA-ICP-MS for the analysis of archaeological glass samples from 3 different locations: Kyjov, Moldava and Visegrad. Measured data were statistically processed by multivariate analysis with focus on classification based on location where the glass was found.

MATERIAL AND METHODS

Characterization of glass samples

The dataset consists of 44 samples; 9 from Visegrad, 25 from Moldava and 10 from Kyjov. These samples are part of the VITREA (ARCHEOLOGICKÝ ÚSTAV AV ČR, 2011) database of archaeological glass samples. Samples found in Visegrad were dated to Post-Medieval times, whereas samples from Moldava and Kyjov are from High Medieval times. These glass samples undoubtedly belong to potassium rich silica glass type; with the average contents of Na_2O = 0.48% and K_2O = 18.73%, except for sample 1149 from Visegrad, where the content of Na_2O is highly above average (11.40%). Colour and transparency were observed as well, since they affect elemental

composition of glass based on additives used during manufacture. These properties are described in Table 1 and Table 2.

Table 1 Colour of samples from 3 different locations

Location	black	brown	colourless	dark blue	green-yel	greenish	grey-green	light green	yellowish	pink	undetermined
Kyjov	0	0	0	1	1	4	0	0	2	1	1
Moldava	1	5	9	0	0	1	1	0	1	0	7
Visegrad	0	0	0	0	0	0	0	2	0	0	7

Table 2 Transparency of samples from 3 different locations

Location	opaque	translucent	transparent	not specified
Kyjov	0	10	0	0
Moldava	7	1	12	5
Visegrad	7	0	2	0

Characterization of measurement

For the measurement, an ICP-MS spectrometer Agilent 7500ce (Agilent Technologies, Japan) with quadrupole mass analyser and ablation system UP 213 (New Wave, USA) was used. The repetition rate of laser was 20Hz, laser beam diameter 65µm, laser beam fluence 10J.cm^{-2}, and wavelength 213 nm. For each sample 5 spots (median of these values was used for statistical analysis) and 49 elements were measured. The following oxides: Na_2O, MgO, Al_2O_3, SiO_2, K_2O, CaO, TiO_2, MnO, Fe_2O_3, CoO, CuO, SnO_2, Sb_2O_3, PbO and elements: ^{7}Li, ^{11}B, ^{45}Sc, ^{51}V, ^{52}Cr, ^{60}Ni, ^{66}Zn, ^{75}As, ^{85}Rb, ^{88}Sr, ^{89}Y, ^{90}Zr, ^{93}Nb, ^{95}Mo, ^{107}Ag, ^{111}Cd, ^{133}Cs, ^{137}Ba, ^{139}La, ^{140}Ce, ^{141}Pr, ^{146}Nd, ^{147}Sm, ^{153}Eu, ^{157}Gd, ^{159}Tb, ^{163}Dy, ^{165}Ho, ^{166}Er, ^{169}Tm, ^{172}Yb, ^{175}Lu, ^{178}Hf, ^{232}Th, ^{238}U were monitored.

Statistical evaluation

A program in R was created for the automatic evaluation of an ICP-MS output file in CSV format; for quantification, total sum content normalization was used. The programming language Python was used for data treatment and statistical data evaluation.

RESULTS AND DISCUSSION

Concentrations of important elements are summarised in Table 3 and oxides in Table 4. These variables were chosen for further analysis based on the application of the Random Forest algorithm for future selection (selection of the key subset of original data in order to reduce the dimensionality). For the implementation of this algorithm Scikit-Learn Python library was used (Pedregosa et al. 2011). Applying this algorithm, a prediction model was build providing predicted importance of all variables. Variable importance is shown in Figure 1.

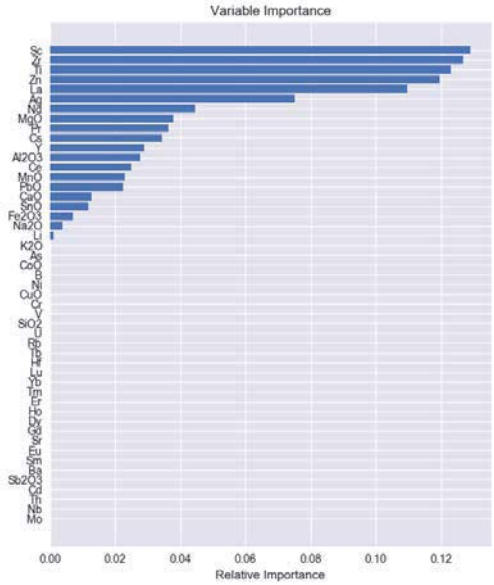
Table 3 Average concentration of important elements (mg/kg)

	Ag	Ce	Ce	Cs	La	Li	Nd	Pr	Sc	Ti	Y	Zn	Zr
Kyjov	2.10	11.86	11.86	0.19	8.11	11.93	4.43	0.34	62.55	967.10	3.60	281.70	82.50
Moldava	0.46	3.80	3.80	2.91	1.60	5.88	1.52	0.08	31.59	530.16	0.98	387.60	38.40
Visegrad	0.00	5.89	5.89	1.67	2.78	15.00	2.22	0.78	2.78	450.22	2.11	55.44	15.89

Table 4 Average concentration of important oxides (%); PbO and SnO are in mg/kg

	Al2O3	Fe2O3	MgO	MnO	Na2O	CaO	PbO	SnO
Kyjov	1.38	0.39	2.50	0.79	0.22	17.49	63.03	24.28
Moldava	0.73	0.18	2.58	0.91	0.21	12.41	68.44	7.10
Visegrad	0.89	0.38	2.13	0.62	1.50	15.03	11.00	23.78

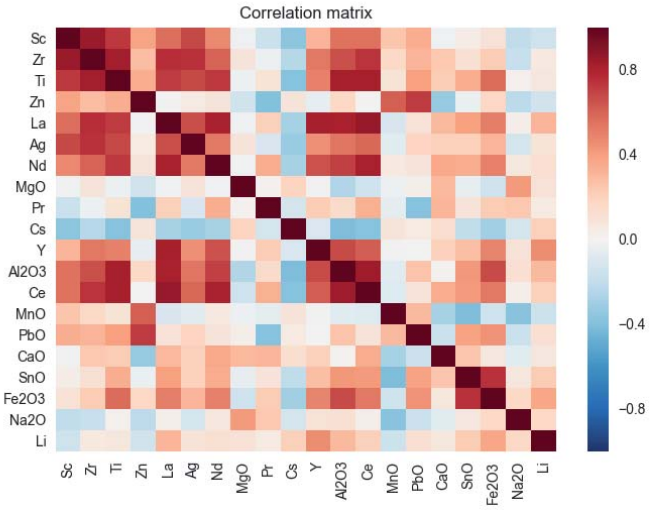
Figure 1 Variable importance calculated by the Random Forest algorithm



Statistical analysis

Initially, an exploratory analysis of the data was carried out. Only the 20 most important elements, according to variable importance, were subjected to a calculation of a correlation matrix. The correlation (Pearson, Kendall Tau and Spearman) was monitored using Pandas library (McKinney 2010). Pearson correlation matrix in Figure 2 shows positive correlation between Na_2O and MgO as well as between rare earth elements. Negative correlation can be seen for example between Fe_2O_3 and MnO; two often used colourants in glass manufacturing.

Figure 2 Pearson correlation matrix



Of the total of 49 variables only 5 of them were actually used for multivariate analysis, since the number of observations (samples) is very low (only 44). These 5 elements (Sc, Zr, Ti, Zn, La) were chosen as 5 most important variables by predictive model of Random Forest algorithm (Figure 1). Importance is calculated based on the ability of each variable to classify samples. Figure 3 shows a box-plot of these 5 elements with differences between archaeological sites. Figure 4 shows a pair-plot, where there are shown relationships between variables; both these variable values and their relationships vary by archaeological sites. From Figure 2 and 3 it is obvious that samples from Kyjov are clearly differentiated based on the content of La. The crucial difference between samples from Moldava and Visegrad lies in the content of Zn and Sc.

Figure 3 Box-plots showing concentration of Sc, Zr, Ti, Zn, La in medieval glass samples according to the archaeological sites

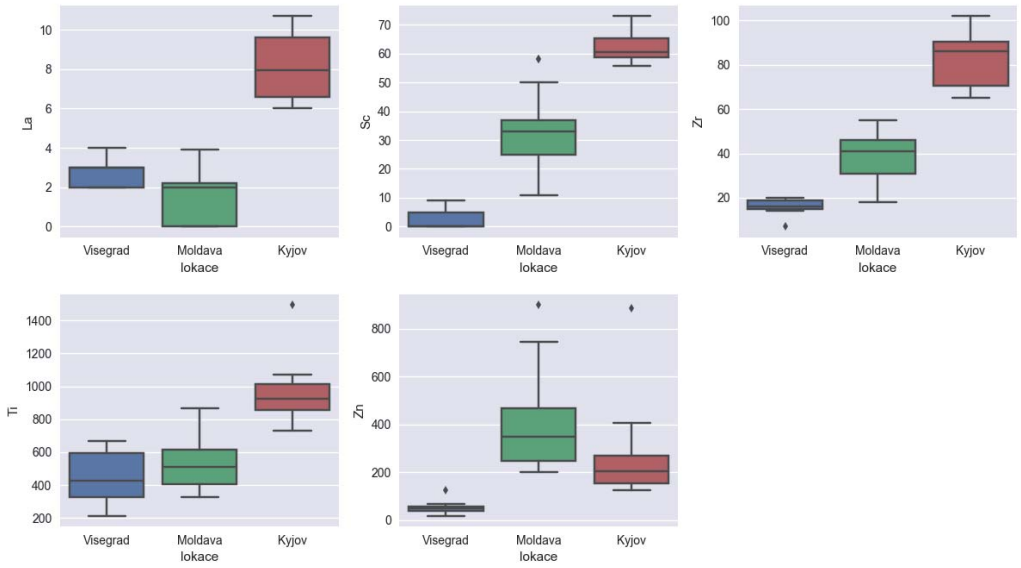
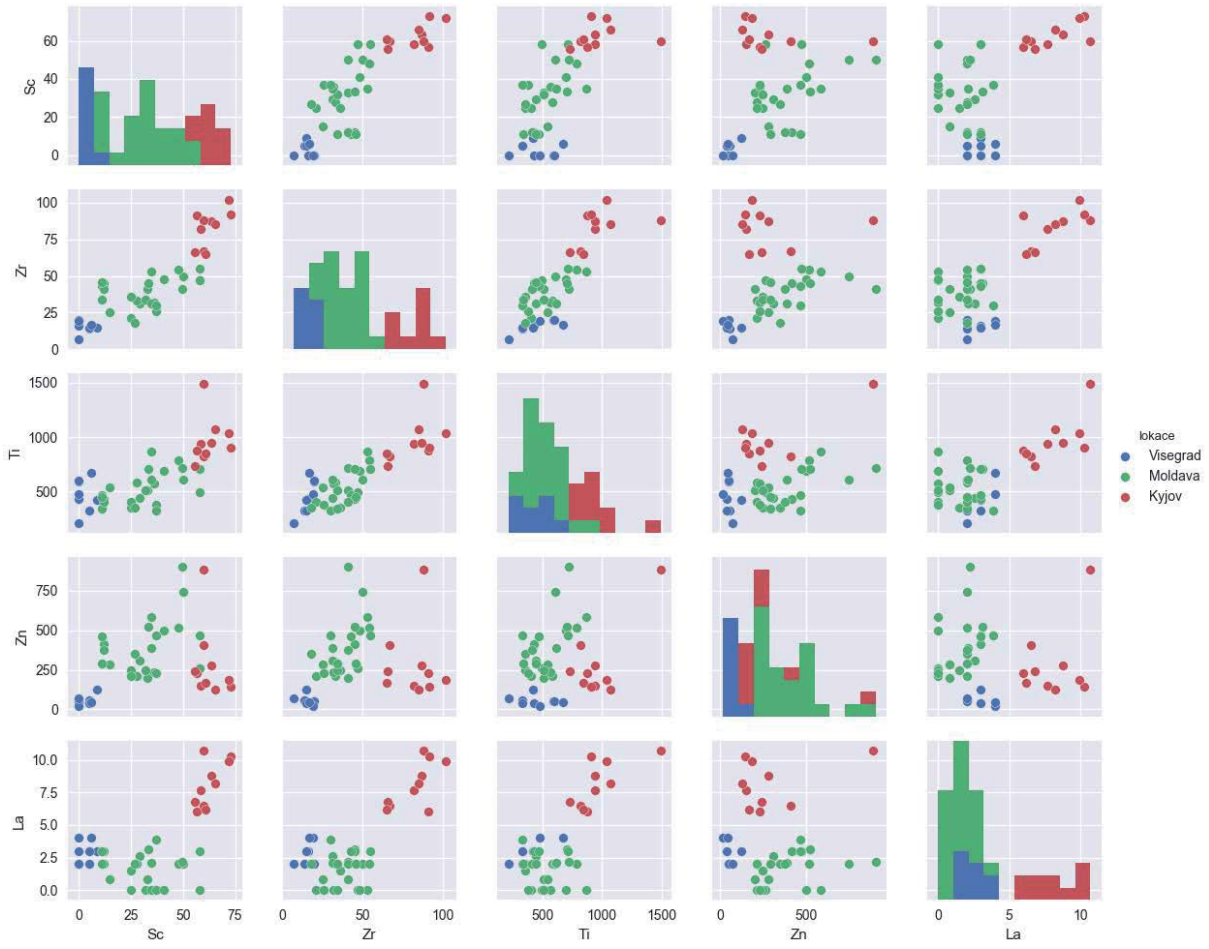


Figure 4 Pair-plot showing interactions between concentration of Sc, Zr, Ti, Zn, La in medieval glass samples according to the archaeological sites



To scale data around value 0, the Standard scaler function from Scikit-Learn Python library was used. PCA (Tipping and Bishop 1999) was carried out to investigate variance between variables. As it is shown in Figure 5, majority of variance can be expressed by first two principal components (99.61%). Based on the scree-plot, the first two components were retained. Figure 6 shows

the scatter-plot of those first two components and labels the data points based on archaeological sites. Samples from Visegrad have much lower values of the first component than samples from Kyjov. Therefore, the first principal component separates glass samples from Visegrad from those from Kyjov. Analogously, the second principal component separates those two groups from samples from Moldava.

Figure 5 Scree-plot of variance explained by principal components

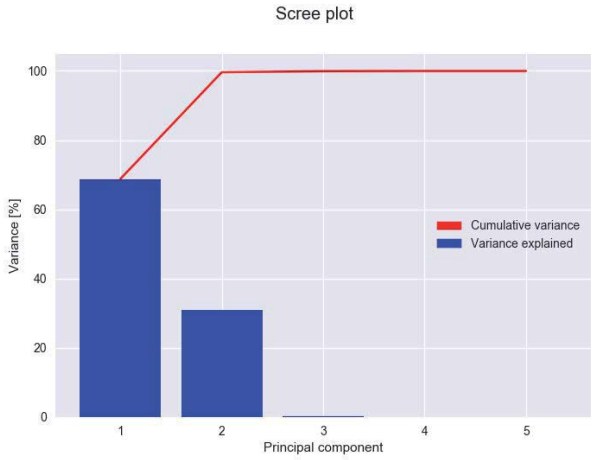


Figure 6 Scatter-plot of the first two principal components grouped by archaeological sites

CONCLUSION

By using multivariate analysis of elemental composition of medieval glass samples, it was possible to discriminate samples from different archaeological sites, regardless of the colour, transparency or type of object. 5 elements (Sc, Zr, Ti, Zn, La) were used for multivariate analysis; these elements were chosen by Random Forest algorithm.

For future research, it would be interesting to use Random Forest algorithm directly for the classification of samples.

ACKNOWLEDGEMENTS

The results of this research have been acquired within CEITEC 2020 (LQ1601) project with financial contribution made by the Ministry of Education, Youth and Sports of the Czech Republic within special support paid from the National Programme for Sustainability II funds.

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DETERMINATION OF THE CONTENT OF CAPSAICIN AND DIHYDROCAPSAICIN IN TWELVE VARIETIES OF CHILLI PEPPERS USING LIQUID CHROMATOGRAPHY WITH UV/VIS DETECTION

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Abstract: The aim of the experiment was to determine the content of capsaicin and dihydrocapsaicin in twelve varieties of chilli peppers (Brown Bhutlah II, Bhut Jolokia Yellow I, Pieto de Moca II, Trinidad 7 POT II, 7 POT White II, Naga Jolokia I, Habanero Orange, Naga Viper II, Bhut Jolokia II, Jalapeno, White Naga Bhut Jolokia I, Carolina Reaper II) using high performance liquid chromatography UltiMate 3000 with UV/VIS detector. Each variety of chilli peppers was prepared without seeds and partitions (septa) and with seeds and partitions (septa) in order to compare yields of capsaicin and dihydrocapsaicin depending on the sample preparation. Based on the experiment, it was shown that chilli peppers with seeds and partitions (septa) had a higher content of capsaicin and dihydrocapsaicin compared to chilli peppers without seeds and partitions (septa). The highest capsaicin and dihydrocapsaicin content was of chilli peppers Carolina Reaper II. From the contents of capsaicin and dihydrocapsaicin was calculated so-called pungency in Scoville units (SHU) in order to compare our results with previous studies.

Key Words: capsaicin, dihydrocapsaicin, high performance liquid chromatography, UV/VIS detection

INTRODUCTION

Chilli peppers are the pungent fruits from plants of genus *Capsicum* in the nightshade family, *Solanaceae*, including 27 wild species and 5 main domestic species: *Capsicum frutescens*, *Capsicum pubescens*, *Capsicum chinense*, *Capsicum annuum*, *Capsicum baccatum* (Gurnani et al. 2016, Nickels 2015). The fruits are commonly used to give a pungent or hot sensation too many different dishes and food products all around the world (Duelund and Mouritsen 2017). The chilli peppers can be used also to produce chilli oil or chilli resin also known as oleoresin (Fernandez-Ronco et al. 2011).

The compounds responsible for the pungency of the chilli peppers belong to group of secondary metabolites known as capsaicinoids. Two major capsaicinoids found in chillies are capsaicin and dihydrocapsaicin. Capsaicin and dihydrocapsaicin represent about 77–98% of total capsaicinoids content in peppers. Other minor capsaicinoids found in chillies are nordihydrocapsaicin and homocapsaicin (Duelund and Mouritsen 2017, Sarpras et al. 2016). Capsaicin was discovered by P. A. Buchholtz in 1816. In 1846 L. T. Tresh isolated the capsaicin in a crystalline state and it was him who named the pungent substance capsaicin (DeWitt and Bosland 2009). Dihydrocapsaicin was described in 1957 (De 2004). Capsaicin is a very stable alkaloid, which is stable under exposure to heat and cold, has no flavour, colour, aroma and is insoluble in water, but easily soluble in fat or some organic solvents (DeWitt and Bosland 2009). Capsaicin is known for his pharmacological, neurological and dietetic effectiveness. It has also significant antibiotic activity and the ability to reduce the cholesterol level in blood (Gurnani et al. 2016). Capsaicin is also capable to kill some types of cancer cells (Anandakumar et al. 2013, Dawan et al. 2017, Prasad et al. 2008) and provide

relief in arthritis and respiratory ailments (Prasad et al. 2008), it is also antioxidant and has anticancer, antiarthritic and analgesic properties (Dawan et al. 2017, Prasad et al. 2008).

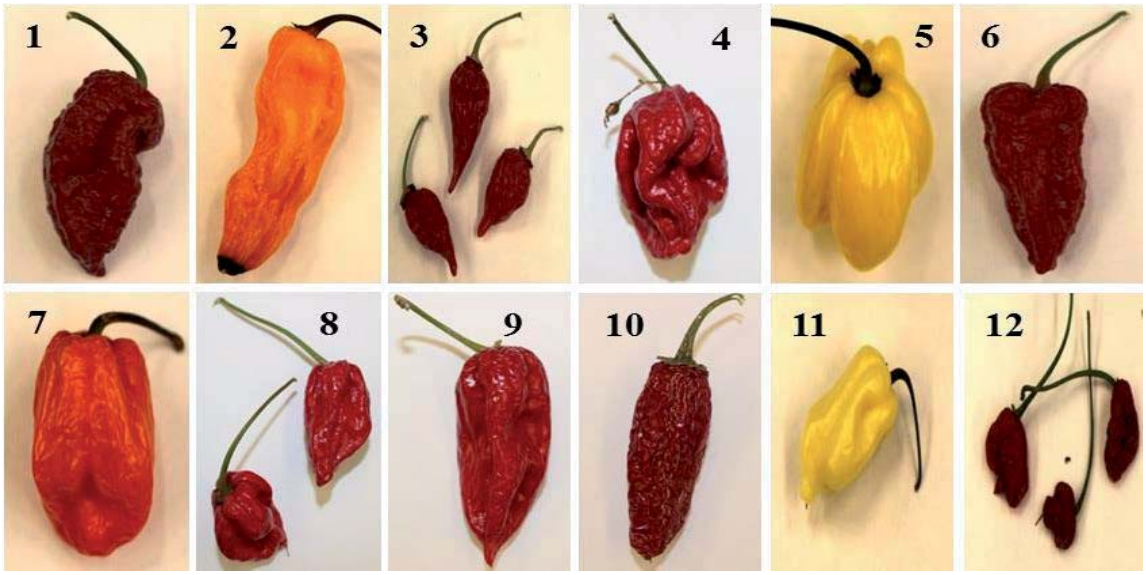
The capsaicin biosynthesis involves two pathways, i.e. the phenylpropanoid pathway, which provides the precursor phenylalanine for the formation of vanillylamine, and the valine pathway which provides the precursor valine for 8-methyl-6-nonanoic acid. Capsaicin is biosynthesized by capsaicin synthetase (CS) through condensation of products of the phenylpropanoid and valine pathways (Prasad et al. 2008). Capsaicinoids accumulation is found specifically in the epidermal layer called dissepiment of the placental tissue (Kybal 1988, Prasad et al. 2008, Sarpras et al. 2016).

The amount of capsaicinoids in chilli peppers depends on variety, age, degrees of maturity, season and agronomic conditions. Generally, in the bigger fruits there is less content of capsaicinoids than in the smaller fruits. Drying affects the content of capsaicinoids (Maradova 2015). Between the colour and amount of chillies there is no relationship (Nickels 2015). The pungency of chilli peppers is expressed by Scoville Heat Units (SHU). Scoville scale was invented by scientist Wilbur Lincoln Scoville in 1912 when he used the organoleptic test. In this test, a certain amount of chilli is extracted with ethanol, which then is diluted repeatedly until the pungent sensation no longer can be detect on the tongue. The number of times the extract has to be diluted is then taken as the pungency in units of SHU (Bosland and Votava 1999, Nickels 2015). Currently the content of capsaicinoids in chilli peppers is measured by HPLC (high-performance liquid chromatography) or GC (gas chromatography) (Duelund and Mouritsen 2017).

MATERIAL AND METHODS

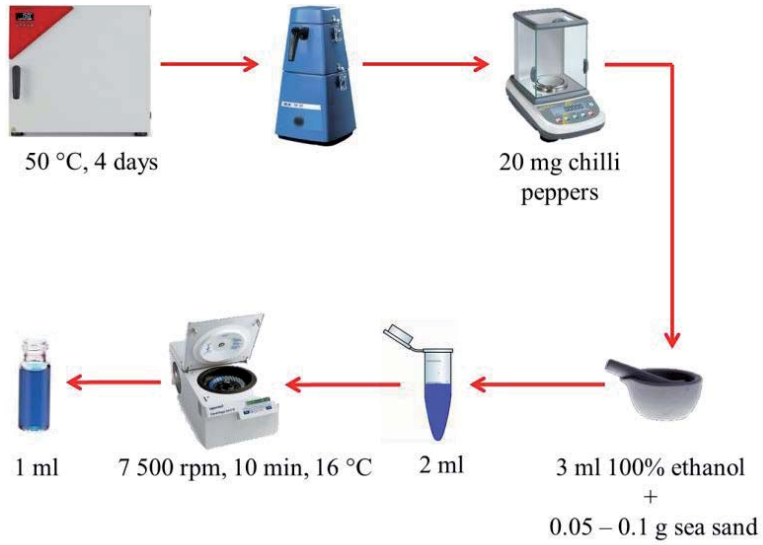
In this experiment twelve varieties of chilli peppers were used (Figure 1).

Figure 1 Varieties of chilli peppers: (1) Brown Bhutlah II, (2) Bhut Jolokia Yellow I, (3) Pieto de Moca II, (4) Trinidad 7 POT II, (5) 7 POT White II, (6) Naga Jolokia I, (7) Habanero Orange, (8) Naga Viper II, (9) Bhut Jolokia II, (10) Jalapeno, (11) White Naga Bhut Jolokia I, (12) Carolina Reaper II.



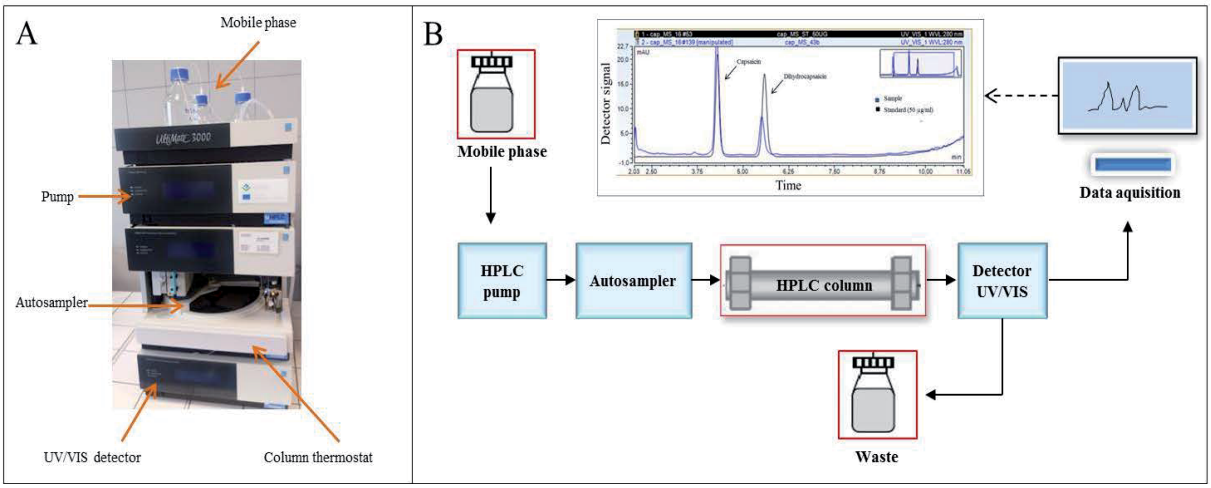
Sample preparation is described in Figure 2. At first, chilli peppers were divided into two parts. The first part of samples has got rid of seeds and partitions whereas in the second part of samples seeds and partitions were preserved. These samples were dried 4 days at 50 ºC (Drying and heating chamber BINDER ED 56, Verkon, Czech Republic). After drying the samples were milled (IKA M20 Universal mill) and from each sample was weighted 20 mg (Analytical Balance EP 240A, Precisa, Czech Republic). Then weighted samples were subsequently homogenized with 3 ml 100% ethanol and 0.05–0.1 g sea sand in friction bowl. Then homogenate was transferred to the microtube (Eppendorf, Germany) and centrifuged at 7 500 rpm at 16 ºC for 10 min. Finally, 1 ml of supernatant was collected from each sample to glass vials (Chromservis s. r. o., Czech Republic).

Figure 2 Scheme of preparing samples of chilli peppers for HPLC analysis.



The capsaicin and dihydrocapsaicin was determined by HPCL UltiMate 3000 (ThermoFisher Scientific, Waltham, USA) with UV/VIS detector (UltiMate 3000 Variable Wavelength Detector). The scheme of high performance liquid chromatograph is shown in Figure 3.

*Figure 3 (**A**) HPLC UltiMate 3000 (**B**) Scheme of high performance liquid chromatograph with UV/VIS detection.*



For the separation of capsaicin and dihydrocapsaicin, the column HYPERSIL GOLD DIM with dimensions 150 x 4.6 mm and a particle size of 5.0 µm was used. The column was equilibrated at 30 °C. Mobile phase A was 0.2% acetic acid and mobile phase B was 100% methanol. Flow rate of mobile phase was 1.0 ml/min. The compounds were eluted with a linear upward gradient: 0–6 min (70% B) 6–9 min (70–100% B) 9–12 min (70% B). The separated substances were detected at a wavelength of 280 nm. Calibration curves for contents of capsaicin and dihydrocapsaicin were linear from 1.00 to 500.00 µg/ml, with correlation coefficients of 0.9989 for both capsaicinoids of chilli peppers without seeds and partitions and 1.00 for both capsaicinoids of chilli peppers with seeds and partitions. All used chemicals were purchased from Sigma-Aldrich (St. Louis, USA). Capsaicinoids contents were re-calculated to Scoville heat units (SHU) by multiplying the pepper dry weight capsaicinoids concentration in parts per million (ppm) by the coefficient of the heat value for each compound. The coefficients are 16.0 for both capsaicin and dihydrocapsaicin.

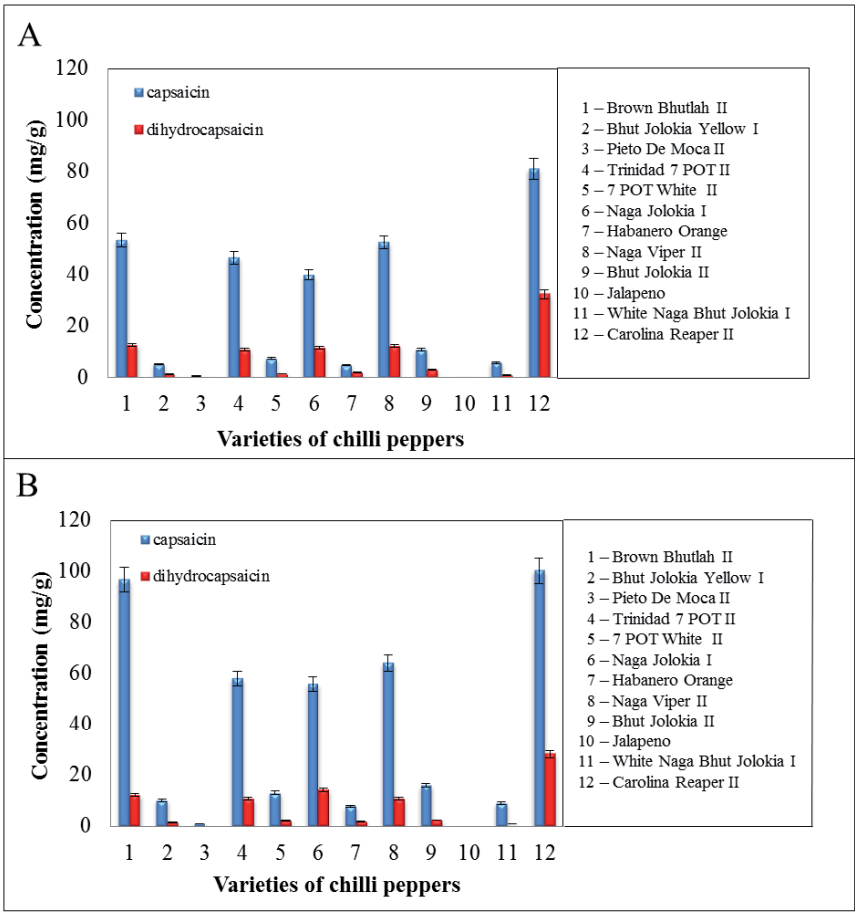
Statistical analyses

Content of capsaicin and dihydrocapsaicin in twelve varieties of chilli peppers were made using standard deviation from three determinations.

RESULTS AND DISCUSSION

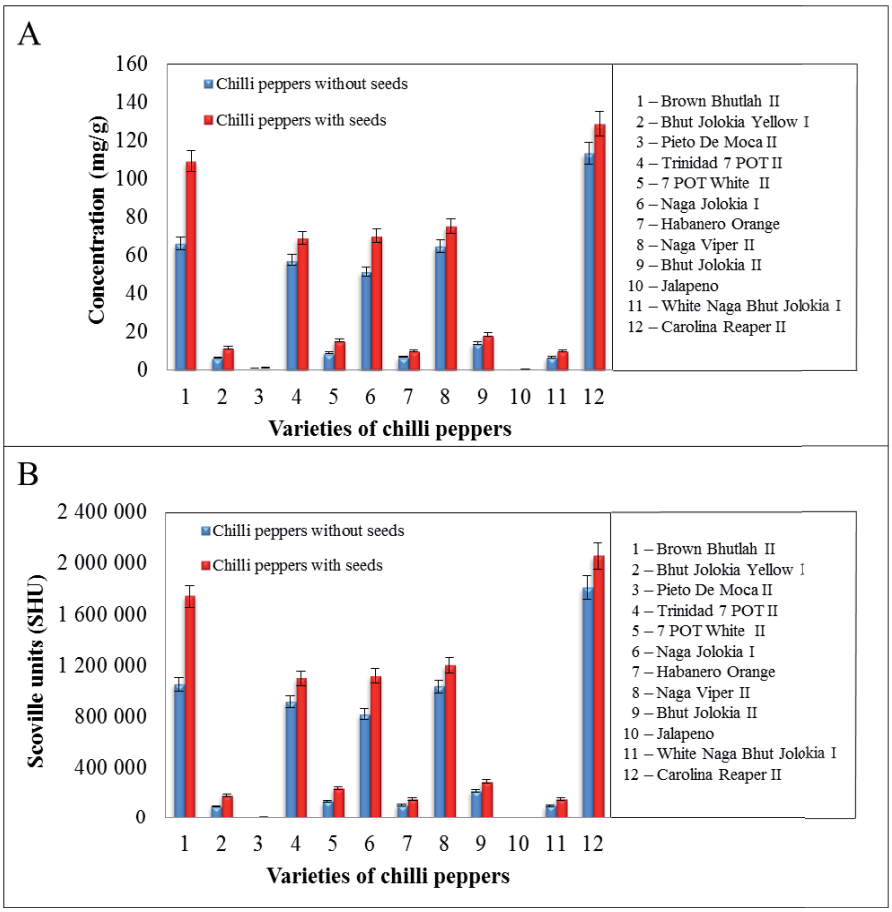
Determination of the presence and content of capsaicin and dihydrocapsaicin were done by high performance liquid chromatography with UV/VIS detection. The results have been recalculated per 1 g of chilli pepper.

*Figure 4 Determination of capsaicin and dihydrocapsaicin (**A**) for the extract of chilli peppers without seeds and partitions, (**B**) for the extract of chilli peppers with seeds and partitions.*



For chilli peppers without seeds and partitions (Figure 4A) we found the highest content of capsaicin (81 ± 4 mg/g) and dihydrocapsaicin (32 ± 2 mg/g) in Carolina Reaper II. The lowest content was in Jalapeno (0.03 ± 0.002 mg/g capsaicin and 0.03 ± 0.002 mg/g dihydrocapsaicin). The contents of capsaicin and dihydrocapsaicin in chilli peppers with seeds and partitions are shown in Figure 4B. The highest content of capsaicin (100 ± 5 mg/g) and dihydrocapsaicin (28 ± 1 mg/g) in chilli peppers with seeds and partitions also was in Carolina Reaper II. The content of capsaicin has increased in all chilli peppers with seeds and partitions whereas the content of dihydrocapsaicin was very various. The content of dihydrocapsaicin slightly increased in four varieties of chilli peppers with seeds and partitions (Bhut Jolokia Yellow I, 7 POT White II, Naga Jolokia I, Jalapeno), in rest of varieties we found less dihydrocapsaicin except Jalapeno. Jalapeno had same level of dihydrocapsaicin in chilli pepper with seeds and without seeds and partitions. Similar results of content of capsaicin and dihydrocapsaicin in Jalapeno were reported in study of (Pena-Alvarez et al. 2009) where, was used different sample preparation and method of determination.

*Figure 5 Determination of total capsaicinoids (**A**) and determination of Scoville units (**B**).*



In Figure 5A there is shown the concentrations of total capsaicinoids (capsaicin + dihydrocapsaicin) in chilli peppers with seeds and partitions and without seeds and partitions. From the figure 5A it is obvious the total content of capsaicinoids was higher in chilli peppers with seeds and partitions than in chilli peppers without seeds and partitions. The biggest difference was in Jalapeno where the content of total capsaicinoids increased by 739%. The least variance was in varieties of Carolina Reaper II (13%) and Naga Viper II (16%). For the remaining varieties, capsaicinoids increased in range from 20 to 84%. From the contents of capsaicinoids was calculated so-called pungency in Scoville units (SHU) (Figure 5B). Recently study (Duelund and Mouritsen 2017) found the pungency of Habanero 247 000 ± 24 000 SHU and Carolina Reaper 1 046 000 ± 34 000 SHU. We found a pungency of Habanero Orange with seeds and partitions 157 837 ± 7 892 SHU and Carolina Reaper II 2 056 026 ± 102 800 SHU.

CONCLUSION

Based on the results of measurement of capsaicin and dihydrocapsaicin we can conclude, that the total content of capsaicinoids depends on a sample preparation, whether extract is prepared from chilli pepper with or without seeds and partitions. The amount of capsaicinoids in chilli peppers also depends on variety, age, degrees of maturity, season and agronomic conditions. The highest concentration of capsaicin and dihydrocapsaicin was determined in Carolina Reaper II so it means it was also the most pungent chilli pepper.

Our future aim will be to test other sample preparation and monitor the change in the concentration of capsaicin and dihydrocapsaicin.

ACKNOWLEDGEMENT
This research was carried out under the project CEITEC 2020 (LQ1601) with financial support from the Ministry of Education, Youth and Sports of the Czech Republic under the National Sustainability Programme II.

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SPECTRAL ANALYSIS OF HUMAN NOREPINEPHRINE TRANSPORTER HOMING PEPTIDES

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Abstract: UV spectrometry is very simple and cheap method for quantitative and qualitative analysis of compounds. Furthermore, it provides intricate information about the bonded π-electron transitions and also non-bonded n-electron transitions. The aim of this work was to identify electron transition bands in two homing peptides of the human norepinephrine transporter (hNET); namely: GASNGINAYL (978 Da) and SLWERLAYGI (1206 Da). Electron transition bands directly indicate structural conformations, particularly the ones associated with double bonds, i.e. conjugated π-bonds of aromatic and peptide bonds. The results show unusual spikes in absorbance in the far UV at low temperature for GASNGINAYL and even more at other temperatures for SLWERLAYGI peptide. The latter supports the hypothesis of a stacking between tyrosine and tryptophan resulting in helix structure. Infrared spectrometry also showed abundant helix structure in SLWERLAYGI but less abundant in GASNGINAYL peptide. Based on π-stacking, an UV spectrometry method can be developed to monitor the helicity of some peptides, such as SLWERLAYGI.

Key Words: UV spectrometry, peptide, tyrosine, tryptophan, targeted therapy

INTRODUCTION

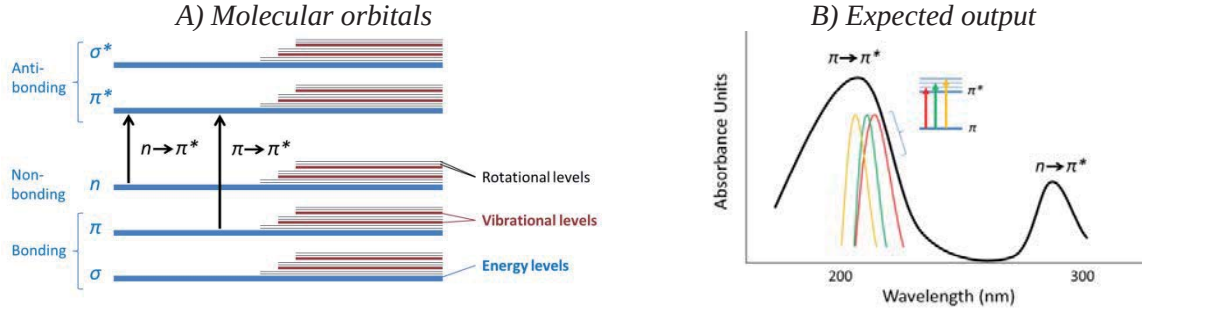
The spectral analysis of amino acids and polypeptides in the UV region was intensively studied more than fifty years ago (Ham and Platt 1952, Wetlaufer 1963, Nielsen and Schellman 1967). UV-Vis spectrometers are used to analyse absorbance of photons that excite electrons to jump from the non-bonding and bonding orbitals to anti-bonding orbitals (Figure 1). Electrons of the π-bond (in double bonds) and free electrons (in the n-orbitals) exhibit unique and quantitative band fingerprints for each molecular structure and in the presence of different solvents, pH and temperature.

The peptide group –CONH– contains four π-electrons and free electrons on the oxygen atom. The energies of the two π-orbitals occupied by electron pairs are -15.04 and -12.68 electron volts (eV). The nonbonding n-orbital at -12.63 eV and the free anti-bonding orbital at +1.24 eV (Simon 1976). Aromatic amino acids such as tryptophan and tyrosine are rich in conjugated π-electrons, and thus have direct influence on absorbance in the UV-Vis region.

Kuipers and Gruppen studied the absorbance of peptide bond at 214 nm (Kuipers and Gruppen 2007). Their findings showed a molar extinction coefficient (ε) of 923 1/M.cm for the peptide bond. This parameter, which is specific for each wavelength, describes how strongly one molar concentration of peptide bond can absorb photons in 1 cm light-path. At 214 nm, tryptophan had highest absorptivity (ε = 29050 1/M.cm). Tyrosine, phenylalanine and histidine reported similar absorptivity (ε = 5375, 5200, and 5125 1/M.cm, respectively) at that wavelength, whereas methionine had similar ε to that of the peptide bond (ε = 980 1/M.cm). Proline had three times higher ε when it is at the *N*-terminus (ε = 2675 1/M.cm) but yet negligible ε inside the peptide chain. Several studies also investigated the peptide bond absorptivity at 205 nm (Scopes 1974, Anthis and Clore 2013). Anthis

and Clore showed ε = 2780 1/M.cm for the peptide bond (Anthis and Clore 2013). They also reported ε for tryptophan (ε = 20400 1/M.cm), phenylalanine (ε = 8600 1/M.cm), tyrosine (ε = 6080 1/M.cm), histidine (ε = 5200 1/M.cm) and other amino acids.

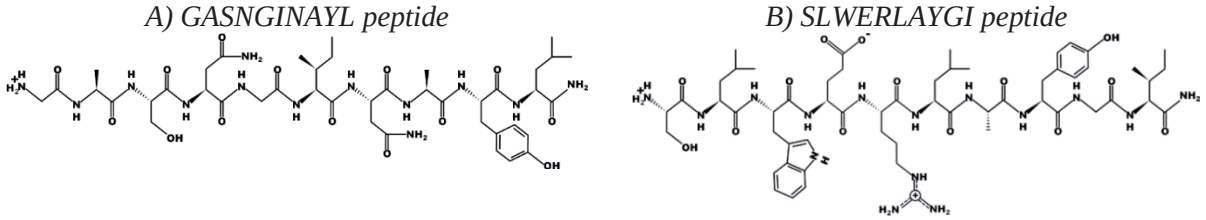
Figure 1 Molecular orbitals and electron transitions detectable via UV-Vis spectrometry



Calculation of sequence-specific ε values for a wide range of wavelengths in the UV spectra can give accurate estimations on the contribution of residues and peptide bonds to spectral bands. While this is applicable for quantitation of large proteins, UV spectrometry can also be used to give insight on structural changes in relatively small peptides of known sequence.

In this study, two homing peptides (shown in Figure 2) are analysed by spectrometry to shed the light on their structural properties. Our objective is to extract structural information from UV spectrum that can help in development of these peptides. Both peptides were derived from α-helix regions in hNET and were shown to have α-helix secondary structure *in silico* (Haddad et al. 2016). hNET protein is one of the most promising therapeutic targets in Neuroblastoma (Matthay et al. 2012). Development of homing peptides for hNET is a new strategy that can replace radio-therapeutic targeting of hNET, and ease the suffering of patients.

Figure 2 Norepinephrine transporter homing peptides structures at pH = 7



MATERIAL AND METHODS

Peptide Synthesis

Peptides were synthesized on Liberty Blue synthesizer (CEM, NC, USA) and stock solutions of 1 mM were prepared in ACS water.

UV-Vis Spectrometry

Specord S600 spectrometer (Analytik Jena AG, Germany) was used for analysis. Temperature of the spectrometer chamber was controlled manually using JUMO dTRON 308 regulator (Analytik Jena AG, Germany). Quartz cuvettes of 1 cm path were used (Hellma, UK). Samples were equilibrated at each temperature for three minutes prior to each reading. ACS water was used as reference. Spectra, measured at 0.5 nm resolutions, were normalized at 280 nm point for peptides at each temperature separately. Peaks were identified manually as highest point(s) with one or two shoulders.

Attenuated Total Reflectance Fourier Transform-Infrared Spectroscopy (ATR-FT-IR)

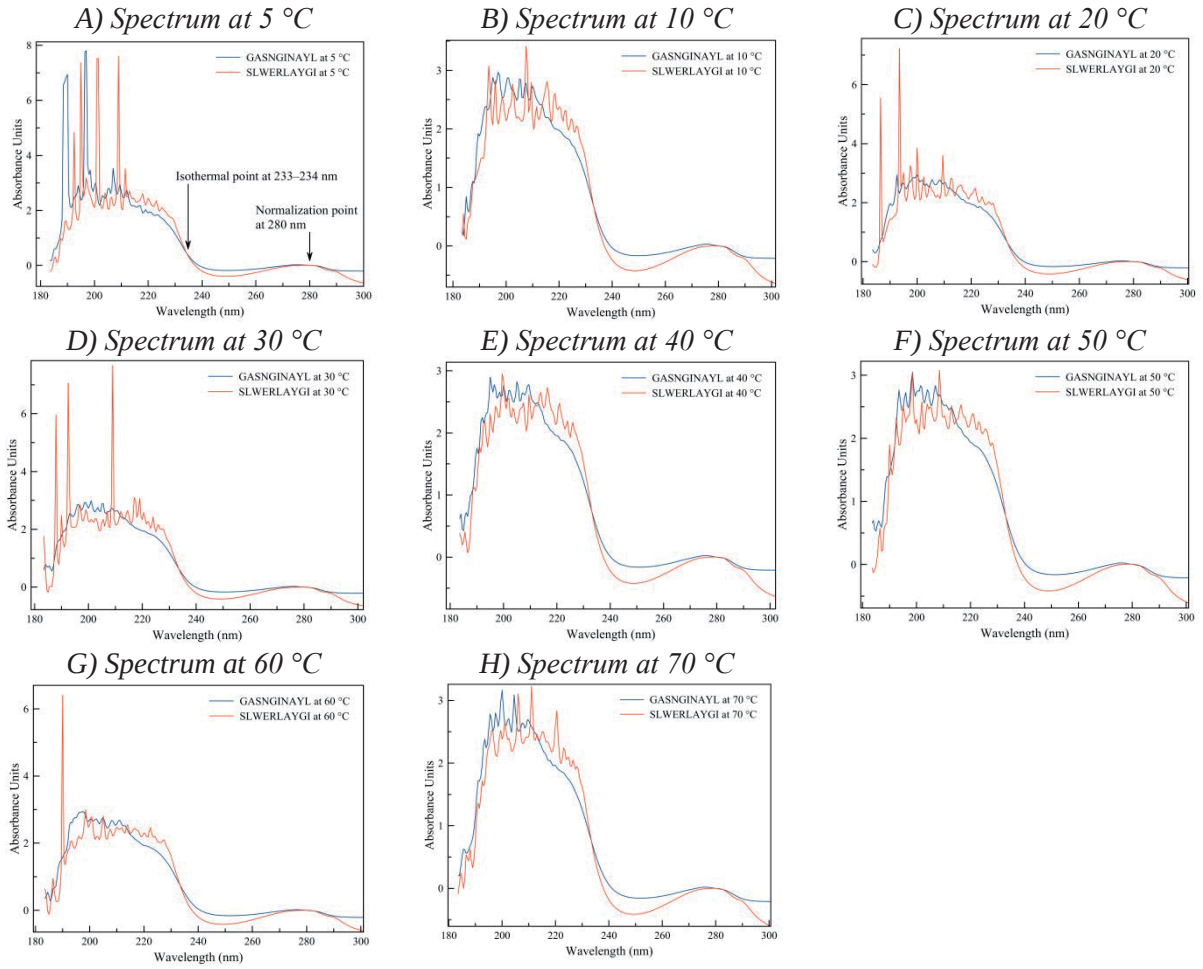
FT-IR spectra were collected using a Nicolet iS10 FT-IR spectrometer with attenuated total reflection (ATR) attachment (Thermo Fisher Scientific, USA), equipped with diamond crystal. Spectra were recorded at 25 °C from 4000 to 650 1/cm at a resolution of 2 1/cm. Each spectrum was acquired by merging 128 interferograms. Peptide samples were directly analysed in lyophilized form.

RESULTS AND DISCUSSION

The double bonds, particularly conjugated double bonds in aromatic structures and peptide bonds, are rich of π-electrons that can be excited in the near and far UV spectrum. The near and far UV spectrum can be used to identify overlapping yet sometimes distinguishable π→π*, n→π* and other electronic transitions. Indeed, the overlapping peaks (as predicted in Figure 1B) around the 220 nm can be attributed to n→π* transition that overlaps the π→π* region in addition to other hidden electronic transitions (Wetlaufer 1963). The shoulder of this peak is slightly red shifted in SLWERLAYGI peptide due to larger number of double bonds in its structure when compared to GASNGINAYL peptide (Figure 3). Researchers use different spectrometric methods to resolve the type of electron transition, such as UV-Vis spectrometry analysis in presence of different solvents, pH titration and substitution with different chemical groups.

The UV spectral peaks for GASNGINAYL peptide at different temperatures are summarized in Table 1. There are several "saturated" spikes in the spectrum particularly at low temperature (Figure 3A). The spikes correspond to 6.61–6.53 eV and 6.33–6.29 eV energy bands at 5 °C (Table 1). We believe these spikes belong to the aggregation and stacking of the tyrosine aromatic rings in the peptides. The fact that these spikes are in the far UV region suggests that they are a result of π→π*, however further confirmation is required.

Figure 3 UV Spectra of the hNET homing peptides



The UV spectral peaks for SLWERLAYGI peptide are summarized in Table 2 at different temperatures. This peptide contains an additional aromatic residue (i.e. tryptophan). As expected, more saturated spikes were identified in low temperature (Figure 3A). The spikes correspond to 6.44–6.42 eV, 6.36 eV, 6.18–6.15 eV and 5.93–5.88 eV energy bands at 5 °C (Table 1). Surprisingly, the spikes do not disappear at higher temperatures (Figure 3C, 3D and 3G). Energy bands of these spikes at 20 °C are 6.68–6.65 eV and 6.42–6.41 eV. And at 30 °C the energy bands

for spikes are 6.61–6.56 eV, 6.44–6.42 eV, and 5.93–5.88 eV. Another spike appears again at 60 °C in the energy band 6.56–6.47 eV. These results demonstrate that the tyrosine and tryptophan stacking is rather a phenomenon inside the peptide and not between peptide aggregates. Unfortunately, we have no data on the spectral behaviour of individual tyrosine or particularly tryptophan under different temperatures. Indeed, tyrosine lost these spikes at higher temperatures; however, it was not possible to know if tryptophan alone exhibits more excited electrons at higher temperature. Esfandiary et al. reported gradient shift in the peaks of aromatic amino acids in the 250–300 nm region that correlate with change in temperature, however such shifts did not exceed 1nm in 10–60 °C range (Esfandiary et al. 2009).

Table 1 Spectrum peak wavelengths (nm) for GASNGINAYL peptide at different temperatures

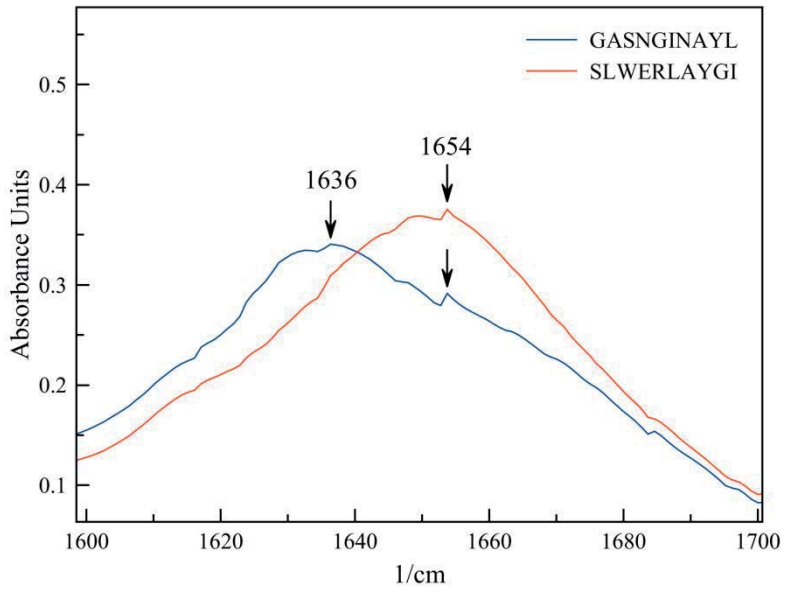
Energy (eV)	70 °C	60 °C	50 °C	40 °C	30 °C	20 °C	10 °C	5 °C
6.76–6.72		184.5	184	184	184	183.5	183.5	
6.70–6.65	185.5–186	186.5	186	186	185.5		185	185
6.61–6.53					188.5		187.5	188.5–190
6.56–6.47	191	190.5	189–189.5	190	191–191.5	190	189.5	
6.46–6.44				192		192.5		
6.46–6.41	193.5	193	193.5	193.5	193–193.5		192–192.5	192.5–193
6.39–6.33	195.5	194.5	195.5–196	195		194.5–195	195	194
6.33–6.29				196.5	196–196.5			196.5–197
6.33–6.23	197.5	196–197.5	198.5	198.5	198.5–199	198.5–199	197–197.5	198.5
6.20–6.18	200	200–200.5	200.5	200		200		200.5
6.18–6.17					201		200.5–201	
6.15–6.12	202.5	202–202.5	201.5–202	202–202.5		201.5–202	202	
6.11–6.09					203.5	203.5		203
6.08–6.03	204.5	204.5–205	204–204.5	205–205.5	205–205.5	205.5	205–205.5	205–205.5
5.99–5.98			207	207–207.5	207		207–207.5	207
5.98–5.93	207.5–208	208–208.5	208.5		208.5–209	208–208.5		
5.93–5.88	209.5–210	210.5–211	211	209–209.5	210.5	210–210.5	210	209.5
5.85–5.81		213	213	212.5–213	212.5–213	212	213–213.5	212
5.78								214.5
5.77–5.69	218			216–216.5	216.5	216.5–217	215–216	216.5–217
5.67–5.66						218.5–219	218.5–219	219
5.65–5.61	219.5–220	220		219.5–220	220	220.5	220.5–221	
5.60–5.55	223		221.5	221.5–222.5	222.5	223–223.5	223.5	223–223.5
5.51–5.50							225–225.5	

ATR-FTIR is a useful method for analysing vibrational frequency of different amide modes. Amide I is one of the most widely used mode in protein structural studies (Adochitei and Drochioiu 2011). Based on infrared spectra of many proteins, bands in the range 1650–1658 1/cm were found to be correlated with α-helix secondary structures while bands in the range 1620–1640 1/cm correlated with β-sheet secondary structures (Haris and Chapman 1992). Another study indicated that the β-sheet range was between 1621 and 1640 1/cm followed by random coil region at 1641–1647 1/cm, helix at 1648 1/cm, turns and bends at 1658–1696 1/cm which is also separated by a β-sheet gap at 1671–1679 1/cm (Wilson et al. 2000). In this study, the amide I band showed a predominant peak in the α-helix range for SLWERLAYGI peptide as expected (Figure 4). Surprisingly, GASNGINAYL peptide showed major amide I peak at the β-sheet range, in addition to a minor spike in the α-helix range. It is unclear if this was a direct result of *C*-terminal amidation during peptide synthesis. The α-helix conformation of SLWERLAYGI peptide allows for stacking of tyrosine and tryptophan which can explain the unusual $\pi \rightarrow \pi^*$ transitions peaks in UV spectra (Figure 3A, 3C, 3D, and 3G). Similar saturated peaks for UV spectra of GASNGINAYL peptide occurs only at 5 °C, possibly due to aggregation. The role of π-stacking is well documented in protein structures. The most common form is an off-centred parallel stacking of the aromatic rings, followed by less common T-shaped stacking (McGaughey et al. 1998).

Table 2 Spectrum peak wavelengths (nm) for SLWERLAYGI peptide at different temperatures

Energy (eV)	70 °C	60 °C	50 °C	40 °C	30 °C	20 °C	10 °C	5 °C
6.76–6.72	184.5	183.5		183.5	183.5		184	
6.68–6.65	186.5	186.5	186.5	185.5		186.5	186–186.5	185.5
6.61–6.56	188		189	188.5–189	188		188.5	187.5
6.56–6.47	191	190	190	191	190		190.5–191.5	189–189.5
6.44–6.42	192.5		192.5	192.5–193	192.5			192.5
6.42–6.41		193–193.5				193.5	193.5	
6.36			195	195		195		195
6.33–6.28	196–196.5	196		197	196.5–197	197.5	196	197
6.25		198.5	198.5				198.5	
6.23–6.20	199.5		200	199.5	199	200		
6.18–6.15	201	200.5–201			201			201–201.5
6.15–6.11	202.5	202.5–203	202	201.5–202	202.5–203	202.5	202.5	
6.09–6.08			204	203.5				204
6.06–6.00	206	205	205		205.5	204.5		206–206.5
6.00–5.98		207.5		206.5–207	207			
5.98–5.95			208.5	207.5			207.5	
5.93–5.88	211	210	210.5	209.5	209	209.5	209.5	209
5.86–5.82	213	212		212	212	212.5–213	211.5	211.5
5.82–5.78		213.5–214	213	213.5–214	214.5			213
5.78–5.70	215–215.5	216.5	216.5	216.5	217–217.5	216	215.5	214.5
5.70–5.66			217.5–218		219		218–218.5	218
5.64–5.60	220.5	220–220.5	220.5–221	221	220.5	221.5	220–220.5	220
5.58–5.55	223–223.5	222.5	223–223.5		222–222.5		222–222.5	223
5.54–5.51	224.5–225	224		224	224	224	224.5–225	225
5.50–5.46	226.5		225.5–226	226	226	226.5	226.5–227	
5.46–5.41	228	227–227.5	228		227.5	228		228–229

Figure 4 ATR-FTIR Spectrum of amide I band for the hNET homing peptides



Circular dichroism (CD) can be used to directly separate the bands in the UV spectra via polarization of light beams. CD spectrometry is the key method for determination of secondary structure in peptides and proteins. More insights on the structure of peptides can be gained by application of molecular dynamic computations as well as wet lab techniques such as Raman spectrometry and nuclear magnetic resonance (NMR). Detailed analysis of the UV spectrum can be done using mathematic derivatives to accurately point the peaks and curvature of spectrum curve (Kus et al. 1996) or the use of deconvolution to resolve hidden peaks (Antonov and Stoyanov 1993).

CONCLUSION

UV spectral analysis provides insights on peptide conformations and structure based on knowledge of electron transitions between molecular orbitals. Using peptide UV spectra at different temperatures, we identified over 25 bands that correspond to peaks in the 185–230 nm range. Also, we highlight some bands that might correspond to π-stacking of tyrosine and/or tryptophan and can be used to monitor the helicity of peptides such as SLWERLAYGI.

ACKNOWLEDGEMENTS

The research was financially supported by the Czech Science Foundation (project GA CR 17-12816S).

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MOLECULAR IMPRINTING TECHNOLOGY FOR TARGETED ANALYSIS OF PROTEINS

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Abstract: Molecular imprinting has appeared to be an effective technique for creating of selective recognition sites in synthetic polymers. This procedure comprises polymerization of monomer in a presence of target molecules (template). The subsequent template removal forms tailor-made cavities that are complementary in shape and size to the template molecules. For protein imprinting, the choice of the suitable polymers is limited and polymerization conditions need to be optimized. In our work, dopamine monomer was chosen for polymer formation due to its nontoxicity, ease of preparation and self-assembly. For the optimization of conditions, lysozyme with molecular weight of 14.3 kDa was used and the functionality was evaluated by fluorimetry. Different concentration of dopamine and lysozyme for polymerization were tested. Under the optimized conditions, the limit of detection for lysozyme was found to be 7.8 µg/ml. Moreover, conditions for polymer formation for a purpose to reduce the overall time of analysis were investigated. The use of dopamine as a monomer in molecular imprinting shown to be beneficial in many aspects.

Key Words: polydopamine, molecularly imprinted polymer, lysozyme

INTRODUCTION

A molecularly imprinted polymer (MIP) is a polymer with selective recognition sites (Mosbach 1994). In the procedure, a template molecule is added into a solution of suitable functional monomers (Bergmann and Peppas 2008). The most common methods of imprinting are bulk imprinting (for small template) and surface imprinting (cells or viruses); other methods are used as an alternative imprinting strategies, e.g. substructure imprinting, substructural analogues, antibody replica, or molding (Schirhagl 2014). A substantial step is template removal, which is especially challenging when imprinting macromolecules. Template can be removed by using various solvents, such as acids or bases, detergents; the polymer can be heated, or digestive enzymes (proteases) could be used. After removing of the imprinted molecule, the cavities formed in the polymer are complementary to the template in size, shape, and orientation of functionalities are left behind, and are capable to selectively recognize the target molecule (Dechtrirat et al. 2012). The optimization of the polymer structure is extremely important. The polymer should have the following properties: stiffness of the polymer structure, high flexibility, good accessibility, mechanical stability and thermal stability (Wulff 1995).

Molecular recognition is a key principle in biology and bioanalysis (Dechtrirat et al. 2012). The first report about molecular imprinting for detection of protein was published in 1985, when organic silane was used as monomer for polymerization on silica beads and enzyme was entrapped (Glad et al. 1985). The following years were addressed to molecular imprinting of proteins due to the fact that proteins could not be always compatible with organic solvents used during polymer preparation (Bossi et al. 2007). Further, proteins easily subject to external influences, e.g. temperature. In the course of molecular polymerization, it is important to think of functional groups that are able to interact with functional monomer.

It is anticipated that MIPs with specificity for proteins will be applied in medicine, diagnostics, proteomics, environmental analysis, sensors and drug delivery (Bossi et al. 2007). Recently, the MIPs have been widely used for extraction, drug delivery, sensors, catalysis, and drug discovery applications based on their high selectivity, stability and adsorption capacity (Yin et al. 2016). Today, laboratory practice is almost dependent on systems utilizing antibodies for specific protein capture in various assays, for isolation, extraction and biosensors (Turner et al. 2006). The disadvantage is that these systems are often expensive and usually suitable only for single use. There is a growing demand for inexpensive, robust and reusable systems that have the desired level of selectivity and specificity.

Recently, the attention has been focused on the dopamine monomer. Its advantage is that it can self-polymerize in an alkaline or oxidative aqueous solution without a cross-linking or initiating agent (Yin et al. 2015). By using dopamine as a monomer, the time of analysis can be reduced due to facile polymer preparation (Yin et al. 2016). The low cost and limitations in the use of chemicals are also beneficial. As an example, Nematollahzadeh et al. 2013 selectively adsorbed human serum albumin on imprinted polydopamine nanolayer on the surface of porous silica particles. Besides, Lin et al. 2013 prepared a boronate-functionalized imprinted monolithic column with polydopamine coating for glycoprotein enrichment. In other study, proteins were imprinted on the surface of amino-modified Fe_3O_4 nanoparticles using dopamine as a monomer (Gao et al. 2014).

The aim of this work was the optimization of polymerization conditions for dopamine monomer. Different concentrations of monomer, template and polymerization conditions were assessed. The detection limit of fluorescence spectrometric detection of a model protein was also determined.

MATERIAL AND METHODS

Chemicals

All chemicals were obtained from Sigma Aldrich (St. Louis, MO, USA). 20 mM Tris-HCl buffer used throughout this experiment was prepared from Trizma® (TRIS base), pH was adjusted to 8.5 with hydrochloric acid (reagent grade, 35%). The pH was measured by using pH meter WTW inoLab (Weilheim, Germany).

Preparation of polydopamine MIP

Molecularly imprinted polymer (MIP) was prepared by self-polymerization according to literature (Gao et al. 2014, Zhang et al. 2012). In brief, a dopamine hydrochloride (monomer) in different concentrations was dissolved in 20 mM Tris-HCl buffer (pH 8.5). Then, the template molecules of lysozyme were mixed with the monomer in a 1 : 1 (v/v) ratio. 50 µl of the polymerization mixture was pipetted into 96-well microplate (Corning, NY, USA) in 6 repetitions. The resultant polymer was then washed 5 times with mixture of 5% acetic acid (HAc) (v/v) and 10% sodium dodecyl sulfate (SDS) (v/w) to remove the imprinted molecules and once with water. Subsequently, sample (lysozyme dissolved in Tris-HCl buffer) was applied for 1 hour; the microplate was shaken on Eppendorf Thermomixer R W/1.5ml Thermoblock (Eppendorf, Hamburg, Germany). The sample was then removed, unbound target molecules, and interferents were washed out with water. The MIP formation process is shown in Figure 1.

Non-imprinted polymer (NIP), serving as a control, was prepared and treated under the same conditions but with absence of template molecules.

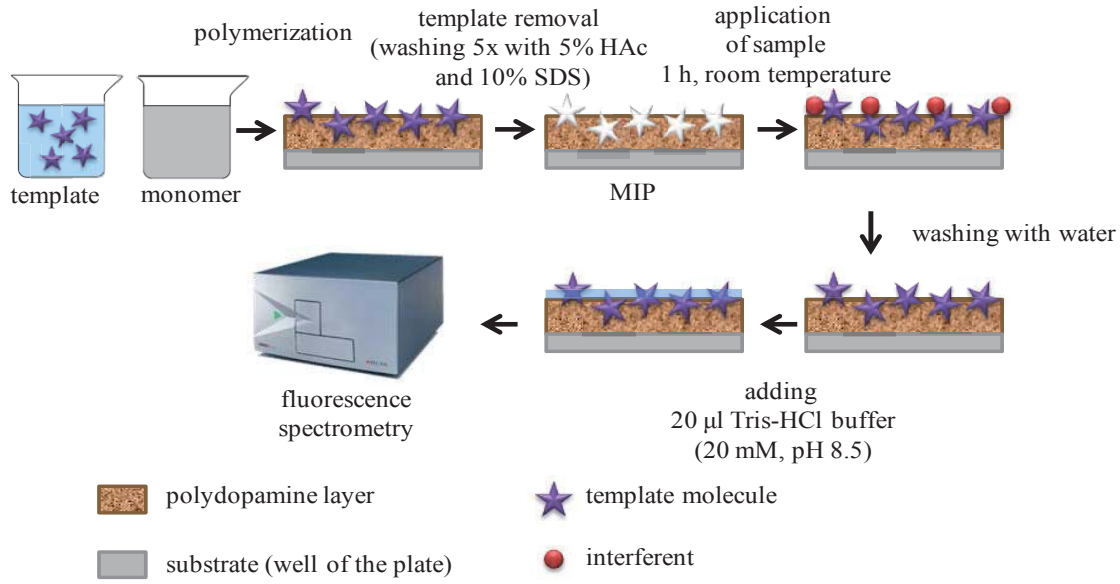
Fluorimetric detection

Fluorescence intensity was measured using fluorimeter Infinite M200 Microplate reader (Tecan, Switzerland). Lysozyme emission (at pH 8.5) was measured at wavelength λ_{ex} 280 nm and λ_{em} 330 nm with gain of the detector set to 100. Before measurements, 20 µl of Tris-HCl buffer was added to the each well.

Statistical analysis

All data were statistically analyzed using Dean-Dixon test (also Q test) and the remote outliers were rejected (with 6 observations at 90% confidence, $Q_{90\%} = 0.56$).

Figure 1 Scheme of MIP polymerization



RESULTS AND DISCUSSION

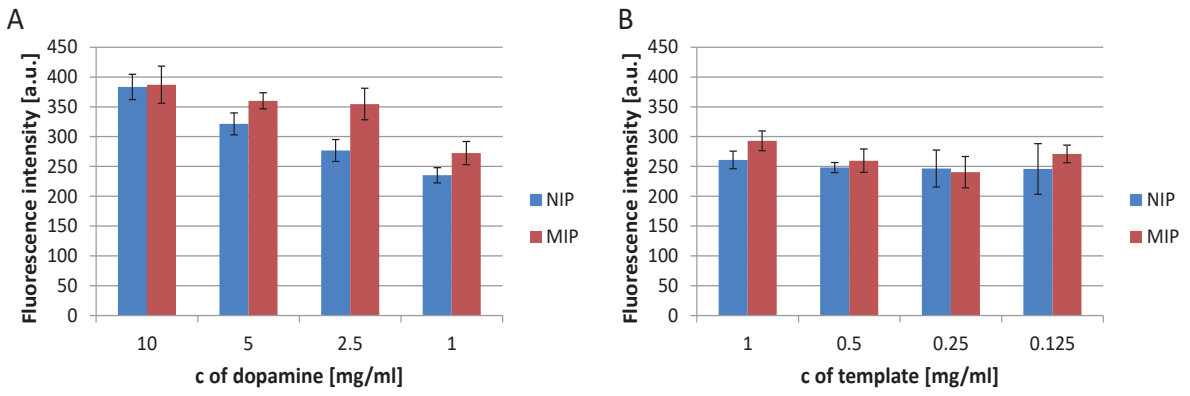
Optimization of monomer concentration

For polymerization, concentrations of dopamine monomer 10, 5, 2.5 and 1 mg/ml were tested. Template concentration of 1 mg/ml was used and concentration of sample was set to 0.125 mg/ml throughout this optimization. Figure 2 A shows fluorescence intensity for different amount of dopamine. Qualitatively, the polymers were evaluated as a relative difference between MIP and NIP. According to our experiment, the best result was achieved in the third case, thus 2.5 mg/ml of dopamine was resulting concentration used further in our experiments.

Optimization of template concentration

In Figure 2 B, the concentrations of template are compared. The initial concentration of 1 mg/ml was sequentially reduced by half. Dopamine concentration was 2.5 mg/ml and sample was 0.125 mg/ml during this experiment. As the most effective, it seems using of template concentration 1 mg/ml (maximal used), where is an evident difference between NIP and MIP, and more template-selective cavities were formed. Other concentrations did not prove to be suitable for the imprinting technique because the fluorescence intensity is comparable between NIP and MIP.

Figure 2 Determination of optimal monomer concentration (A) and template concentration (B)

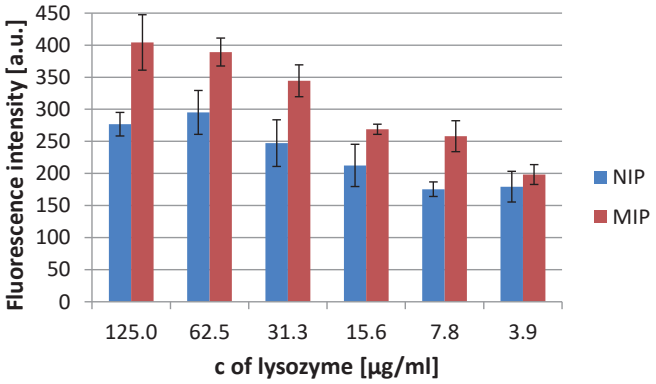


Determination of detection limit

Subsequently, a limit of detection of the created imprinted polymer with optimized concentration of monomer 2.5 mg/ml and template 1 mg/ml was determined. We have examined

concentrations of sample in range from 125.0 to 3.9 µg/ml. Figure 3 shows differences between NIP and MIP among these concentrations. With lower concentration of applied sample, fluorescence intensity declines. For each concentration, there is an obvious difference between NIP and MIP. However, for concentration of 3.9 µg/ml we cannot definitely determine NIP from MIP due to the fact that the error bars overlap. Therefore, the lowest value that we are able to detect using fluorimeter Infinite M200 Microplate reader is 7.8 µg/ml.

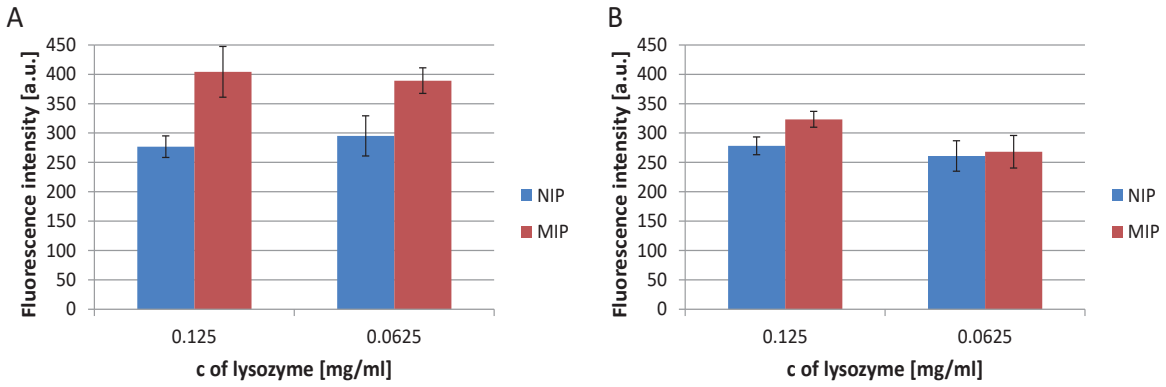
Figure 3 Limit of detection for lysozyme determined using fluorimetry



Optimization of polymerization conditions

The advantage of dopamine polymer is self-polymerization and shorter time of polymerization against most of the polymers. As proposed elsewhere, the polymerization time can be reduced with use of different techniques, such as UV light (Du et al. 2014) or increased temperature (Zhou et al. 2014). We tried to shorten the overall time of the analysis by polymerization of imprinted polymers in a laboratory oven Memmert UE 400 (Schwabach, Germany). NIP and MIP were prepared as mentioned above. We have tested the polymerization at 40°C for 4 hours and compared to our results when the polymer was dried overnight. In both cases, samples with concentration of lysozyme 0.125 mg/ml and 0.0625 mg/ml were applied. As shown in Figure 4 A, we can see the difference between NIP and MIP in both concentrations when the polymer was dried overnight. Nevertheless, in Figure 4 B, there is difference between NIP and MIP only in concentration of applied lysozyme 0.125 mg/ml. This result indicates that the polymerization at the elevated temperature is effective but yields to decreased sensitivity. However, the conditions may be further investigated for dopamine polymerization, more preferably with lower temperature and longer time of drying.

Figure 4 Comparison of dopamine dried overnight (A) and at temperature 40°C for 4 hours (B)



CONCLUSION

In this work were optimized conditions of polymerization dopamine monomer. The best results were achieved with monomer concentration of 2.5 mg/ml; optimal concentration of template (lysozyme) was 1 mg/ml. Under these conditions, fluorimetric method was used for determination of lysozyme due to its simplicity, low costs and effectivity. We were able to measure the protein

in concentration as low as 7.8 μg/ml. To accelerate the dopamine polymerization, conditions 40°C for 4 hours were tested. Under these conditions, the polymerization was effective; however the created polymers did not attain such functionality as when they were dried at room temperature overnight.

ACKNOWLEDGEMENTS

The research was financially supported by the project CEITEC 2020 (LQ1601) with financial support from the Ministry of Education, Youth and Sports of the Czech Republic under the National Sustainability Programme II.

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hNET AS A TARGET FOR NEUROBLASTOMA NANOMEDICINE

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Abstract: Chemotherapy often results in various side effects, which can negatively affect health. Neuroblastoma, one of the most common types of childhood cancer, is but one of the examples, where side effects of chemotherapeutic treatment lower the quality of patient's life. Modern way how to fight that is to enclose cytotoxic drug into some nanocarrier and its targeting to receptors overexpressed in membranes of cancer cells. Apoferritin (Apo), a natural protein cage, is very suitable as a nanocarrier, as it has no toxicity, immune system does not react to it, and drug can easily be loaded into its cavity. We enclosed ellipticine, clinical tested anti-cancer drug, into Apo cavity (creating ApoElli). The percentage of encapsulation was 61% and size and transmission electron microscopy analysis showed the preserved Apo ~12 nm icosahedral structure after this encapsulation. Then we modified Apo outer surface with *in silico*-modelled peptides with hNET affinity and tested its toxicity and hemolytic activity. ApoElli modified with anti-hNET peptides was able to internalize into neuroblastoma cells and to deliver the drug. However, it proved to be safe for human RBC, unlike pure ellipticine, which caused observable hemolysis at the same concentration.

Key Words: ellipticine; hemolysis; nanoconstruct; neuroblastoma; toxicity

INTRODUCTION

Neuroblastoma is the most common cancer among children; the average age of diagnosis is 17 month (Davenport et al. 2012). This cancer is arising in adrenal medulla or paraspinal ganglia and affects development of sympathetic nervous system. Over 50% of tumours are present in abdomen, neck, chest or pelvis (Vo et al. 2014). It is well known for its wide variety of clinical behaviour depending on the site of primary tumour, presence or absence of metastatic disease sites etc. (Park et al. 2010). Diagnosis is performed by INSS (International Neuroblastoma Staging System), whose criteria were first formulated in 1986 (Brodeur et al. 1993). Initial tests include CT or MRI to evaluate primary tumour, localize it, and define its size and possible spreading. Pathological confirmation is performed from biopsy of tumour tissue or from neuroblastoma tumour cells in bone marrow sample (Park et al. 2010). Treatment methods include surgery, chemotherapy, radiotherapy and biotherapy, as well as observation with selected circumstances (Maris et al. 2007). Approved drugs for neuroblastoma are for example doxorubicin hydrochloride, vincristine sulphate, Clafen®, Cytoxan® or CEM®. Side effects of chemotherapeutic drugs can be very serious; some of them might appear years after successful treatment, such as 65% of childhood patients treated with doxorubicin hydrochloride who suffer from its cardiotoxicity in adulthood (Hutchins et al. 2017).

One way how to protect organism against these side effects is to close these drugs into some nanocarrier. For this experiment, apoferritin (Apo) was selected. It is a regular, uniformly self-assembling nano-sized protein cage with excellent biocompatibility and unique structure that allows encapsulation of small molecules into its inner core (Belletti et al. 2017). It has many positive

characteristics, the mechanism of drug loading can be based on passive penetration process through pores (Linder 2013) or active pH-dependent disassembly/reassembly protocol (Dostalova et al. 2017). Apo has a long lifetime and has affinity for tumour cells, though it has the ability to bind at human TfR1 receptor, which is over-expressed in rapidly proliferating cells (Li et al. 2010).

Apo surface can be further modified (Blazkova et al. 2013) and so can be directed to different cell types which increases its specificity. In this paper, we modified Apo surface with gold nanoparticles and use them to further modify the surface with peptides with high affinity for human norepinephrine transporter (hNET). The affinity of various peptide sequences for this receptor was tested using homology modelling (Haddad et al. 2017), and two different peptides were chosen for *in vitro* studies. This modification allowed to increase the Apo specificity and allow transport into cells through norepinephrine transporter receptor overexpressed on the membranes of neuroblastoma cells (Haddad et al. 2017).

The ability to deliver drug molecules to neuroblastoma cells was tested using ellipticine (Elli). It is an alkaloid that was first isolated from *Ochrosia elliptica*. Preclinical and clinical studies showed that ellipticine has ability to arrest growth of several cancer cell types (Poljakova et al. 2009), but has multiple toxic side effects, including hemotoxicity (Auclair 1987). The mechanism of action has not been precisely described , but it combines DNA damage by inhibition of topoisomerase II, generation of cytotoxic free radicals, regulation of Bcl-2 family protein, rescue of mutant p53 activity and initiation of mitochondrial apoptosis pathway (Kuo et al. 2005). However Elli is a possible mutagen (Stiborova et al. 2001) and has no specificity, so can also eliminate healthy cell. To protect healthy cells from effects of Elli and also specify place of toxic effect, we encapsulated Elli into Apo creating ApoElli.

MATERIAL AND METHODS

Chemicals

All chemicals of ACS purity were obtained from Sigma-Aldrich (St. Louis, MO, USA), unless stated otherwise. The pH was measured using pH meter WTW inoLab (Weilheim, Germany).

Encapsulation of Elli into Apo and modification of its surface with hNET peptides

200 µl of 1 mg/ml Elli (dissolved in 150 : 1 1 M HCl) was added to 20 µl of 50 mg/ml horse spleen Apo and 100 µl of ACS water. The solution was mixed for 15 min. 0.66 µl of 1 M sodium hydroxide was added to increase the pH and encapsulate the Elli inside Apo (creating ApoElli). The solution was mixed for 15 min. To remove non-encapsulated Elli molecules, solution exchange was performed three times (6000 g and 4 °C for 15 min). 25 µl of 1.3 nm gold nanoparticles was added to the sample and the solution was mixed for 12 h, creating ApoElli+Au. To remove unbound gold nanoparticles, solution exchange was performed two times. Next, 2.8 µl of 1.25 mg/ml anti-hNET peptide A (GASNGINAYLC, creating ApoElli+hNET pA) or anti-hNET peptide B (SLWERLAYGIC, creating ApoElli+hNET pB) was added to the sample. The sample was mixed for 1 h at 600 rpm and 45 °C. Solution exchange was performed two times to remove unbound peptide molecules. Samples were stored at 4 °C until used.

Characterization of nanocarrier

To evaluate Elli concentration in ApoElli and its encapsulation efficiency, absorbance at wavelength of 420 nm and fluorescence with excitation wavelength of 420 nm and emission wavelength of 450 nm were measured using Tecan Infinite 200 PRO (Tecan, Männendorf, Switzerland).

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 was diluted with ACS water (1 : 200), 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.

Visualization of the nanocarrier shape was performed using transmission electron microscopy (TEM) with negative staining technique. For this purpose, an organotungsten compound, Nano-W (Nanoprobes, Yaphank, NY, USA) was utilized. 4 µl of samples was deposited onto 400-mesh cooper grids coated with a continuous carbon layer. Dried grids were imaged by TEM (Tecnai F20; FEI, Hillsboro, OR, USA).

Toxicity of peptides and nanocarrier

MTT toxicity test were performed with neuroblastoma cell lines NB4, and SH-SY5Y to evaluate the cytotoxicity of anti-hNET peptides A and B (in concentration range of 0.98-1000 µM), Elli, ApoElli, ApoElli+hNET pA and ApoElli+hNET pB (in Elli concentration range of 0.078-80 µM). 5000 cells in 50 µl of medium were seeded in each well of 96-well plate. The cells were incubated at 37 °C for at least 8 h. 50 µl of samples diluted in culture medium was added to cells, with one well receiving only culture medium. The plates were incubated at 37 °C for 12 h, after which 10 µl of 5 mg/ml of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dissolved in PBS (pH 7.4; 0.137 M NaCl + 0.0027 M KCl + 0.0014 M KH_2PO_4 + 0.0043 M Na_2HPO_4), was added to every well. After further incubation at 37 °C for 3 h, the solution was removed and 100 µl of dimethylsulfoxide was added to disrupt the cells and release purple formazan. Absorbance of the samples at 570 nm was measured by Tecan Infinite 200 PRO.

Hemolytic assay was performed to evaluate hemotoxicity of Apo, Elli, ApoElli, ApoElli+hNET pA and ApoElli+hNET pB). Fresh blood is centrifuged at 3000 rpm for 10 min. Blood plasma was removed and red blood cells (RBCs) were repeatedly washed by 150 mM sodium chloride and centrifuged at 3000 rpm for 10 min, until supernatant cleared from haemolytic RBCs. After that, the RBCs were diluted with PBS and 150 µl was mixed with 150 µl of various concentrations (0.0468-0.375 mM) of the tested Elli nanoformulations. PBS was used as negative control and 0.1% Triton X-100 was used as positive control. All samples were incubated at 37 °C for 1 h and centrifuged at 3000 rpm for 10 min. Absorbance of 50 µl supernatant at 540 nm was measured using Tecan Infinite 200 PRO. The percentage of hemolysis was calculated using formula:

$$\%\text{hemolysis} = [(A_t - A_c)/(A_{100\%} - A_c)] \cdot 100 \quad (1)$$

A_t stands for absorbance of supernatant from sample, A_c represents absorbance of supernatant from negative control and $A_{100\%}$ stands for absorbance of supernatant from positive control.

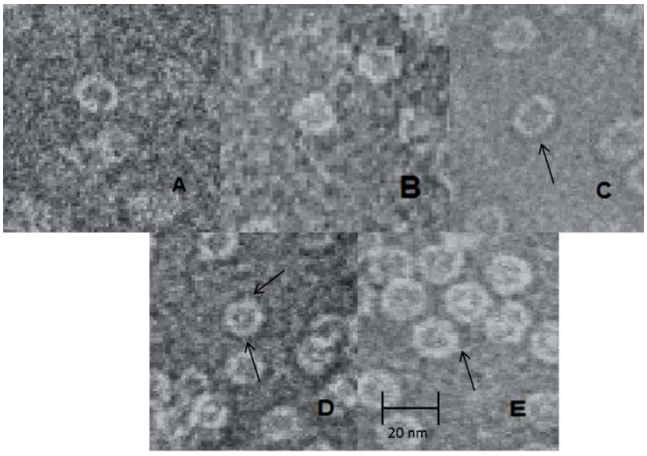
RESULTS AND DISCUSSION

Table 1 Average size of the tested nanocarriers

Sample	Average size (nm)
Apo	12.0
ApoElli	15.7
ApoElli+hNET pA	18.2
ApoElli+hNET pB	43.8

Average Elli encapsulation efficiency into Apo was 61%, as evaluated by absorbance measurement. The average size of the nanoparticles was measured to further confirm the surface modifications (Table 1). While the average size of empty Apo is ~12 nm, encapsulation of Elli increased it to 15.7 nm. This could be caused by less rigid assembly after filling of the cavity or by drug molecules attached to the outer surface of the nanocarrier. Modification with anti-hNET peptide A increased the size to an average of 18.2 nm, proving that if there were drug molecules on the outer surface, their presence did not hamper binding of targeting peptides. The biggest size, around 43.8 nm, was measured for ApoElli modified with anti-hNET peptide B. Since during the homology modelling the peptide B was found to bind to other peptide B molecules, this was probably caused by formation of multiple ApoElli connected by these peptides.

Figure 1 TEM visualization, A –Apo; B – ApoElli; C – ApoElli +Au, arrow shows Au nanoparticles, D – ApoElli+hNET pA, arrows show visible peptide tails E – ApoElli+hNET pB, arrows show visible peptide tails



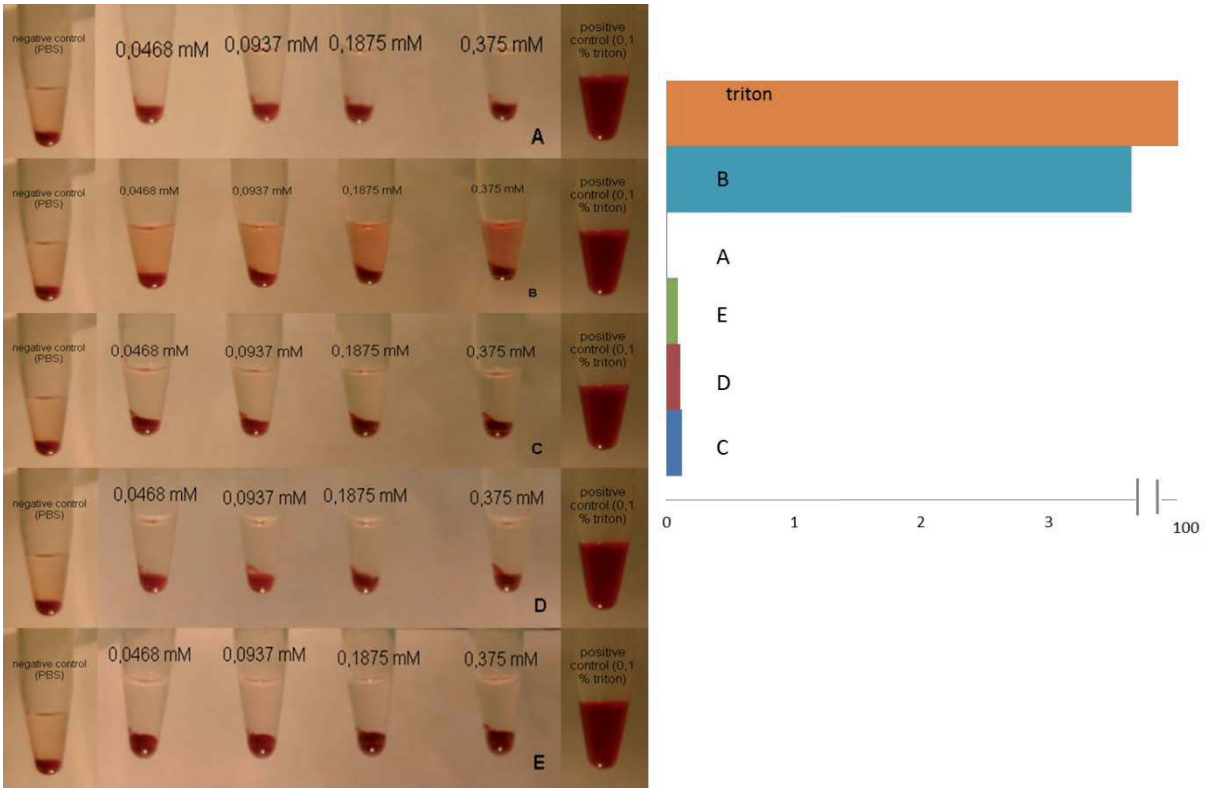
To evaluate the structure of nanoparticles, TEM visualization was used (Figure 1). The structure of unloaded Apo (Figure 1A) shows icosahedral cage with empty cavity. On the next picture is Apo loaded with Elli (Figure 1B, ApoElli) where a filled cavity can be clearly observed. Gold nanoparticles on ApoElli surface can be seen as a dark ring around ApoElli (arrow on Figure 1C, ApoElli+Au). Peptides present on the surface of ApoElli+hNET pA and ApoElli+hNET pB were observed as small protein tails on the icosahedral cage (arrows on Figures 1D and 1E, respectively). However, all pictures showed typical apoferritin structure in its stable and assembled state, proving that the performed surface modifications did not lead to disassembly of its structure.

Table 2 MTT toxicity test

Cell line	Sample	$24IC_{50}$ (μM)
SH-SY5Y	anti-hNET peptide A	330.0
	anti-hNET peptide B	203.0
	Elli	8.8
	Apo	ND
	ApoElli	16.5
	ApoElli+hNET pA	16.5
	ApoElli+hNET pB	20.7
NB4	anti-hNET peptide A	ND
	anti-hNET peptide B	216.0
	Elli	7.3
	Apo	ND
	ApoElli	16.3
	ApoElli+hNET pA	16.2
	ApoElli+hNET pB	15.5

To evaluate the cytotoxicity of these nanocarriers for neuroblastoma cells, MTT toxicity test was performed using two different neuroblastoma cell lines, SH-SY5Y and NB4. The peptides themselves proved non-toxic for these neuroblastoma cells lines, where their $24IC_{50}$ was 30 fold higher than that used for ApoElli targeting. Empty Apo was did not show any toxicity. Samples containing Elli reached $24IC_{50}$ at low dosage, and encapsulated Elli reached $24IC_{50}$ with concentration 2.5 fold higher than pure Elli. These results show that Apo nanocarrier was able to deliver Elli cargo to cells, where its structure was disassembled due to acidic endosomal environment and Elli was released.

Figure 2 Hemolysis test; Letter describe samples, A – Apo, B – Elli, C – ApoElli, D – ApoElli+hNET pA, E – ApoElli+hNET pB, graph shows percentage of hemolysis by highest concentration of samples



Hemolytic test was performed using fresh RBCs. 0.1% Triton was used as a positive control, as it hemolysed 100% of RBCs. PBS was used as a negative control. Empty Apo had no hemolytic effect at all (Figure 2A). Pure Elli caused observable hemolysis, as it hemolysed 3.6% of RBCs at its highest concentration (Figure 2B). Elli encapsulated into Apo (Figure 2C) and its modifications with anti-hNET peptides A (Figure 2D) and B (Figure 2E) were not hemolytic for RBCs. Percentage of hemolysis caused by these nanoformulations was below 1% at all tested concentrations. It can be seen, that ApoElli with peptide B was a little less hemolytic then ApoElli and ApoElli with peptide A.

CONCLUSION

We managed to create possible nanocarrier loaded with anti-cancer drug ellipticine. By closing Elli into this protein carrier Apo we can protect healthy cells as with modification of Apo surface we are able to specifically target it to cancer cells. In this paper we encapsulated Elli into Apo and modified its outer surface with gold nanoparticles and anti-hNET peptide A and hNET peptide B for neuroblastoma targeting. We characterized the size and structure of these particles and proved that these modifications did not affect the structure of Apo and so its function. We also demonstrated safety of this nanocarrier by itself, but also when loaded with Elli and modified with targeting peptides. Elli itself proved toxic for both cancer and red blood cells, even in low concentration. Whereas the encapsulation in Apo keeping the toxicity for neuroblastoma cancer cells while eliminating the toxicity for red blood cells, although the highest concentration applied to red blood cells was almost 25 fold higher than that used on neuroblastoma cancer cells. By modifying of Apo surface, we proved that the transport of cytotoxic drug with many side effects can be more specific to affect only target cancer cells. Still, further experiments are needed to evaluate the targeting and *in vivo* behaviour of this nanocarrier.

ACKNOWLEDGEMENTS

The research was financially supported by the Grant Agency of the Czech Republic (GA CR 17-12816S) and CEITEC 2020 (LQ1601).

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NEW OPTION FOR DECREASING OF CONCENTRATION LIMIT OF DETECTION IN ELECTROPHORESIS

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Abstract: A hyphenated method consisting of transient electrokinetic dosing on-line coupled to isotachophoretic analysis was developed for the pre-concentration, pre-separation and analytical determination of a model substance – anionic herbicide glyphosate (gly) – in aqueous samples containing very low concentration of the analyte of interest. Various parameters were investigated in the framework of an optimisation study; the aim was to reach minimal concentration limit of detection decreasing in minimum time. The developed method consists of 2 phases. In the first one, a sample with addition of convenient buffer (dosing electrolyte) is electrokinetically dosed to the isotachophoretic column with proper leading electrolyte. During the dosing time, a moving-boundary electrophoresis zone of accumulated sample is created in the column and it is slowly moving through column. The accumulation of zone is proportional to the time and driving current and to the composition of dosing electrolyte. After some time of accumulation of the zone, dosing electrolyte is replaced with terminating one. Now, the regular isotachophoretic separation and analysis starts. The electrolyte composition and the dosing time were thoroughly optimized and 14 fold of accumulation was reached in 25 minutes in comparison to classical isotachophoretic analysis. The method is simple and applicable to all commercial isotachophoretic analysers.

Key Words: isotachophoretic analysis, glyphosate, separation, ampholyte, electrolyte

INTRODUCTION

In the environment, balanced conditions are created naturally for the existence of all living organisms, including pests and weeds. To protect the crops from these unwanted phenomena, herbicides and pesticides are still often used in agriculture, but the trend is to use preparation with good bio-degradation abilities that do not create long-term environment pollution and do not leave toxic residua in produced food. Thus, monitoring of the behaviour of these substances and their impact on the environment and human health is required on a regular basis (Zákon č. 17/1992 Sb., o životním prostředí).

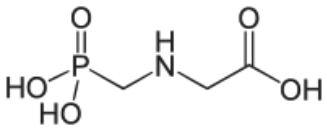
The isotachophoretic analysis (ITP) is one of chemical methods suitable for monitoring of such substances, for example in water. This method belongs to the family of electromigration separation method. Thanks to the current level of instrumentation, it is possible to measure the quantitative amount of the substance of interest in the sample directly by the means of a suitable program. At the same time, qualitative analysis is done - it is possible to find out what kinds of substances are present in liquid sample.

In isotachophoretic analysis, the sample is separated according to its electrophoretic mobility: speed of the charged particles in the homogeneous electric field. The sample is inserted to the system consisting of two electrolytes with different mobility. A highest mobility electrolyte is called the “leading electrolyte” (LE) and lowest mobility one is referred to as the “terminating electrolyte” (TE) (Klouda 2003). When the voltage is inserted to the system, the charged components start to migrate at different speed and organize themselves according to their mobilities. After the separation phase they migrate in their zones according to their mobilities from the highest to the lowest one (Pospíchal and Glovinová 2001). To obtain good results, it is necessary to choose a suitable electrolyte system in which the analysis will precede and in which the appropriate

separation-distribution of the monitored analytes will be achieved. The choice of the electrolyte system can also influence the sensitivity, speed and accuracy of the analysis (Klouda 2003).

The analyte of interest, glyphosate (systematic name N-phosphonomethylglycine) is a broad-spectrum herbicide applied to plant leaves. Glyphosate is an ampholytic substance; its structure contains three functional groups: the basic amine and acidic carboxy and phosphonyl group (see Figure 1) (Newman et al. 2016).

Figure 1 The structural formula of glyphosate (Available from http://www.wikiwand.com/cs/Roundup)



Substances used as herbicides and pesticides play crucial roles in our lives and can be found for example as residua in food or as pollutants in the environment. (Pospíchal and Glovinová 2001). The research performed in the framework of the Friends of Earth Europe projects included, among other things, analyses of 182 volunteers across 18 European Union countries and it was found out that 80 volunteers had glyphosate in their bodies (Institute of Science in Society, 2015).

The aim of this work was to develop and validate a new isotachophoretic method for the determination of glyphosate in aqueous samples that would allow much better analyte accumulation and thus enable the determination of much lower concentrations of this herbicide in water, and at the same time to keep the time of analysis at practically short level.

MATERIAL AND METHODS

All experiments were performed using a commercially available isotachophoretic instrument (CS Isotachophoretic analyzer, Labeco, Slovak Republic, see Figure 2). Standard of N-phosphonomethylglycine was bought from Sigma Aldritch, St. Louis, Missouri, USA. Deionised water used for the preparation of model samples was prepared in Milli-Q Ultrapure Water Systems, Merck, Darmstadt, Germany. The operating electrolytes used for individual analyses are given in Table 1.

The validation of the measurement for both old and new method were performed during the same day without any changes of instrumentation. Thus the cLOD and cLOQ (limit of quantification) were constant under these conditions.

Figure 2 Schematic diagram of the isotachophoretic analysis (VN-High voltage power supply)

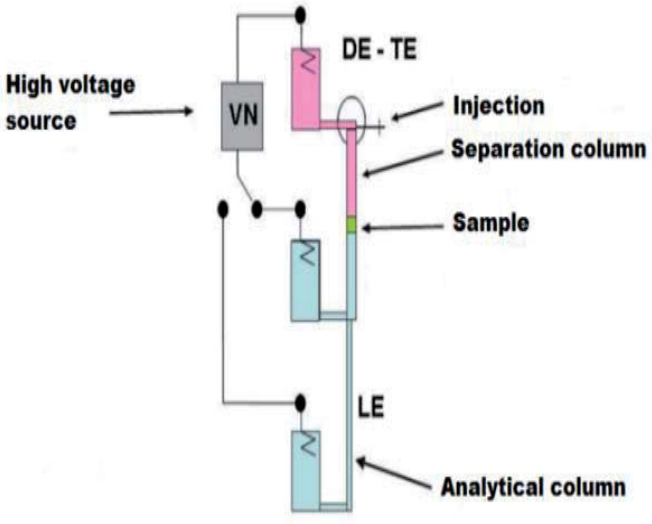


Table 1 Operating electrolytes used for individual analyses

Parameter	Basic primary /LE	Acid primary DE	Terminating TE
Solvent	Water	Water	Water
Anion	Cl^-	$C_6H_5COO^-$	$C_6H_5COO^-$
Concentration	0.01 mol/l	0.0001 mol/l	0.01 mol/l
Cation	Bala	Bala	Bala
Concentration	0.02 mol/l	0.02 mol/l	0
Additive	PEG; TRITONE		
Concentration (%)	10; 1	0; 0	0; 0

Legend: Abbreviations: LE – leading electrolyte; DE – dosing electrolyte; TE – terminating electrolyte; Bala – beta alanine All chemicals were obtained from Sigma-Aldrich and were analytical grade.

RESULTS AND DISCUSSION

Presented method of lowering concentration limit of detection (cLOD) is based on the transient electrokinetic dosing of the analyte to the column, i.e. sufficient (maximal) amount of the sample must introduced to the column during some period of time at a given electric current.

This amount is proportional to the driving current and to the transference number of the analyte in the dosing electrolyte, which is given by the ratio of conductivities of the sample and whole electrolyte. Sample conductivity is a product of effective mobility of sample ion species and their concentrations. Effective mobility is a function of ionic mobility and pH. Thus, there are four variables to be optimized and one given parameter: time, current, conductivity of DE, pH and the concentration of ions in the sample, respective.

Time

Time of the dosing (at a constant driving current) is limited by the geometry of column and the LE concentration – the total time of the sample passage through the column, which is constant, can be divided into the separation and dosing time. Thus, the dosing time for the given column, current and sample must be evaluated experimentally. Here, the separation electrolyte quality is important; better and shorter separation time in a given column gives more time for the dosing.

Driving current

Maximum attainable current is given by column ability to dissipate Joules heat, i.e. it is given by geometry and physical properties of the column and by conductivity (concentration) of the electrolyte. Again, the current must be set up on maximal value experimentally.

pH of the DE

Proper value of pH is setting up a dosing speed and selectivity. pH should be set up such a way that transference number of the sample (effective mobility) is maximal and the transference number of disturbing substances low. In the case of known mobilities of the separated substances this parameter can be optimized successfully by computer simulation.

Conductivity of DE

This parameter should be kept as low as possible. The approach of minimal manipulation was adopted, where a sample buffered to a proper value of pH is used as a DE. A buffer addition setting up the pH should not increase the given conductivity of the sample – DE too much.

During all experiments, the DE was buffered by ampholytes in its electrical point (pI), so the conductivity was not increased. Different ampholytes with different pI -pH were used to set up different selectivity. Histidine (HIST), pI 7.4, and Bala, pI 5.5, were used as DE buffers. 0.02 mol/l Bala was selected as final buffer for our analyses, because this amino acid is more acidic and is not dosing the hydrogen carbonate. 0.01 mol/l benzoic acid was used as the terminating electrolyte, because this acid is available in extreme clean grade and that means that unnecessary impurities are not concentrated in the system.

0.01 mol/l HCl and 0.02 mol/l Bala were used as leading electrolytes. The samples containing glyphosate and marker dye SPADNS in different concentrations were injected into the system.

In the first step glyphosate cLOD was determined by classical ITP analysis, the obtained value was 0.0005 mol/l.

Figure 3 Zone length of the glyphosate-to-concentration graph. TE: 0.01 mol/l C_6H_5COOH, LE: 0.02 mol/l HCl + 0.02 mol/l Bala, SAMPLE: GLY + SPADNS

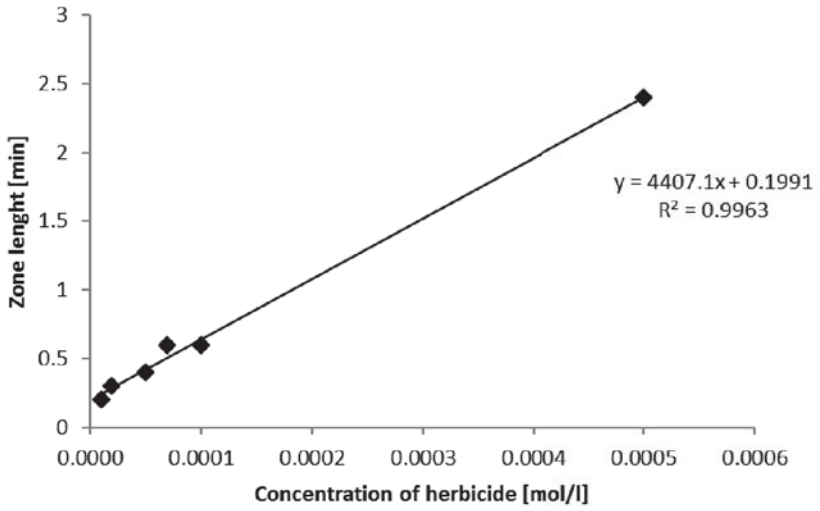
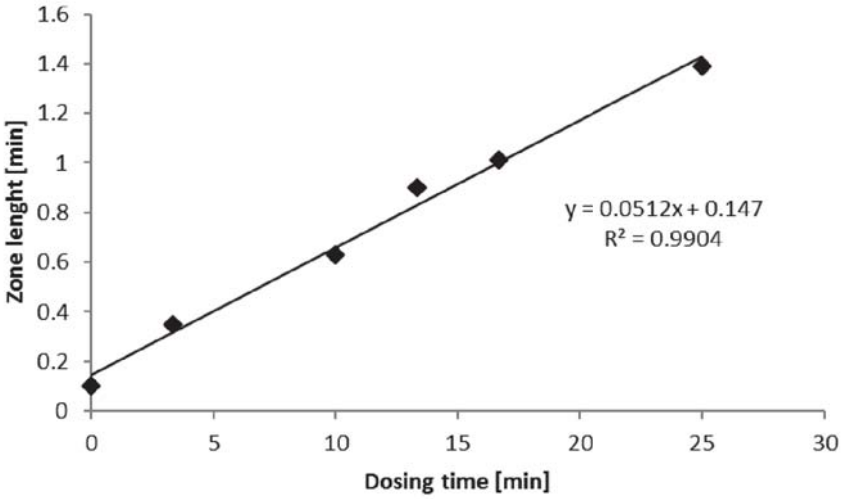


Figure 4 Zone length of the glyphosate-to-dosing time graph. TE: 0.01 mol/l C_6H_5COOH, LE: 0.02 mol/l HCl + 0.02 mol/l Bala, DE:0.0001 mol/l C_6H_5COOH + GLY 0.00005 mol/l



The cLOD (see Figure 3) in the framework of the method optimization, cLOD concentration was dosed to the column for 1500 seconds when the boundary reached physically half of the column. Thus, the dosing time was thoroughly optimized and 14 fold accumulation was reached in 25 minutes.

After that the dosing electrolyte was changed for terminating one and classical ITP analysis with conductivity detection was performed. The dependence of the zone length on the dosing time at the concentration 0.00005 mol/l and maximum dosing 1500 second (see Figure 4). The cLOD was calculated from the calibration curve as a 3Sd/slope at zero concentration of analyte**.**

CONCLUSION

With the increasing need of environmental monitoring it is necessary to investigate suitable electrolyte systems and develop new quick and robust methods which would enable to perform the analyses in the shortest possible time and give us reliable data.

The new method consists of 2 phases. In the first one was a diluted sample with addition of convenient buffer (dosing electrolyte) dosed to the ITP column with proper leading electrolyte. Then dosing electrolyte was replaced with terminating one. After that started the regular ITP analysis. The electrolyte composition and the dosing time were thoroughly optimized and 14 fold accumulation in comparison to classical ITP analysis was reached in 25 minutes.

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ANTIMICROBIAL ACTIVITY OF CDTE QDS MODIFIED WITH LANTHANIDES ON *PSEUDOMONAS AERUGINOSA*

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Abstract: The aim of this study is to obtain data based on an experimental procedure and check the effectiveness of some antibacterial agents against pathogenic bacteria. In the present experiment, two different nanoparticles (Cadmium Telluride Quantum dots with Lanthanides: Gadolinium and Terbium) were used to check their antibacterial properties on *Pseudomonas aeruginosa*. The Cadmium Telluride Quantum dots without the Lanthanides, Gadolinium nitrate and Terbium nitrate were also tested on those bacteria as control. In the present experiment, the following methods were employed: disc-diffusion test, the determination of the growth properties of the bacteria and comparison of absorbance after treatment with antimicrobial agent. From the results it has been found that the tested Cadmium Telluride Quantum dots with Lanthanides (Gadolinium and Terbium) have good antimicrobial effects. Additionally, GdQDs show stronger antibacterial effect than Tb QD and other tested compounds.

Key Words: Pseudomonas aeruginosa, antimicrobial activity, lanthanides, quantum dots

INTRODUCTION

Bacterial infections are one of the major threatening for human health. A serious problem is the treatment of diseases causing resistant bacteria and bacteria forming a biofilm (*Pseudomonas aeruginosa*). More than 70% of nosocomial pathogens have become resistant to the drugs considered to be their first line treatment (Muto et al. 2003).

In hospitals antibiotic resistance is an important issue (Cook et al. 2004). In the last 50 years, the number of bacterial strains resistant to antibiotics has increased almost uniformly around the world. The bacteria started to be resistant to antimicrobial agents by changing their chromosomes and exchanging their genetic materials through plasmids (Lutsar et al. 1997). Due to excessive use of these drugs, antibiotic resistance increases rapidly (Andersson and Levin 1999).

Pseudomonas aeruginosa is one of the important nosocomial pathogens worldwide. Nosocomial infections caused by this organism are often hard to treat. *Pseudomonas aeruginosa* has intrinsic resistance and remarkable ability to acquire further resistance mechanisms to multiple groups of antimicrobial agents (Strateva and Yordanov 2009).

Nanoparticles with unique chemical and physical properties have shown an increasing importance in biomedical and pharmaceutical applications. Inorganic nanomaterials are regarded as good candidates to replace traditional organic antimicrobial agents, because they have large specific surface area and high bioactivity. A number of nanoparticles with antimicrobial activities have been reported recently. QD are crystalline clusters synthesized from semiconductor materials. Due to their wide potential, the study of antimicrobial activity of QDs go up logically (Lu et al. 2008). Lanthanide complexes are of increasing importance in diagnosis and therapy, due to the versatile chemical and magnetic properties (Teo et al. 2016).

Due to the increasing antibiotic resistance CDTE QDs modified with Lanthanides have been manufactured and their antimicrobial activity on *Pseudomonas aeruginosa* was investigated in this study.

MATERIAL AND METHODS

Chemical compounds

All the reagents for quantum dots synthesis, standards, and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) in ACS purity, unless noted otherwise. Media for cultivation of microorganisms were purchased from OXOID CZ (ThermoFisher Scientific - CZ).

Preparation of CDTE QDS modified by a Gadolinium-schiff base complex (GDQDs) and Terbium-schiff base complex (TBQDs)

The Schiff base, [(2-[(E)-2-pyridylmethyleneamino]-N-[2-[(E)-2-pyridylmethylene-amino]ethyl]ethanamine)] was prepared according to previous experiment (Kopel et al. 2014). In a separate beaker, methanol was mixed with an aqueous solution of gadolinium nitrate, which was subsequently added to the Schiff base solution. The prepared Gd-SB solution was stored at 25 °C until use. Microwave preparation of the CdTe QDs was carried out according to our previous study (Moulick et al. 2015) with necessary modifications. Next, the Gd-SB solution was added to the prepared CdTe QD solution, followed by heating using 300 W under microwave irradiation to prepare the GdQDs. The sample and control particles were filtered through 0.22 µm membranes and subsequently dialysed against deionized water several times to remove the unreacted initiators. Then, the particles were dispersed in deionized water for further characterization and use. TBQDS were produced in a similar way like GDQDs where Terbium nitrate was used in place of gadolinium nitrate.

Collection of wound swabs from the patients with bacterial infections

The smears, collected from infected wounds with the agreement of patients from Trauma Hospital in Brno, were sampled by rolling motion at the wound using a sterile swab sampler. All patients were divided into two subgroups, on the grounds of infection severity: deep and superficial wound. A detailed description of comorbidities and duration of treatment was obtained. Patients were classified according to the Classification of surgical wounds – SSI (surgical site infections). Infected wounds were sampled by using disposable tampon swabs maximizing collection of representative microflora. Tampons were subsequently stored in transport medium (inorganic salts, sodium thioglycolate, 1% agar, activated charcoal). The important part of our work-flow process comprised sampling in duplicates with further transport in both aerobic and anaerobic conditions to preserve bacterial viability (Chudobova et al. 2015).

Cultivation of clinical specimens

Four types of selective nutrient media (blood agar enriched by 10% NaCl, Endo agar, blood agar without any other component, and blood agar with amikacin) we employed for further microbiological selection. Petri dishes, containing the above mentioned media were subsequently incubated according to conventional protocols, as described elsewhere, to maintain suitable conditions for growth of all types of bacteria. These Petri dishes were incubated for 24–48 h at 37 °C supplemented by TGY medium (1 g L−1glucose, 5 g L−1tryptone, 2.5 g L−1yeast extract). Subsequently, individual colonies were collected from each Petri dish and stored in 1 µL of enriched media. These samples were processed and utilized for both – MALDI-TOF MS identification and PCR with subsequent sequencing. The glycerol stocks were prepared from bacterial cultures and 80% glycerol for long-term storage and further use (Chudobova et al. 2015).

Cultivation of bacterial strains

The pathogenic bacterial strain *Pseudomonas aeruginosa* from the infected wounds of the patients were cultivated. The composition of cultivation medium was as follows: Mueller Hinton broth 21 g and 1000 mL distilled water with 18 MΩ. Mueller Hinton agar 38 g into the 1 000 mL of distilled water. The pH of the cultivation medium was adjusted to pH 7.4. Prior experiments, the cultures were diluted by Phosphate-buffered saline (PBS) to OD_{600} nm = 0.5 McF standard

(Chudobova et al. 2015). PBS was prepared with NaCl 8 g, KCl 0.2 g, Na_2HPO_4 1.44 g, KH_2PO_4 0.2 g and 1000 mL of miliQ water (Chudobova et al. 2015).

Analysis of the inhibition zones using agar microdilution method

To determine the antimicrobial effect of the compounds on the bacterial culture of *Pseudomonas aeruginosa* the measurement of the inhibition zones was performed. Agar surface in Petri dish was covered with a mixture of 0.5 McF standard bacterial cultures (100 µL of 24 h bacterial cultures in the exponential phase of growth, and 3 mL of MH broth). Discs (Ø 0.6 cm) were filled with 10 µL of 2 mM antimicrobial agents. 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) at 37 °C for 24 h. After 24 hours of incubation, the inhibition zones were measured and photographed in each Petri dish (Chudobova et al. 2014).

Determination of growth properties

The second procedure for the evaluation of an antimicrobial effect of antimicrobial agents employed apparatus Multiskan EX (Thermo Fisher Scientific, Germany) for analysis of bacterial growth curves. The diluted cultures (OD_{600nm} = 0.5 McF and next dilution 1:100 with MH medium) were pipetted into a microplate (total volume of 200 µL) alone as a control variant, or with various concentrations of antimicrobial agents. The concentrations of compounds were 0; 15.6; 31.25; 62.5; 125; 250; 500; 1000 µM. Measurements were carried out at time 0, then each 30 min for 24 hours at 37 °C, at a wavelength of 600 nm (Chudobova et al. 2014).

Statistical analysis

Software STATISTICA (data analysis software system), version 10.0 (Tulsa, Oklahoma, USA) was used for data processing. The general regression model was used to analyse differences between the measured values. To reveal differences between the cell lines, Tukey's post hoc test within homogenous groups was employed. Unless noted otherwise, $p < 0.05$ was considered significant (Berney et al. 2007, Milosavljevic et al. 2017).

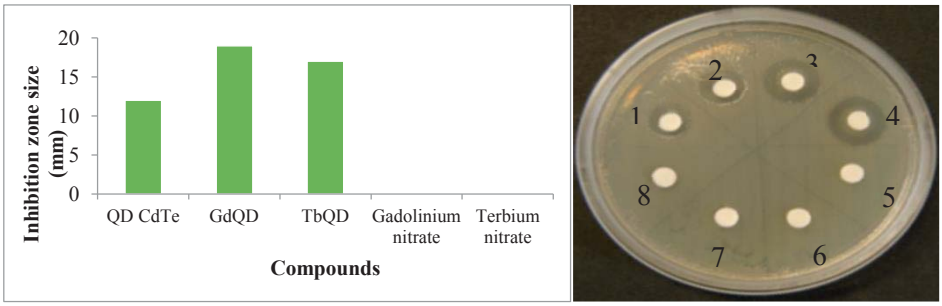
RESULTS AND DISCUSSION

The antibacterial activity of CdTe QDs modified with Lanthanides has been determined using disc-diffusion method and diameter size of the inhibition zone (mm) was showed (Figure 1). CdTe QDs modified with Lanthanides were applied on *Pseudomonas aeruginosa*. The influence of CdTe QDs modified with Lanthanides on pathogenic bacteria has been shown in Figure 1, 2 and 3. The highest inhibitory effect after 24 hours of incubation can be seen after the application of GdQD on *P. aeruginosa*.

Disc-diffusion method

The graphics and pictures below show the results of inhibition zones after the application of circular discs with antibacterial compounds against the pathogenic bacterial strain. Bacterial pathogen (OD_{600} = 0.5 McF) was exposed to a 2mM antibacterial agent and the incubation was carried out at 37 °C for 24 hours.

Figure 1 Results of the disc-diffusion test on Pseudomonas aeruginosa



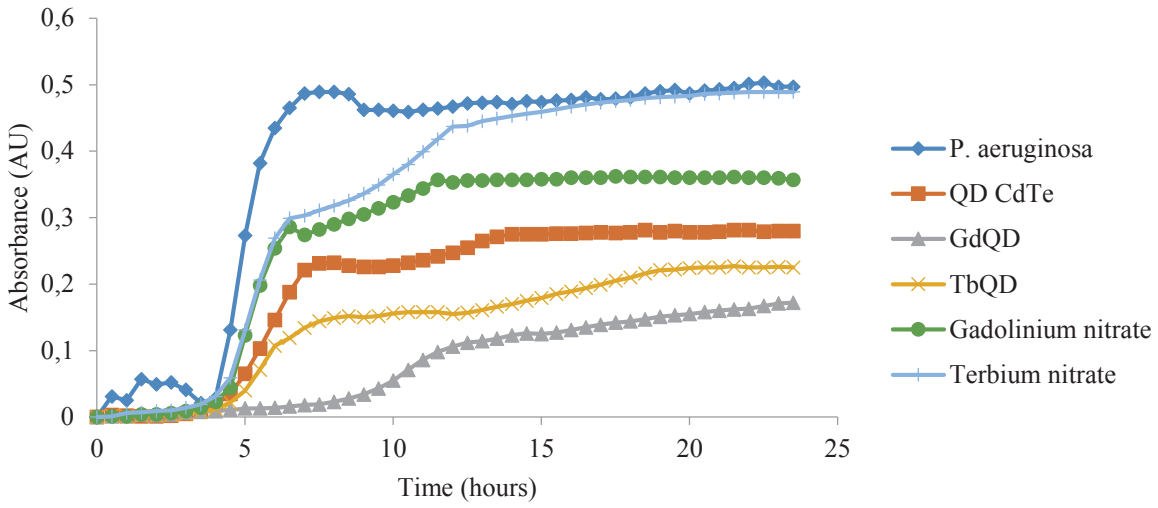
Legend: The discs on the plate: 1. QD CdTe; 2. TbQD; 3. TbQD; 4. GdQd; 5. Gadolinium nitrate; 6. Gadolinium nitrate; 7. Terbium nitrate; 8. Terbium nitrate

It can be noticed with the graphic and picture above, that the two compounds with lanthanides have the best results, with a significant difference between the QD CdTe and them. Although GdQD is working better than TbQD against *Pseudomonas aeruginosa.*

Determination of the growth properties of bacteria

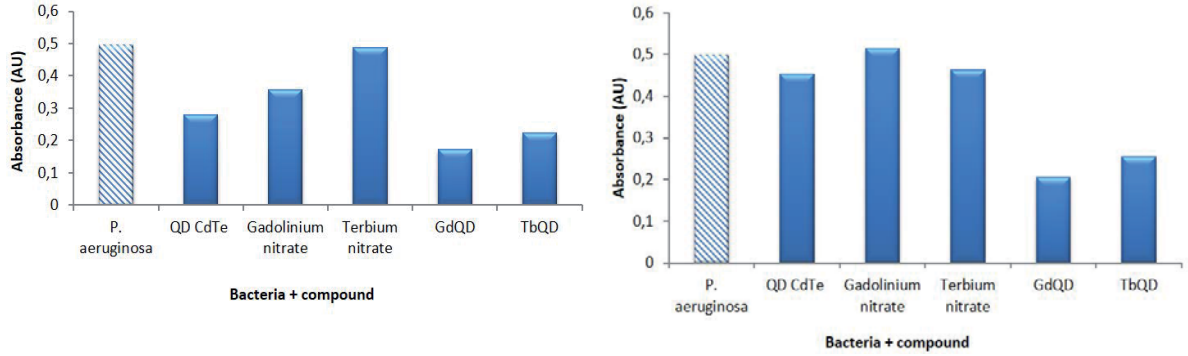
The growth curves of bacteria alone and after application with antimicrobial agent show that GdQD is the compound with higher effectiveness against *P. aeruginosa* in measurement by using Multiscan EX.

Figure 2 Growth curves after application of different compounds on Pseudomonas aeruginosa (concentration of the compound is 250 µM)



Comparison of the absorbance after 24 hours by Multiscan EX. The pathogenic bacteria were treated with and without different antimicrobial agents. In the Figure 3 it can be seen that each antimicrobial compound inhibited bacterial growth. The most effective in this case was GdQD on *Pseudomonas aeruginosa*.

Figure 3 Measurement of absorbance of Pseudomonas aeruginosa after 24 hours



Legend: The concentration of compounds, which was used in the experiment of measurement of Absorbance: the figure left 250 µM for each compound; the figure right 500 µM for each compound

CONCLUSION

The aim of this experiment was to study the antimicrobial effects of Cadmium Telluride Quantum dots with Lanthanides (Gadolinium and Terbium) and the chemicals themselves without Quantum dots against pathogenic bacterial strain (*Pseudomonas aeruginosa*). The antimicrobial activity of prepared CdTe QD with Lanthanides was measured by a disc-diffusion method, measurement of growth properties and comparison of absorbance measured after 24 hours by Multiscan EX. In view of the results obtained with the experiments carried out, it can be established as a conclusion that the QDs with Lanthanides are working well against the pathogenic

bacteria (*Pseudomonas aeruginosa*). Additionally, GdQDs showed stronger antibacterial effect than Tb QD and other tested compounds. The use of CdTe QD in combination with Lanthanides (mostly Gadolinium) appears to be a good way for the reduction of bacterial infection.

ACKNOWLEDGEMENTS
The research was financially supported by IGA grant, no. IP 10/2017.

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APOFERRITIN-MEDIATED DOXORUBICIN INTERNALIZATION THROUGH TRANSFERRIN RECEPTOR 1

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Abstract: This work is aimed at the possibilities of targeted drug delivery into the tumour tissue. This approach can greatly reduce the otherwise serious side effects of conventional treatment – systemic toxicity. For this purpose, ubiquitous protein cage apoferritin was employed as a carrier of cytotoxic drugs. Its molecule size of 10–12 nm allows it to employ the effect of increased permeability and retention as well as to avoid renal clearance. The cellular uptake of this carrier is known to be mediated *via* the transferrin receptor 1 (TfR1), which is overexpressed on metabolically highly active cells, such as cancer cells. Therefore, apoferritin's ability to deliver drug molecules to site-of-action was tested using cell lines with high, medium and low expression of TfR1. The optimal conditions for studying the expression of TfR1 using western blotting were as follows: lysate of 50 000 cells applied in non-reducing non-denaturing buffer and the concentration of the primary antibody of 1.0 µg/ml. The properties of encapsulated doxorubicin were not affected by apoferritin, thus preserving its toxicity for cells with high level of TfR1expression (30% growth inhibition of these cells after 24 h of treatment). The suitable usage of apoferritin as a nanocarrier for chemotherapeutic delivery was confirmed in this work.

Key Words: apoferritin, cancer, doxorubicin, nanomedicine

INTRODUCTION

Even with advances in health care, more and more people die of cancer every year. The main guilt lies on unhealthy life style, the environment we live in, and also on genetic predisposition. Every 5th woman and every 4th man in developed countries dies of cancer. Although these figures are alarming, not only the mortality itself but also the quality of life of patients with diagnosed and treated cancer should raise the question. Although we are able to treat various types of cancer, each of the administered treatment is accompanied by many side effects that negatively affect not only the physical but also the psychological aspects of patients' lives.

Chemotherapy can be divided into groups based on various factors including their chemical composition and function. This work is focused on anthracycline doxorubicin, one of the most commonly used chemotherapeutics (Erkekol 2011). Doxorubicin has been used for the treatment of cancer for over 30 years. Although its ability to kill fast-dividing cells and to slow development of disease is known for several decades, its use is limited by its high toxicity. Like most drugs, doxorubicin enters the cells through passive diffusion. It mainly accumulates in the liver, most likely due to the role of liver in drug metabolism. Up to 40% of patients with this treatment suffer from some form of liver damage. One of the other reasons why dose of doxorubicin should be limited is its

cardiotoxicity. Patients are negatively affected to varying degrees from chronic to acute conditions. Doxorubicin is responsible for structural changes in cardiomyocytes in the heart, especially their magnification (Carvalho et al. 2009).

Systemic toxicity of anti-cancer treatment can be limited by its targeting to tumour cells only. This can be limited by encapsulation of chemotherapeutic drugs into a suitable carrier that can be targeted to specific glycoproteins overexpressed on the membranes of tumour cells. One type of such antigens is the transferrin receptor 1(TfR1). This work deals with the employment of natural antigen for these receptors – apoferritin, which uses these transferrin receptors for internalization in cells. Apoferritin is suitable for use as nanocarrier of anti-tumour drugs to tumour cells, due to its properties. Unlike other, artificial nanocarriers, apoferritin's particles are homogeneous and have a uniform size of approximately 12 nm (Iwahori et al. 2005). Its main advantage is its ability of reversible dissociation and association depending on the surrounding pH, whereby small-molecule drugs can be easily and efficiently encapsulated into the internal cavity of the apoferritin without use of any organic solvents that are needed for encapsulation in other nanocarriers. Its safety and biodegradability are also ensured, due to its natural presence in organisms (Zang et al. 2017).

In this study, the conditions for the study of TfR1 expression in various tumour cell lines were optimized. The results obtained were verified by short- and long-term *in vitro* testing of the anti-tumour effect of apoferritin loaded with doxorubicin.

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).

Transferrin receptor 1 expression study

Quantitative expression of transferrin receptor 1 (TfR1) in cell lysates was studied using western blot. 50 000 cells were lysed with RIPA buffer and 10 µg of the lysate proteins was separated on 12.5% SDS PAGE. The proteins were transferred to the Immun-Blot® PVDF membrane (Bio-Rad, Hercules, CA, USA). Anti-Transferrin Receptor antibody 13E4 (ab38171, Abcam, Cambridge, UK) was used, diluted in antibody buffer [1 mg/ml BSA in phosphate buffered saline (PBS)] in 1 : 2000, 1 : 1500, 1 : 1000 and 1 : 500 ratio, respectively.

Study of the short-term effect of apodox on cells with varying levels of TfR1 expression

The encapsulation of doxorubicin into apoferritin (creating apodox) and doxorubicin concentration measurements were performed according to previous publication (Dostalova et al. 2017). Internalization of doxorubicin / apodox into cells and their short-term effects on them were monitored by ambient and fluorescence microscopy using the IX 71S8F-3 (Olympus, Tokyo, Japan). 1×10^5 UKF-NB4 (high TfR1 expression), PC-3 (medium TfR1 expression), and MDA-MB-231 (low TfR1 expression) cells in 1 ml of IMDM (for UKF-NB4) or RPMI 1640 (for PC-3 and MDA-MB-231) medium was seeded in a 12-well culture plate and incubated for 21 h. After the incubation, the medium was replaced with 200 µl of fresh medium containing 34 µM doxorubicin / apodox. The doxorubicin / apodox-treated cells were incubated for additional 2 h. Living cells were stained with CellRox® Green (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's instructions. Cell morphology was monitored under ambient light, fluorescein isothiocyanate filter (excitation of 460–495 nm, emission of 510–550 nm, dichroic mirror at 505 nm) was used to visualize oxidative stress, and fluorescence of doxorubicin was monitored using the Texas Red filter (excitation of 545–580 nm, emission of 610 nm, dichroic mirror at 600 nm) at 200-times magnification. All photos were uploaded and edited using the Stream Basic software.

Study of the long-term effect of apodox on cells with variying levels of TfR1 expression

To monitor the long-term effect of doxorubicin and apodox on tumour cell lines with varying levels of TfR1 expression, the XCELLigence RTCA DP (Roche Diagnostics, GmbH, Basel, Switzerland) was used. The background impedance was measured with 100 µl of culture media with doxorubicin / apodox. Then, 1.5×10^4 cells in 100 µl of culture medium were plated in 16-well

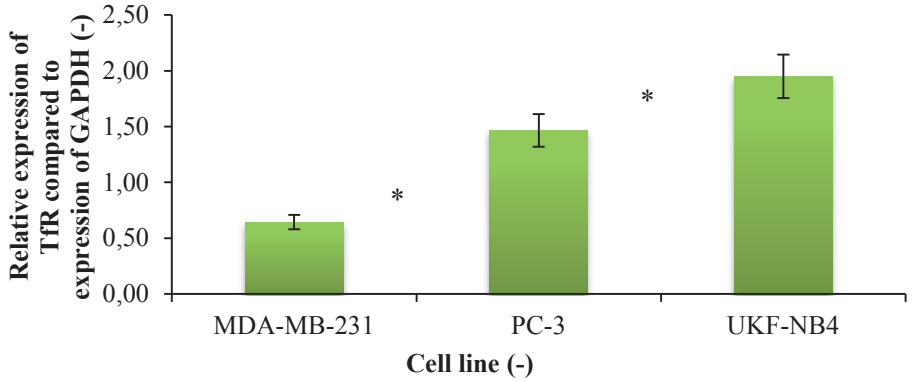
culture plate (Roche Diagnostic GmbH). Incubation was carried out at 37 °C in an atmosphere containing 5% CO_2. Cell proliferation was monitored every 30 s for 10 min, then every 30 min for 24 h. The experiments were performed in triplicates.

RESULTS AND DISCUSSION

Apoferritin is a protein cage composed of 24 subunits self-assembled by ferritin subunits in medium that does not contain iron. Ferritins are present in almost all living organism where their main function is the storage and transfer of iron (Zang et al. 2017). Ferritin employs TfR1 to internalize into cells (Suzumoto et al. 2012). According to literature, TfR1 is extensively expressed by some neoplastic cells on the surface of their cytoplasmic membrane to satisfy their higher metabolic needs (Peer et al. 2007).

To test the possibility of TfR1 use in nanomedicine, its expression in various cancer cell lines was evaluated (Figure 1). For this purpose, following cell lines were used: MDA-MB-231 (breast cancer cell line), PC-3 (prostate cancer cell line) and UKF-NB4 (neuroblastoma cell line). Significant differences in TfR1 expression were observed among these cell lines. UKF-NB4 showed the highest TfR1 expression (relative TfR1 expression of 1.9). PC-3 showed medium levels of TfR1 expression (1.4). The lowest levels of TfR1 expression were found in MDA-MB-231 cells (of 0.6).

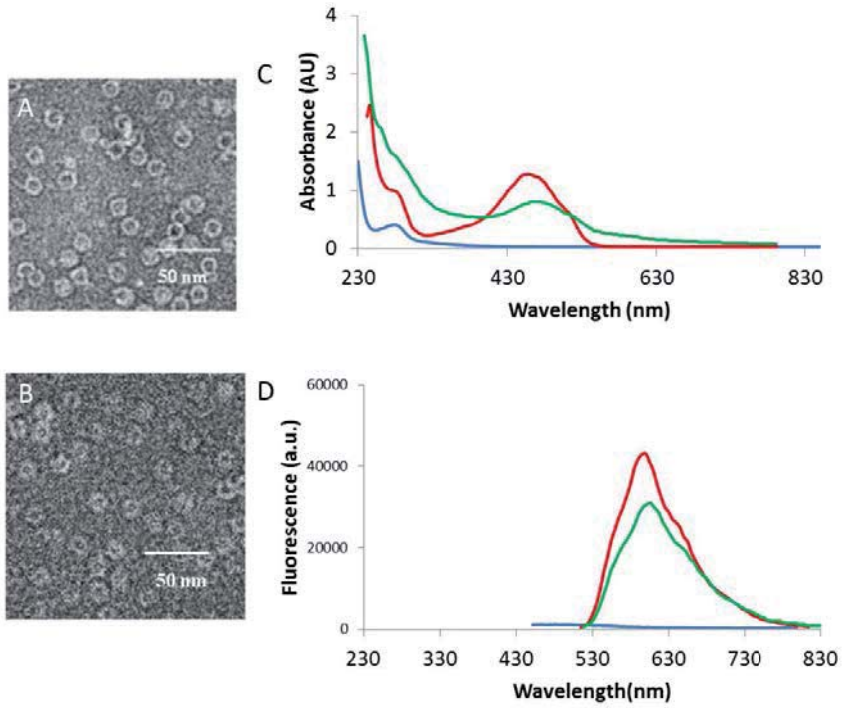
*Figure 1 Relative expression of TfR1 in different cell lines. MDA-MB-231- breast tumor; PC-3 - prostate cancer androgen independent; UKF-NB4 - neuroblastoma. * Determines a statistically significant difference ($p < 0.05$) in TfR1 expression between UKF-NB4 cells and other cell lines*



Even though apoferritin's cavity is naturally used to store iron ions, it can be artificially used to store and transfer any molecule of suitable molecular weight. Nanoparticles with a diameter below 100 nm can use the enhanced permeability and retention (EPR) effect in tumour tissues, so they can easily get into irregular, leaky tumour vessels with relatively large pores. In contrast, nanoparticles below 10 nm can be removed by normal vascular extravasation and renal clearance (Svenson 2013). Due to its size, apoferritin can employ the EPR effect of tumours for accumulation in cancerous tissue (Suzumoto et al. 2012) and TfR1 can then be employed in order to penetrate inside individual tumour cells (Peer et al. 2007). Moreover, apoferritin is suitable as a nanocarrier also due to its biocompatibility, high symmetry, solubility and stability, uniformity and ease of genetic and chemical manipulation (Zang et al. 2017).

The structure of the hollow protein cage was verified using a transmission electron microscope (Figure 2A). The size of the molecule was proven to be as expected, 10–12 nm in diameter (Suzumoto et al. 2012). The size did not increase after encapsulation (Figure 2B) and apoferritin retained its structure of icosahedral cage, although it can be seen that its cavity was filled by doxorubicin molecules, compared to apoferritin which showed empty cavity. However, the increase of its negative surface charge from -19.8 mV to -26.0 mV demonstrated that some doxorubicin molecules were probably bound to the surface of apoferritin during encapsulation and were not only encapsulated in the cavity. The amount of these doxorubicin molecules on the outer surface of apoferritin was low enough to not change the overall size of apoferritin (10–12 nm).

Figure 2 (A) – TEM apoferritin; (B) – TEM apodox; (C) – Absorbance spectra of apoferritin (blue colour), doxorubicin (red colour) and apodox (green colour); (D) – Fluorescence spectra of apoferritin (blue colour), doxorubicin (red colour) and apodox (green colour)



Furthermore, the optical properties of apoferritin, doxorubicin and apodox were characterized. The absorption spectrum (Figure 2C) clearly showed that apoferritin molecules absorbed only in UV area (with a specific absorbance of aromatic amino acids at 280 nm). Doxorubicin showed specific absorbance with maximum at 480 nm. Apodox showed absorbance spectrum containing both peaks characteristic for apoferritin and doxorubicin, although absorbance of encapsulated doxorubicin was lower than that of free doxorubicin. After filtration of non-encapsulated doxorubicin molecules, encapsulation efficiency was measured as 77%.

Doxorubicin also showed fluorescence after excitation at 480 nm (Figure 2D). It retained its fluorescent properties after encapsulation in apoferritin (creating apodox), which could be useful for detection of doxorubicin distribution *in vivo*. Due to the conditions for detection of doxorubicin, apoferritin fluorescence was examined at excitation wavelength of 480 nm. It is clear that apoferritin under these conditions showed no fluorescence.

Study of the short-term effect of apodox on cells with varying levels of TfR1 expression

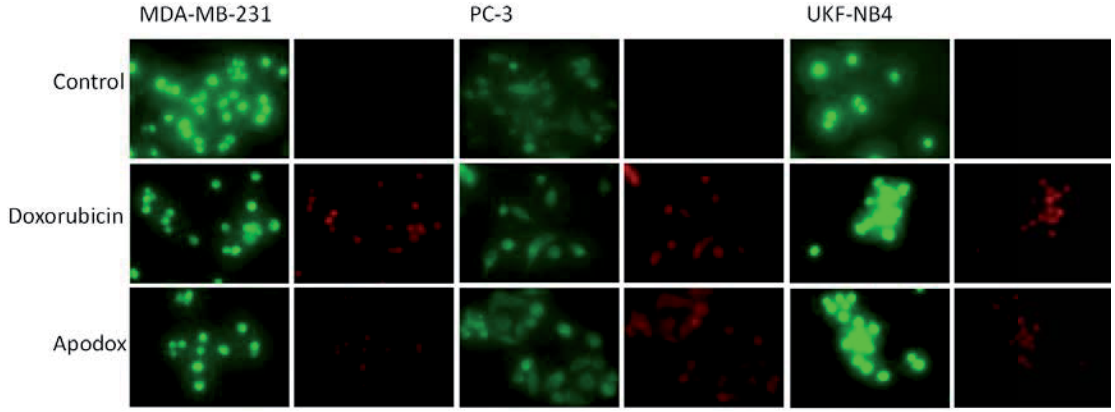
Thanks to the optical properties of doxorubicin, fluorescence microscopy was employed to evaluate the internalization of doxorubicin and apodox into tumour cells with different expression of TfR1 and their influence on oxidative stress of these cells (Figure 3). Oxidative stress is one of the main mechanisms by which doxorubicin and therefore apodox eliminate tumour cells (Shafiei-Roudbari et al. 2017).

No autofluorescence after excitation at 480 nm was detected in control sample of untreated cells. Free doxorubicin fluorescence differed among the cell lines. The highest fluorescence (39 a. u.) was observed in the tumour cell line with high TfR1 expression (line UKF-NB4). Medium doxorubicin fluorescence (35 a. u.) was observed in cell line PC-3 and lowest fluorescence (31 a. u.) was observed in cell line MDA-MB-231.

Oxidative stress is produced as a by-product of metabolism, by oxidation, degradation and detoxification of reactive intermediates. Therefore, this condition was observed not only in treated cells, but also untreated ones. After application of apodox and free doxorubicin, an increased oxidation stress level was expected. The level of oxidative stress caused by free doxorubicin was highest in case of UKF-NB4 (32 a. u.). Medium level of oxidative stress was observed in MDA-MB-231 cell line

(8 a. u.), while PC-3 cell line showed similar level of oxidative stress after doxorubicin treatment as in untreated cells.

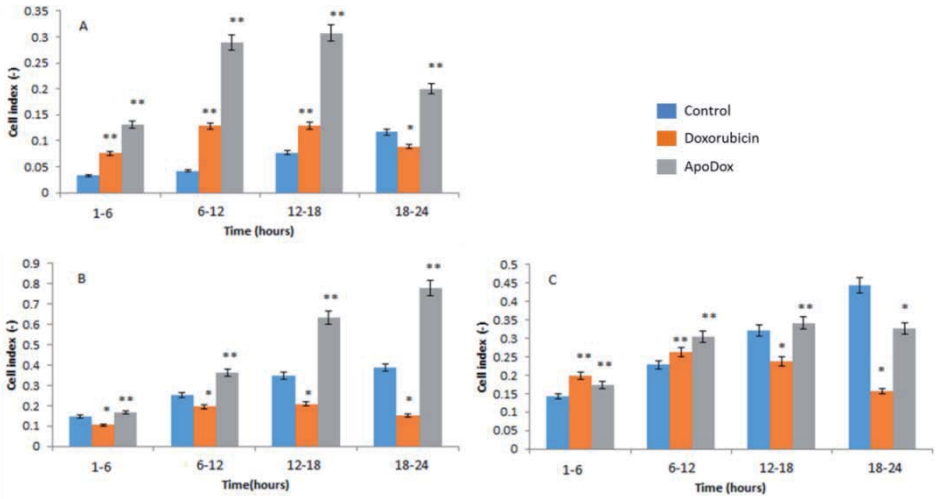
Figure 3 Fluorescence microscopy showing fluorescence (red colour) of doxorubicin and doxorubicin encapsulated in apoferritin (apodox) internalized into tumour cell lines with low (MDA-MB-231), medium (PC-3) and high (line UKF-NB4) expression of TfR1. Fluorescence microscopy showing the degree of oxidative stress (green colour) after treatment with doxorubicin and apodox



In the case of apodox treatment, the dependence of internalization of apodox into the cells was assumed to be higher. The difference between the cancerous cell lines with the highest and lowest TfR1 expression was statistically significant. The higher the expression of TfR1, the higher was the observed apodox fluorescence (33 a. u. for UKF-NB4, 29 a. u. for PC-3 and 27 a. u. for MDA-MB-231). However, oxidative stress showed different trend where highest level was observed in UKF-NB4 cells (26 a. u.), followed by MDA-MB-231 cells (4 a. u.), while PC-3 cells showed no level of oxidative stress when compared to untreated cells.

Study of the long-term effect of apodox on cells with varying levels of TfR1 expression

*Figure 4 Cell proliferation rates at 6 h intervals at the MDA-MD-231 (A); PC-3 (B) and UKF-NB4 (C) cell line untreated and treated with doxorubicin and apodox. * Determines a statistically significant ($p < 0.05$) decrease in the growth of the treated cells as compared to that of control cells. ** determines a statistically significant increase ($p < 0.05$) in the growth of treated cells compared to that of control cells*



Since the internalization of free doxorubicin and apodox showed the same trend in the short-term study, and the fluorescence of doxorubicin at its high concentrations may not be reliable, the long-term effect of doxorubicin and apodox on these cells was further studied (Figure 4).

The growth of all of the tested cell lines was inhibited after 24 h of doxorubicin treatment (25% for MDA-MB-231, 63% for PC-3 and 66% for UKF-NB4). However, their growth during treatment with apodox was significantly dependent on their TfR1 expression. Both MDA-MB-231 (Figure 4A)

and PC-3 (Figure 4B) cell lines showed increase in their growth after treatment with apodox. This was probably caused by hormetic effect, increase in cellular growth caused by adaptive response of cells to low concentration of toxic molecules (Mattson 2008). However, cells with high TfR1 expression were apparently able to internalize enough apodox to inhibit their growth by 30% after 24 h treatment. The significant differences caused by different TfR1 expression show reliable natural targeting of apoferritin nanocarrier and therefore its suitability for use in nanomedicine.

CONCLUSION

The experiment presented in this work dealt with the evaluation of the ability of naturally occurring and versatile protein apoferritin to deliver anti-tumour drugs selectively to cancer cells *via* transferrin receptors. Overall, the presented nanocarrier showed the optimal size in the range of 10–12 nm, required for passive targeting to tumours *via* EPR effect, while avoiding removal from the body through renal clearance. The various degrees of internalization of apodox into cells with different TfR1 expression and its long-term effect on these cells have been observed. This has confirmed the presumption of transferrin receptor function as a mechanism by which the nanocarrier penetrates metabolically highly active cancer cells.

ACKNOWLEDGEMENTS

The research was financially supported by the Czech Science Foundation (GACR 17-12816S) and CEITEC 2020 (LQ1601).

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EFFECT OF THE SELECTED PHENOLIC AND FLAVONOID COMPOUNDS OF BLACK PEPPER AND CARAWAY SEEDS ON PROSTATE CELLS

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Abstract: In this study, the effect of selected phenolic and flavonoid compounds of black pepper and caraway seeds on prostate cells (PNT1A, 22RV1 and PC3) was observed. Synthetic standards of 3,4-dihydroxybenzaldehyde and naringenin chalcone, identified previously by HPLC-MS in black pepper seeds extracts, and neochlorogenic acid and apigenin, identified in caraway seeds extracts, were applied. For the evaluation of the potential inhibitory effect of selected compounds on PNT1A, 22RV1 and PC3 cells, the clonogenic assay and the microscopic observation of cells were done. The results of clonogenic assay showed that phenolic compounds had the strongest inhibitory effect on 22RV1 and PC3 cells, while the flavonoid compounds had the strongest inhibitory effect on PNT1A cells.

Key Words: spices, clonogenic assay, phenolic compounds, flavonoid compounds, prostate cell lines

INTRODUCTION

Prostate cancer is the second cause of cancer death for men (Atabi et al. 2017). A number of studies which show the anti-carcinogenic effects of piperine (Samykutty et al. 2013), curcumine (Nakamura et al. 2002, Chendil et al. 2004, Wei et al. 2012) or capsaicine (Surh and Kundu 2011) in prostate cancer cells have been conducted. In the context of previous research our experiment was focused on the studying of anti-carcinogenic ability of selected phenolic and flavonoid compounds identified by HPLC-MS analyses in methanolic extracts of blank pepper seeds and caraway seeds. Previously published studies were focused on anti-cancerogenic effect of piperine, originated from black pepper seeds, on prostate cancer cells (Ouyang et al. 2013, Samykutty et al. 2013). Regarding the effect of caraway seeds phenolic extracts on prostate cells, no studies have been published yet. The selection of the most common phenolic and flavonoid compounds in caraway and black pepper seeds was based on our previous experiment (Lackova et al. 2016). This experiment showed that the most abundant phenolic and flavonoid compounds in black pepper are 3,4-dihydroxybenzaldehyde and naringenin chalcone, respectively. In caraway seeds, the most abundant phenolic compound was identified a neochlorogenic acid, whereas the most abundant flavonoid compound was an apigenin (Lackova et al. 2016). 3,4-dihydroxybenzaldehyde has anti-inflammatory and antioxidant effects, decreased proliferation effect on human cancer and induced apoptosis properties (Banerjee et al. 2016). Naringenin chalcone has inhibitory effects on some cancer cells (Zhang et al. 2016). Neochlorogenic acid has demonstrated the antioxidant and chemopreventive activity in some cancer cells (Banerjee et al. 2016). Apigenin inhibits tumor growth and angiogenesis induced by different cancer cells (He et al. 2012). Nevertheless, the effect of these four compounds has not been investigated in the prostate cells yet.

Based on our previous study (Lackova et al. 2016), we decided to determine the effect of selected phenolic and flavonoid compounds on the prostate carcinoma cell lines. In this experiment,

prostatic cells were exposed to selected phenolic and flavonoid compounds followed by clonogenic assay and cell observation performed under a microscope in time-dependent manner.

MATERIAL AND METHODS

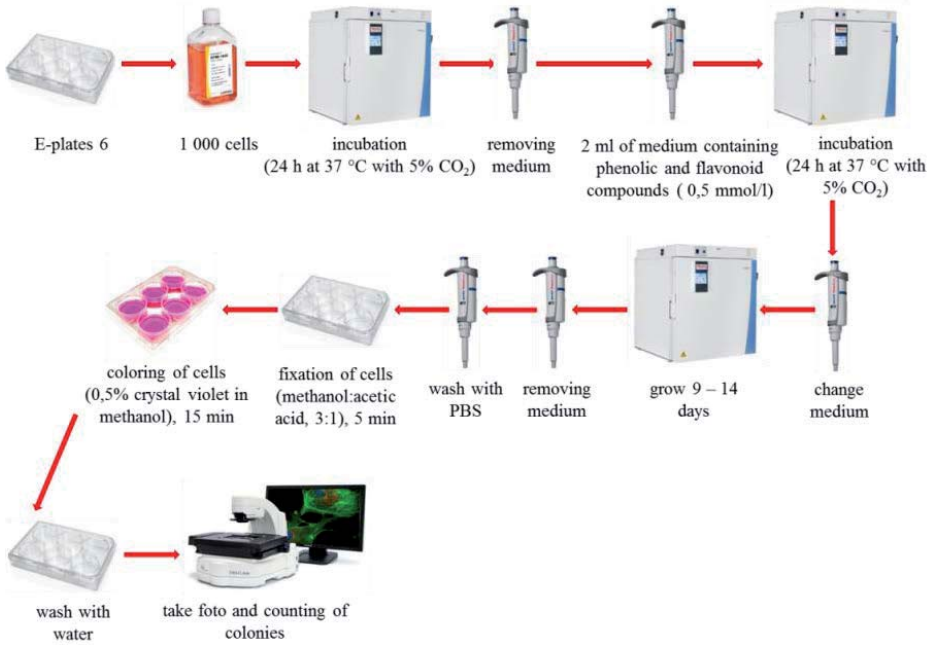
Selected phenolic (3,4-dihydroxybenzaldehyde, neochlorogenic acid) and flavonoid (naringenin chalcone, apigenin) compounds were used. Apigenin and neochlorogenic standards were purchased from Extrasynthese (Genay, France). Naringenin chalcone standard was purchased from Phytolab (Vestenbergsgreuth, Germany). Three types of prostatic cells, PNT1A (immortalization of normal adult prostatic epithelial cells), 22RV1 (androgen dependent) and PC3 (androgen independent) cells were used. All cell lines used in this study were purchased from Health Protection Agency Culture Collection (Salisbury, UK). PNT1A, 22RV1 and PC3 cell lines were cultured in RPMI-1640 medium with 10% fetal bovine serum, supplemented with penicillin (100 U/ml) and streptomycin (0.1 mg/ml). Cells were then harvested, washed four times with PBS, pH 7.4, and counted using Countess IIFL Automated Cell Counter (Life Technologies, Carlsbad, CA). Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) in ACS purity.

The preparation of samples containing phenolic and flavonoid compounds for observation of cells under a microscope (EVOS FL Auto Cell Imaging system, TermoFisher Scientific, USA) was performed at 0, 1, 3, 6 and 12 hours of treatment (Figure 1). Images at a 400 µm magnification were obtained. Experiments were performed in duplicate.

Figure 1 Scheme of samples preparation for a microscopic observation

After observation of cells under a microscope, a clonogenic assay was performed (Figure 2). Cells were washed with Milli-Q water. Images were obtained with Canon EOS 650D (Canon, Óta, Japonsko). Experiments were performed in duplicate.

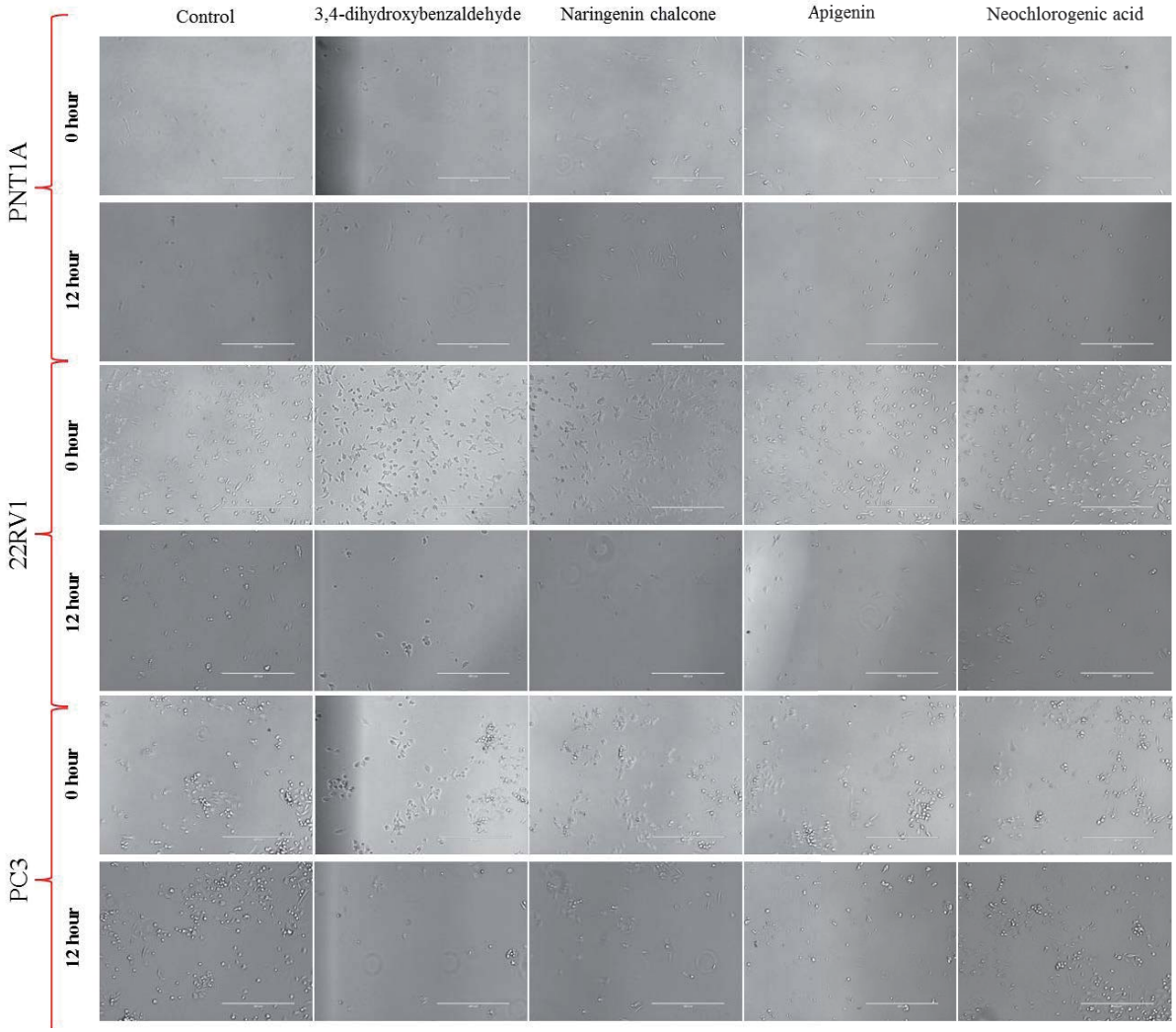
Figure 2 Scheme of samples preparation for a clonogenic assay



RESULTS AND DISCUSSION

The results of cells observation under a microscope (Figure 3) showed that PNT1A cells did not exhibit significant changes in growth over the 12-hour period compared to controls. PNT1A cells were growing very slowly in control samples. Nevertheless, different results were obtained for 22RV1 and PC3 cells when compared with PNT1A cells. The selected phenolic and flavonoid compounds have caused an inhibitory effect on 22RV1 and PC3 cells. In both cases, there was a gradual accumulation of cells and their inhibition compared to the control at time 0 hours.

Figure 3 Results of observation of cells under a microscope from 0 to 12 hours



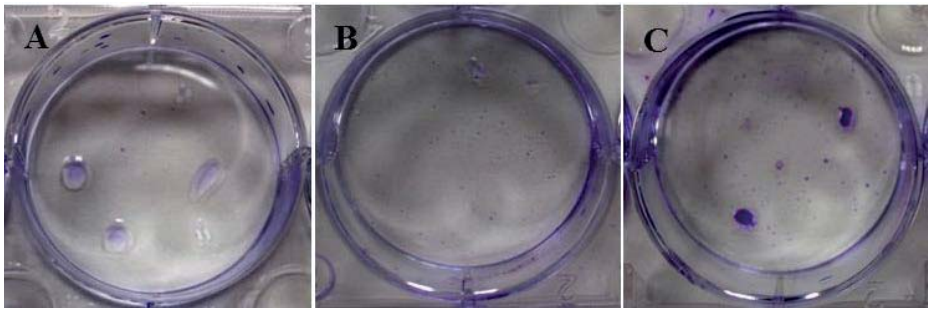
A clonogenic assay assesses the number of colonies growing after the treatment with the test compound, in our case with selected phenolic and flavonoid compounds. In all cell lines, the reduction of the number of colonies after the treatment with phenolic and flavonoid compounds was observed in comparison to control cell lines. The highest number of colonies, compared with control, was observed for PNT1A cells treated with naringenin chalcone. Conversely, the lowest number of colonies was observed when apigenin was applying, compared with control. For 22RV1 cells, the highest number of colonies was observed after the application of 3,4-dihydroxybenzaldehyde, and the lowest number of colonies was observed after the application of neochlorogenic acid, compared with control. For PC3 cells, the results were similar as in the case of 22RV1 cell lines. The highest number of colonies, compared with control for PC3 cells, was observed when naringenin chalcone was applied, while the lowest number of colonies was observed after the treatment with neochlorogenic acid.

Table 1 Results of clonogenic assay for PNT1A, 22RV1 and PC3 cells

	PNT1A cells		22RV1 cells		PC3 cells	
Compounds	**Number of colonies***	**%**	**Number of colonies***	**%**	**Number of colonies***	**%**
Control	21.5	100	132.5	100	164.5	100
3,4-dihydroxybenzaldehyde	2.5	12	44.5	34	40.5	25
Naringenin chalcone	19.0	88	31.0	23	46.5	28
Apigenin	1.5	7	34.0	26	31.5	19
Neochlorogenic acid	3.0	14	2.5	2	24.5	15

*Legend: * average of two measured values*

*Figure 4 Growing colonies in controls (**A**) for PNT1A cells, (**B**) for 22RV1 cells, (**C**) for PC3 cells*



CONCLUSION

The aim of the experiment was to investigate inhibitory effect of selected phenolic and flavonoid compounds of black pepper and caraway seeds on three prostate cells (PNT1A, 22RV1 and PC3). From black pepper, 3,4-dihydroxybenazldehyde and naringenin chalcone were used. From caraway seeds, neochlorogenic acid and apigenin were used. The results of observation of cells under a microscope showed that all four applied compounds had inhibitory effect on all cell lines used in the study. The results of clonogenic assay showed that the lowest number of colonies was observed for PNT1A treated with apigenin compared with control. For 22RV1 and PC3 cells, the lowest number of colonies compared with control was observed after the treatment with neochlorogenic acid.

This data serves as a pilot study for a larger experiment evaluating the effect of 3,4-dihydroxybenzaldehyde, neochlorogenic acid, naringenin chalcone and apigenin on prostate cell lines.

ACKNOWLEDGEMENT

This research was carried out under the project CEITEC 2020 (LQ1601) with financial support from the Ministry of Education, Youth and Sports of the Czech Republic under the National Sustainability Programme II and MENDELU IGA IP 33/2017.

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DETERMINATION OF HYDROXYPROLINE USING ION-EXCHANGE LIQUID CHROMATOGRAPHY WITH VIS DETECTOR AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTOR

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Abstract: The first aim of the experiment was an optimization of the method for determination of hydroxyproline in a pig skin using high-performance liquid chromatography with fluorescence detector (HPLC-FLD) and ion-exchange liquid chromatography with VIS detector (IEC-VIS). On the basis of the experiments performed, it was found that HPLC-FLD method is three times more sensitive than IEC-VIS method. For IEC analysis of hydroxyproline, the limit of detection (LOD) was 4.10 µg/ml whereas the limit of quantification (LOQ) was 13.50 µg/ml. For HPLC analysis of hydroxyproline, the limit of detection (LOD) was 1 ng/ml whereas the limit of quantification (LOQ) was 3 ng/ml. The second aim was the testing of influence of different volume of 6M HCl on extraction of sample (50 mg) for analysis using HPLC-FLD. Here it was found that the best volume was 250 µl 6M HCl.

Key Words: hydroxyproline, HPLC-FLD, IEC-VIS, microwave hydrolysis, pig skin

INTRODUCTION

Hydroxyproline ($C_5H_9O_3N$) is a non-proteinogenic amino acid produced by hydroxylation of the amino acid proline via prolyl hydroxylase enzyme (Gorres and Raines 2010). Hydroxyproline and proline are a main part of collagen of animals (Zhang and Duan 2017) and humans (Sugioka et al. 2017). Hydroxyproline organize the triple-helical structure of collagen and played key role in collagen stability (Shoulders and Raines 2009). Content of hydroxyproline in biological fluids is used as a parameter of collagen catabolism (Lv et al. 2017), especially bone resorption or tissue degradation (Kimura et al. 2017). Different changes in hydroxyproline metabolism play major roles in the pathophysiology and pathogenesis of various diseases (Srivastava et al. 2016). The elevated level of hydroxyproline was observed in several disorders, i.e. graft versus host disease, keloids, vitiligo, cases of depression and stress (Srivastava et al. 2016, Dong et al. 2017). Decreased level of hydroxyproline is a marker of poor wound-healing (Srivastava et al. 2016). Degradation of collagen may accelerate the increased amount of reactive oxygen species (ROS) (Shi et al. 2016). Hydroxyproline is a potential oxidative biomarker for diagnosis of fibrosis in hepatitis C (Attallah et al. 2007), adiposity and cardiometabolic health (Jennings et al. 2016).

Several methods are usually used for determination of hydroxyproline from different types of matrices, including high-performance liquid chromatography with fluorescence detection (Ren et al.

2017), high-performance liquid chromatography with tandem mass spectrometric detection (LC/MS/MS) (Shiota et al. 2017), hydrophilic interaction chromatography with tandem mass spectrometry and ion-exchange liquid chromatography with post-column derivatization (Rigas 2012, Zhu et al. 2014).

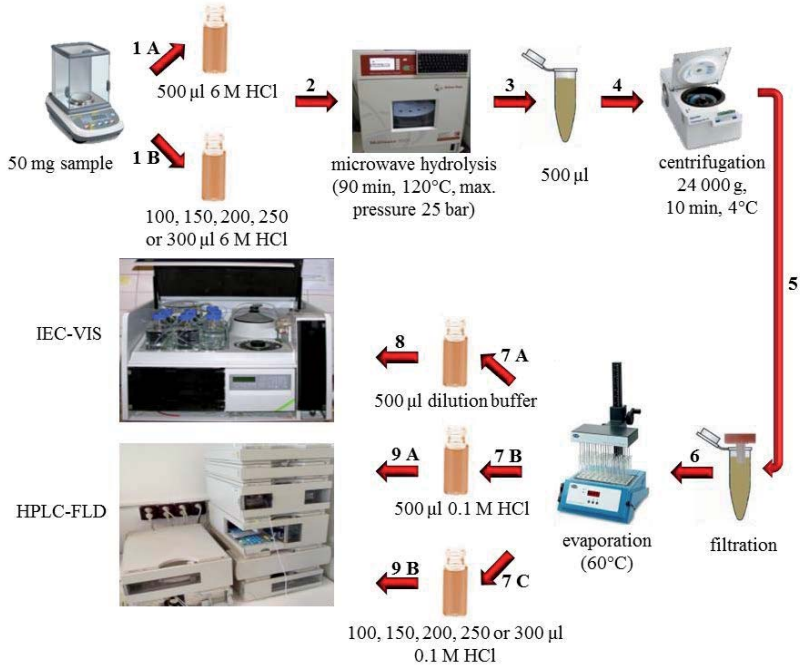
We have focused this work on optimization of two chromatographic methods, namely, ion-exchange liquid chromatography with VIS detector and high-performance liquid chromatography with fluorescence detector.

MATERIAL AND METHODS

A pig skin was used in this experiment. The preparation of samples for high-performance liquid chromatography with fluorescence detector (HPLC-FLD) and ion-exchange liquid chromatography with VIS detector (IEC-VIS) were a bit different. For the first aim of this experiment, the sample preparation included the following process numbers (Figure 1): 1 A, 2, 3, 4, 5, 6, 7 A, 7 B, 8 and 9 A. As an alternative sample preparation procedure, we chose different volumes of 6M HCl for sampling 50 mg of a sample. This sample preparation method was used for HPLC-FLD analysis. For the second aim of this experiment, the sample preparation included the following process numbers (Figure 1): 1 B, 2, 3, 4, 5, 6, 7 C and 9 B.

The dilution buffer consisted of thiodiglycol (5 ml/l), sodium azide (0.1 g/l), citric acid (14 g/l) and sodium chloride (11.5 g/l).

Figure1 Scheme of the pig skin sample preparation for IEC-VIS and for HPLC-FLD analysis and scheme of optimization of the pig skin samples preparation for HPLC-FLD analysis



For the primary determination of hydroxyproline, an ion-exchange liquid chromatograph AAA-400 (Ingos, Czech Republic) with post column derivatization by ninhydrin and an absorbance detector in visible light range (VIS) were used. A glass column with an inner diameter of 3.7 mm and a length of 350 mm was filled manually with strong cation exchanger (LG ANB, Ingos, Czech Republic) with ~12 µm particles and 8% porosity. The column was equilibrated at 60 °C. A double-channel VIS detector with an inner cell of 5 µl was set to two wavelengths: 440 and 570 nm. A prepared solution of ninhydrin was stored under nitrogen atmosphere in the dark at 4 °C. The elution of amino acids was carried out by buffer containing 10.0 g of citric acid, 5.6 g of sodium citrate, and 8.4 g of sodium chloride per one liter of solution (pH 2.7). The flow rate was 0.25 ml/min. The reactor temperature was set to 120 °C. All chemicals were purchased from Ingos (Ingos, Czech Republic).

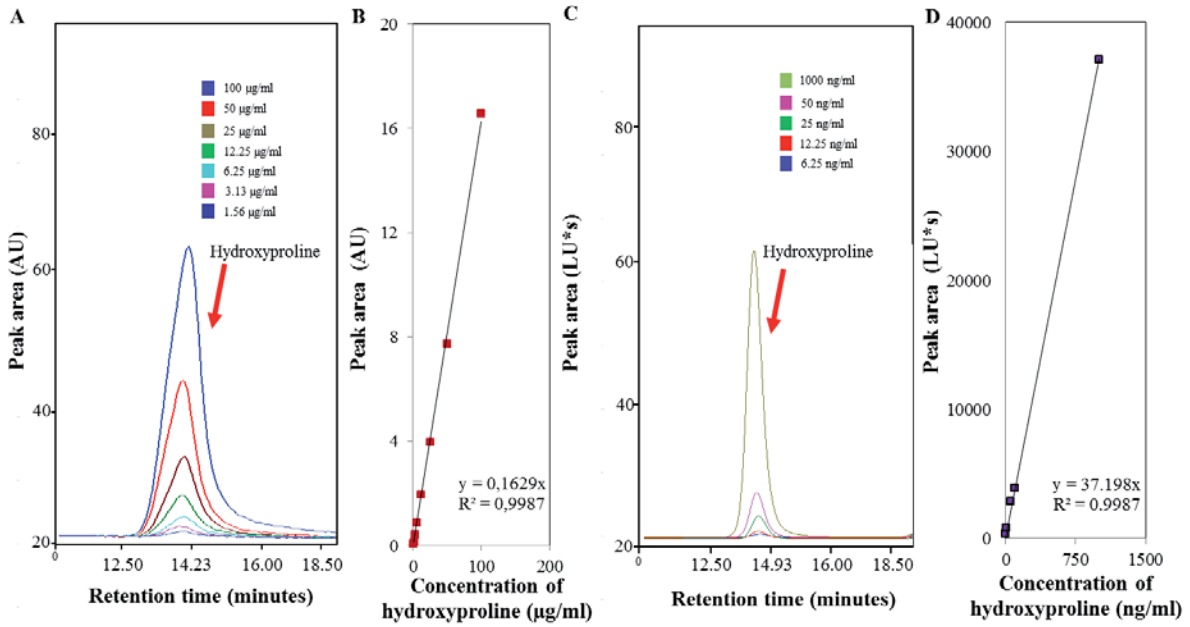
For alternative determination of hydroxyproline, a high-performance liquid chromatograph HP 1100 Series with FLD detector (HP, Germany) was used. The HPLC chromatographic system was controlled with ChemStation software (rev. A 07.01). The column effluent was monitored with a diode-array detector at 338 nm (10 nm bandwidth) and a fluorescence detector at $340_{ex}/450_{em}$ nm using the OPA reagent for precolumn derivatization. For the separation of amino acids, the column Zorbax Eclipse AAA (Agilent Technologies, USA) with dimensions 150 x 4.6 mm and a particle size of 3.5 µm was used. The column was equilibrated at 40 °C. A mobile phase A consisted of 40 mM Na_2HPO_4 at pH 7.8 (5.5 g of NaH_2PO_4 monohydrate + 1 l of H_2O, adjusted to pH 7.8 with 10M NaOH solution) and a mobile phase B was acetonitrile/methanol/water (45:45:10 v/v). The flow rate of mobile phase was 2 ml/min. The compounds were eluted with a linear upward gradient: 0.0 min (0% B) 0.8 min (57% B) 10.0 min (100% B) 12.5 min (0% B) to 14.0 min. All chemicals were purchased from Sigma-Aldrich (St. Louis, USA).

RESULTS AND DISCUSSION

A determination of the presence and concentration of hydroxyproline was held by a high-performance liquid chromatography with fluorescence detection and an ion-exchange liquid chromatography with VIS detector. The content of hydroxyproline in a pig skin was expressed as mean ± standard deviation from three replicates.

Two calibration curves were prepared in the ranges 1.56 – 100.00 µg/ml for IEC-VIS and 6.25 – 1000.00 ng/ml for HPLC-FLD. The calibration curve for IEC-VIS showed a good linearity with correlation coefficient R^2 = 0.9987 and R.S.D. = 2.1% (Figure 2A, B). The calibration curve for HPLC-FLD showed a good linearity with correlation coefficient R^2 = 0.9987 and R.S.D. = 1.75% (Figure 2C, D).

*Figure 2 (**A**) Chromatograms from calibration dependence of hydroxyproline determined by IEC-VIS, (**B**) calibration curve measured within the range from 1.56 to 100.00 µg/ml under the experimental conditions, (**C**) chromatograms from calibration dependence of hydroxyproline determined by HPLC-FLD, (**D**) calibration curve measured within the range from 6.25 to 1000.00 ng/ml under the experimental conditions*



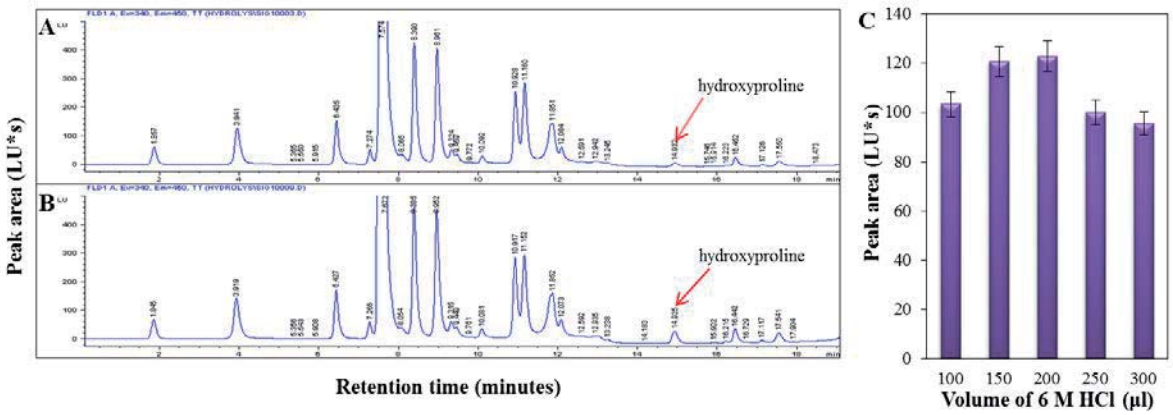
After comparing the results of HPLC-FLD and IEC-VIS methods, it was clear that HPLC-FLD provided higher assay sensitivity than IEC-VIS. A higher sensitivity of HPLC-FLD is demonstrated by a calibration curve where the calibration range is in an order of ng/ml, whereas the calibration range for IEC-VIS method is in an order of µg/ml. Another advantage of HPLC-FLD is that FLD is a selective detector and therefore HPLC-FLD could be used as a confirmatory method. HPLC-VIS

results always need to be confirmed using any other method, for example LC-MS. This may be the subject of further experiments. Another advantage is the sample volume, which is 250 µl for IEC-VIS and 5 µl for HPLC-FLD, respectively. Therefore, only HPLC-FLD was used for further experiments. The initial preparation of the sample was replaced and different volumes of 6M HCl were optimized for a load of 50 mg of a sample.

The representation of influence of different volumes of 6M HCl for mineralization of a pig skin sample is shown in figure 3C. For each vial containing 50 mg of a pig skin, the following volumes of 6M HCl were added: 100, 150, 200, 250 and 300 µl. The results shown in figure 3C indicate that the best yield of hydroxyproline was acquired using a volume of 200 µl of 6M HCl compared to the other volumes of 6M HCl.

In figures 3A and 3B, the HPLC-FLD chromatogram of real samples of a pig skin is shown. The chromatogram in figure 3A shows the presence of hydroxyproline in a 50 mg real sample dissolved in 200 µl of 6M HCl. Hydroxyproline concentration was 4 ng/ml. The chromatogram in figure 3B shows the presence of hydroxyproline in a 50 mg real sample dissolved in 200 µl of 6M HCl spiked with 10 ng/ml of a standard hydroxyproline. Hydroxyproline concentration was 14 ng/ml.

*Figure 3 Chromatogram of real sample of a pig skin (50 mg), mineralized in 200 µl of 6M HCl, (**A**) without spike and (**B**) spiked with standard of hydroxyproline (10 ng/ml). (**C**) Influence of added different volume of 6M HCl to the 50 mg of real samples of a pig skin on the concentration of hydroxyproline after microwave mineralization.*



CONCLUSION

Based on the experiment, the comparison of two tested methods, HPLC-FLD and IEC-VIS, showed that the HPLC-FLD method is much more sensitive than the IEC-VIS method for the detection and quantitation of hydroxyproline. The HPLC-FLD sensitivity versus IEC-VIS sensitivity was three orders better (µg/ml versus ng/ml). Another advantage is the used sample volume, which is 250 µl for IEC-VIS and 5 µl for HPLC-FLD. From the results of the optimization of the 6M HCl volume used in dilution of 50 mg of a real sample, it was found that the best for the highest yield of hydroxyproline from the real sample was a 200 µl of 6M HCl. The hydroxyproline concentration 4 ng/ml in the real 50 mg sample was optimized with the use of 200-µl 6M HCl. It was proved, that HPLC-FLD is a suitable method for analysis of hydroxyproline in low concentrations and sample amounts and can be used as a detection method for analysis of real samples of a skin for further experiments.

ACKNOWLEDGEMENT

This research was carried out under the project CEITEC 2020 (LQ1601) with financial support from the Ministry of Education, Youth and Sports of the Czech Republic under the National Sustainability Programme II and MENDELU IGA TP3/2017.

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HOW MUCH IS NOT ENOUGH? PEPTITE-BASED IDENTIFICATION AND QUANTITATION OF PROTEINS

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Abstract: Protein identification and quantitation is routinely based on oligopeptides longer than six amino acids. Here, we examined theoretical tryptic digests of five contrasting model proteomes and evaluated potential benefits of shorter peptide sequences for proteome analyses. Our results indicate that pentapeptides should not be excluded and shorter sequences may present valid targets in a dedicated targeted analysis.

Key Words: protein identification, protein quantitation, oligopeptides, tryptic peptides

INTRODUCTION

In the last two decades, mass spectrometry has been established as a method of choice to identify and quantify proteins. The so-called "Bottom-up" shotgun approach is by far the most common method to do that. In this technique, a protein or a protein mixture is digested and the resulting peptides are analyzed. The key to this technique is the detection of a unique peptide that is exclusive to a given protein and can not originate from a homologous sequence in a different one. However, evolution and gene duplication events resulted in homologous sequence motifs that are shared between different proteins and it is thus often impossible to distinguish between them. In this article, we provide a comparative analysis of tryptic protein digests of five representative model organisms and discuss the implications for targeted quantitative analyses and peptide-based identifications.

MATERIAL AND METHODS

Proteome sequences

Proteome sequences were downloaded from the Ensembl (*H. sapiens*), EnsemblBacteria (*E. coli*), EnsemblFungi (*S. cerevisiae*), Araport (*A. thaliana*) and NCBI (*P. palmivora*) databases.

Analysis of tryptic peptides

Tryptic digests were generated with Protein Digestion Simulator (https://omics.pnl.gov/); MS Excel was used for the data filtering and Skyline 3.7 (https://skyline.ms/) to assess the peptide uniqueness.

Protein identification

The spectra from Arabidopsis seedlings (Baldrianova et al. 2015) were processed against Araport 11 database by Sequest HT 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. The search against concatenated database of unique tetra-, penta- and hexapeptides had the following parameters: Enzyme - trypsin, no missed cleavage; Mass tolerance - 35 ppm (MS) and 0.1 Da (MS/MS); Modifications - no modification allowed. Data were processed and visualized by ProteomeDiscoverer 2.2 (Thermo).

RESULTS AND DISCUSSION

In silico trypsin digestion

There are 22 known proteinogenic amino acids and thus there could be 22^n possible combinations for peptide sequence that contains n amino acids (Černý et al. 2013). However, peptides are not random combinations and thus not all possibilities are found in nature. In fact, our data show that only a minor fraction is actually employed in real protein structures. We analyzed sequences of all putative tryptic peptides three to seven amino acids in length that could originate in the tryptic digest of five contrasting species: bacteria *Escherichia coli*, yeast *Saccharomyces cerevisiae*, plant pathogen *Phytophthora palmivora* and *Homo sapiens* (Table 1). Trypsin is the most commonly used enzyme for a protein digestion with high substrate specificity and cleaves next to arginine or lysine. It was believed that trypsin does not cleave before proline but this so-called Keil rule has been challenged in the past. Our putative tryptic peptides were thus generated to include any amino acid sequence that could be present in the fully tryptic digest with no missed cleavages or "Proline Rule". This means that there could exist $20\times22^{(n-1)}$ unique combinations.

Table 1 Putative tryptic peptide combinations. Calculated number of all possible combinations and peptides found in silico in fully tryptic digests of five evolutionary distant model proteomes. Numbers in thousands.

Peptide length	n. combinations	*E. coli*	*S. cerevisiae*	*P. palmivora*	*A. thaliana*	*H. sapiens*
3	8.8	1.1	1.1	2.4	2.2	3.4
4	176.0	6.1	8.7	13.0	12.8	15.4
5	3520.0	9.8	20.3	53.2	56.8	62.0
6	70400.0	8.9	20.0	62.6	68.4	76.9
7	1408000.0	8.5	17.7	57.9	64.9	71.0

The ratio of theoretical amount to real amount of amino acid combinations has a logarithmic dependence on peptide length

As expected, the highest percentage of the available combinations is found for tripeptides (up to 38% in human proteome; Figure 1), and the smallest proteomes (*E. coli*, 5,494; *S. cerevisiae*, 6,692 proteins) contain significantly lower amounts of amino acid combinations in their respective peptide sequences. We assessed the occurrence of peptide sequences and found that either LLR or LLK is the most abundant tripeptide in the analyzed species. The ranking of longer tryptic peptides was not similar which is well in line with the evolutionary distance of these five organisms. The occurrence of different oligopeptides increases with the peptide length but the dependence curve has a hyperbolic shape and does not follow $20\times22^{(n-1)}$ dependence.

How many amino acids do we need for a proteotypic peptide?

Shorter peptides are often excluded from protein identification and quantitation analyses because they can be often found in multiple different proteins. The default settings for proteomic software restricts the peptide length by limiting m/z ratio to 350 - 400. This represents at least four amino acids in a singly-charged peptide for a protein identification and the quantitation limits are even higher (e.g. Novak et al. 2015, Cerna et al. 2017). We examined our putative tryptic digests and the results are summarized in the Table 2 and Figure 2.

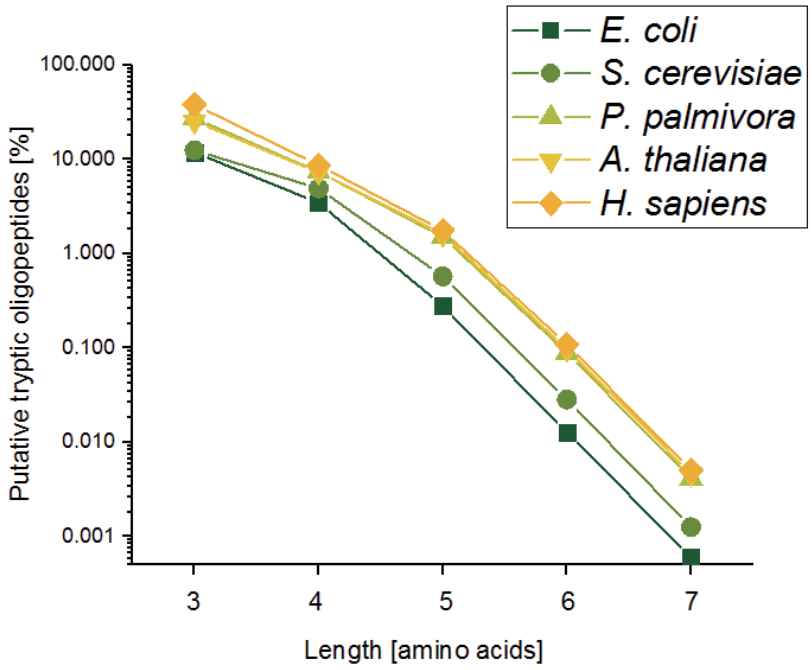
We have found that tetrapeptides may represent a significant portion of proteotypic peptides in a prokaryotic cell and should not be excluded in any proteomic analyses of an organism with a known genome. For instance, unique tetrapeptides in *A. thaliana* can be employed to identify and quantify over 2,400 proteins. However, without any prior knowledge of a peptide uniqueness, more than four independent tetrapeptides should be used and thus their usefulness is limited. The size of *A. thaliana* proteome (~48,000) and that of *P. palmivora* (~35,000) are quite similar and this similarity is reflected in statistics. The probability that a pentapeptide or a hexapeptide in *P. palmivora* or *A. thaliana* is unique is around 70% and 90%, respectively. In contrast, the human proteome database contains over 104,000 protein models. We did not consider proteoforms in our analyses and duplicate peptide sequences

originating from the same gene were always removed. However, the ratio between unique and non-unique peptide sequences in *H.sapeins* digest is still low and seven in ten heptapeptides are not unique. *A. thaliana* and *H.sapiens* have a similar amount of protein-coding genes and it is tempting to speculate that our limited knowledge of plant genome/proteome produces bias in our data.

Table 2 Putative tryptic oligopeptides three to seven amino acids in length (AA) and their uniqueness in model proteomes. Only sequences that are found in three and less different protein models are listed.

	Occurrence	*3 AA*	*4 AA*	*5 AA*	*6 AA*	*7 AA*
E. coli	Unique	381	3090	8821	8533	8218
	2	59	1380	791	254	203
	3	19	749	134	64	49
S. cerevisiae	Unique	404	3017	17208	19189	17200
	2	51	1730	2418	639	386
	3	8	1194	453	64	44
P. palmivora	Unique	1209	3060	35727	55514	53144
	2	380	1265	10110	4767	3208
	3	127	1055	3683	1097	712
A. thaliana	Unique	1097	2768	37040	60615	59752
	2	336	1194	11425	5946	3972
	3	90	1002	4376	1112	706
H. sapiens	Unique	1143	3125	14763	23367	22526
	2	565	1085	10562	16812	16164
	3	312	652	7929	11384	10387

Figure 1 The number of existing amino acid combinations correlates with a proteome size

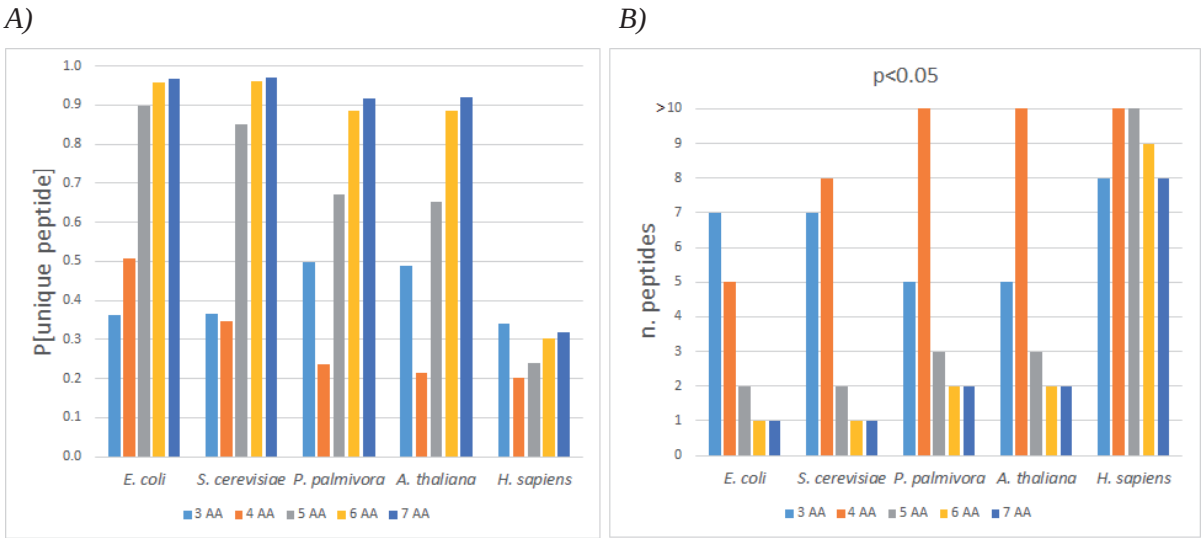


Is it possible to detect tripeptides and tetrapeptides in LC-MS analyses?

Standard proteomics databases dedicated to the targeted protein identification do not contain short peptide sequences (e.g. Desire et al. 2006). However, we believe that this should be changed and thus we reanalyzed raw LC-MS data of *A. thaliana* seedlings (Baldrianova et al. 2015) employing a modified FASTA database containing only tryptic peptides of four to six amino acids. A single LC-MS run processed with the original settings contained 775 protein families representing 1507 protein

identifications. The inclusion of shorter peptides to the identification list provided additional 30 protein families, representing a low but significant increase in protein IDs. However, all newly identified peptides were only doubly charged hexapeptides. Shorter peptides were not detectable in this dataset. This was not unexpected because only precursors over 350 m/z were selected for fragmentation. Further, the MS/MS based untargeted identification is not very practical for shorter sequences. The number of peaks in the MS2 fragmentation pattern is low and an automatic scoring of these spectra does not work properly. Smaller peptides are also more likely to be lost during the desalting step. We believe that a metabolomic-based approach that is optimized for lower molecular masses could be beneficial to further improve the proteome coverage. In this context, we should note that the new release of the NIST metabolomic database contains entries for over 400 dipeptides and 800 tryptic peptides (Yang. 2017).

Figure 2 How many peptides do we need for a confident identification and quantitation? (A) The probability of uniqueness of a random peptide sequence of given length. (B) Minimal number of peptides of given length that is required for confident ($p<0.05$) identification and quantitation of a protein. These calculations do not include proteoforms.



CONCLUSION

Our results indicate that the length restriction in protein identification and quantitation should be lowered and known unique oligopeptides included in analyses. However, we note that the present-day proteomic workflow may not be optimal for shorter peptides and that techniques dedicated to small molecule analyses could improve the proteome coverage.

ACKNOWLEDGEMENTS

The research was financially supported by the European Regional Development Fund, Project Phytophthora Research Centre Reg. No. CZ.02.1.01/0.0/0.0/15_003/0000453 and IGA grant no. IP 15/2017.

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SPIROPYRAN-ZINC INTERACTION CHARACTERIZED BY FLUORESCENCE SPECTROMETRY AND CAPILLARY ELECTROPHORESIS WITH LASER-INDUCED FLUORESCENCE DETECTION

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Abstract: Zinc is an important biogenic element which is able to connect with indicators such as a spiropyran. The spiropyran is known for its fluorescent character and selectivity towards zinc ions (and some other metal ions, e.g. cadmium). This complex, especially with zinc, gives a high fluorescence intensity which was analyzed by fluorescence spectra and by capillary electrophoresis with laser induced fluorescence detection. In addition, the influence of visible light irradiation on zinc release was tested. The spiropyran specificity to zinc was also investigated by coupling with copper and cadmium.

Key Words: the spiropyran, zinc, fluorescence intensity, vis irradiation

INTRODUCTION

Zinc is a very important biogenic element which plays many roles in human body and other living organisms. In terms of the human body, it is a cofactor of lots of enzymes and an important component of insulin molecule and insulin metabolism. Further, zinc ions participate in metabolism of saccharides, proteins, and phosphorus (Maret 2013). It is predominantly an intracellular element. It is possible to detect and determine zinc using many methods, e.g. UV spectroscopy, atomic force microscopy (Taranath et al. 2015), methods using extractions, atomic absorption spectrometry or spectrophotometry (Smith et al. 1979). Zinc ions can be also detected by fluorescence probes such as FluoZin (Kimura andKoike 1998). Here, a sensoris meant a molecule which binds other molecules, elements, hormones, and other substances and is able to transmit them under certain conditions (Pijanowska et al. 2003).

One of the newly synthesized receptors is a spiropyran. The spiropyran is a receptor which is fluorescent and photoregenerable. It is selective towards zinc(II) ions. Creation of a complex is connected with a structure isomerization in a response to electromagnetic irradiation. After creation of metastable merocyanine, the phenolic oxygen with a negative charge causes the ability to bind metal ions (e.g. zinc) (Natali et al. 2010).

Due to the fluorescent character of the spiropyran, it is possible to use fluorescence spectrometry to measure fluorescence intensity. The advantage of fluorescence intensity detector is that it has much larger range of application than absorbance scan and differences between single measurements are more perceptible and it is much easier to evaluate them (Strickler andBerg 1962). The next step to complete information of fluorescence intensity is using capillary electrophoresis

with laser induced fluorescence detection (CE-LIF). This method is universal, highly effective and very sensitive and accurate (Huang et al. 2006).

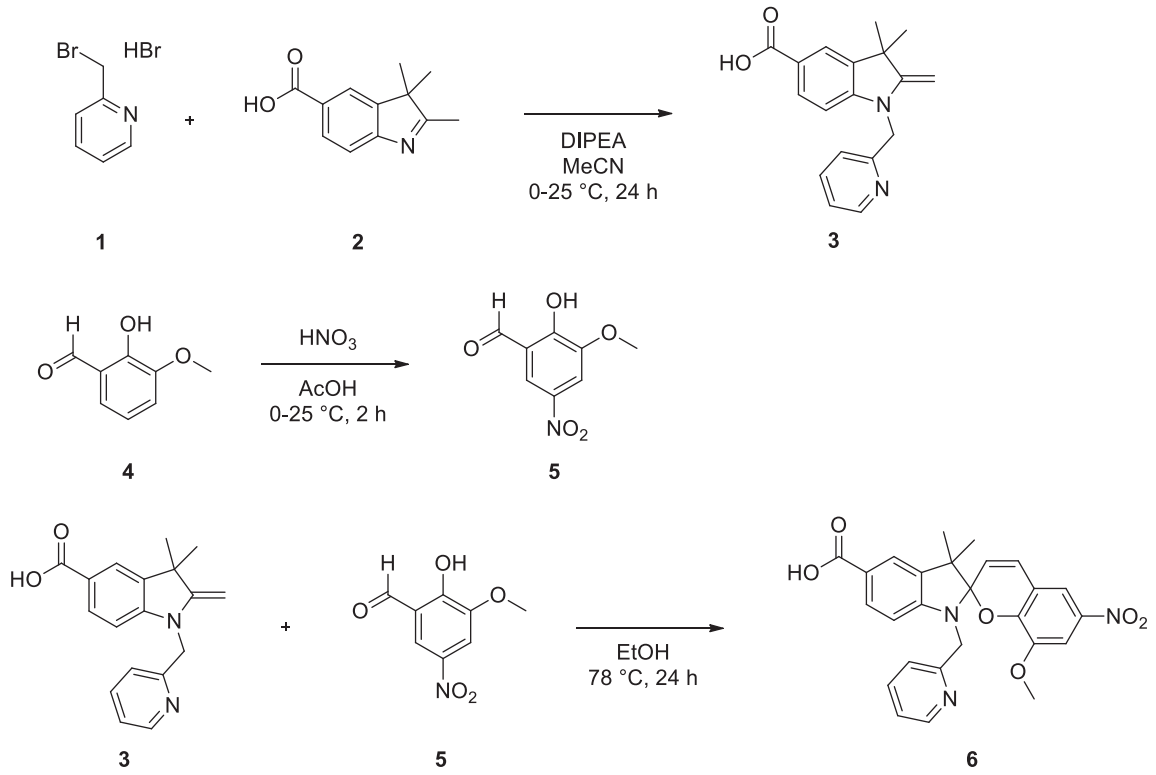
In this study the photosensitive spiropyran was synthesized. Effect of zinc complexation (and other metals) and effect of visible light (vis) irradiationwere characterized by fluorescence spectra. The fluorescence signal was analyzed also by CE-LIF.

MATERIAL AND METHODS

The spiropyran synthesis

All chemicals of ACS purity were obtained from Sigma-Aldrich (St. Louis, USA) unless otherwise stated. The spiropyran was prepared from commercially available starting material in three steps (Figure 1). First, 2,3,3-trimethyl-3H-indole-5-carboxylic acid **(1)** was alkylated with 2-bromomethyl pyridine **(2)** in presence of *N*,*N*-diisopropylethylamine (DIPEA) leading to compound **3** (Natali et al. 2010). In parallel, *ortho*-vanilin**(4)** was nitrated with nitric acid in acetic acid providing 3-methoxy-5-nitrosalicylaldehyde **(5)** (Darwish et al. 2014).Then a condensation reaction of compounds **4** and **5** in ethanol led to desired the spiropyran **6** (Natali et al. 2010).

Figure 1 The scheme of the spiropyran synthesis



Fluorescence spectrometry analysis

Dimethyl sulfoxide (DMSO) was chosen as an appropriate solvent for fluorescence spectrometry analysis. Before analysis DMSO was deprived of zinc ions by using Chelex 100 Resin (Bio-Rad, USA). The amount of Chelex in DMSO constituted of about 10% of the total volume. The whole solution was then once more treated by Chelex. Then spiropyran (1 mg/ml) in DMSO was mixed with equal moles of $ZnCl_2$, $CdSO_4$ or $Cu(NO_3)_2$. The fluorescence spectra were measured on fluorimeter Infinite M200 PRO microplate reader (Tecan, Austria). The data were compiled by fluorimeter software i-control 1.9 (Tecan). All samples were monitored using excitation wavelength λ_{ex} 490 nm.

Visible light irradiation

White LED (6000 K, Roithner Lasertechnik, Austria) was used to illumine the samples before measurement. Different times of irradiation were tested (1 and 5 min).

Capillary electrophoresis with laser-induced fluorescence detection (CE-LIF)

Samples of spiropyran with and without zinc were analyzed by CE-LIF. The capillary electrophoresis separation was implemented on 7100 CE System (Agilent, Germany) with a fluorescence detector (ZetaLIF, Picometrics, France) and a solid-state laser (λ_{ex} = 488 nm) as an excitation source. The uncoated fused silica capillary (Polymicro Technologies, USA) with inner diameter 75 µm, the total length 64 cm, and effective length 43cm was used. All data evaluation was performed with Agilent ChemStation software. The 20 mM borate buffer (pH 9.2) was used as a background electrolyte. The hydrodynamic injection by 30 mbar for 5 s and the separation voltage of 25 kV was employed.

RESULTS AND DISCUSSION

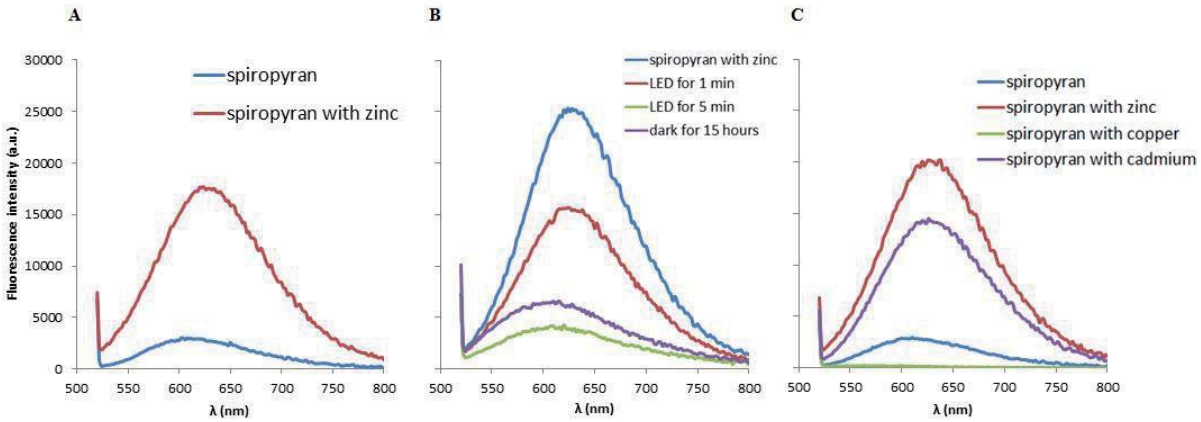
The spiropyran interaction with zinc ions

First of all, the fluorescence intensity of spiropyran itself with fluorescence intensity of the spiropyran complexed with zinc was compared. The fluorescence intensity maximum for spiropyran was at 605 nm and for the complex at 625 nm. As it is obvious (Figure 2A), the spiropyran complexed with zinc has much higher fluorescence intensity than the spiropyran alone. In the presence of zinc ions, the fluorescence intensity increased six times. Compared to commonly used zinc-sensitive fluorescent probes (e.g. FluoZin-3(de Silva et al. 1997)), the here presented probe belongs to the group of stimuli-responsive materials and therefore after irradiation by visible light, the captured zinc ions are released.

Visible light irradiation

Subsequently the influence of vis light irradiation on zinc release and re-complexation was investigated (Figure 2B). Samples of complexes were illuminated by white LED for 1 and 5 min causing decrease of fluorescence intensity by 40 and 80 %, respectively. This observation corresponds to zinc ions release from the complex. Then the sample was placed in lightproof box in order to survey re-complexation. As was shown in Fig 2B, not even after 15 hours the fluorescence intensity did not reach the original value. This fact indicates very slow or partial re-complexation of the zinc ion to the spiropyran.

Figure 2 Development of fluorescence intensity depending on the conditions



Legend: A – difference between fluorescence intensity of the spiropyran (blue) and the spiropyran with zinc (red), B – fluorescence intensities depending on enlightenment changes, C – differences between fluorescence intensity of the spiropyran with other metals

Specificity study

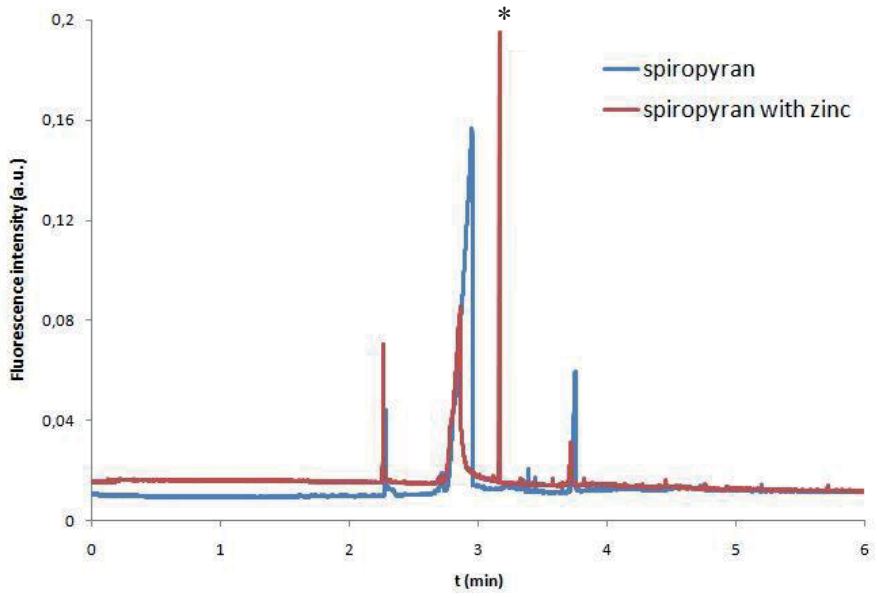
Finally, the spiropyran interaction with other metals was investigated (Figure 2C). In case of copper, no fluorescence signal was observed, because copper does not react with the spiropyran at all. The situation changes in case of cadmium which forms a complex with fluorescence intensity higher than the spiropyran alone however intensity reaches about 70% of fluorescence intensity in comparison with the spiropyran–zinc complex. These results are in agreement with the work

by Natali et al. where the interaction with a variety of ions (mono-, di-, and tri-valent) was tested; however the exact explanation of the selectivity was not wholly explained yet.

CE-LIF analysis

Preliminary results obtained by CE-LIF shoed (Figure 3) that the solution of spiropyran alone led to formation of peaks in migration times of 2.3 min, 2.7 min and 3.7 min. In case of the spiropyran-zinc complex, another peak with migration time of 3.1 min appeared (*).

Figure 3 The typical electropherogram of the spiropyran and the spiropyran with zinc



CONCLUSION

Aim of this study was to investigate the influence of presence of zinc ions and influence of illumination on the spiropyran fluorescence intensity.

As found, the spiropyran formed a highly fluorescent complex with zinc ions. This complex dissociated by LED illumination, but its reassociation in darkness was much slower. The spiropyran was also able to bind cadmium but not to copper. As observed, it is convenient to use the spiropyran as a probe of zinc, because it is easy to determine this complex and utilize this knowledge in other studies. Future work includes not only detail investigation of the probe sensitivity and selectivity

ACKNOWLEDGEMENTS

This research was carried out under the project CEITEC 2020 (LQ1601) with financial support from the Ministry of Education, Youth and Sports of the Czech Republic under the National Sustainability Programme II.

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EVALUATION OF CHLORIDES TRANSPORT PARAMETERS IN NATURAL SOILS BASED ON LABORATORY STUDIES

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Abstract: This study pays attention to the possibilities of using column tests in order to determine parameters of contaminant migration through soils. The column tests were performed in the Trautwein apparatus by the constant head method. In this research solution of chlorides prepared on the basis of distilled water has been used as a conservative marker. Based on breakthrough curves, velocity of flow, dispersion coefficient, longitudinal dispersivity and Peclet number were calculated for each sample tested using CXTFIT-STANMOD package. Obtained Peclet numbers (Pe=0.82, Pe=0.57, Pe=19.01, respectively) have indicated that advection is dominant mechanism of chloride transport in sand whereas diffusion dominates during the chlorides transport through silt loam. Velocity of chlorides flow in silt loam samples were almost equal to 5×10^{-7} m/s, whereas in sand was equal to 3.1×10^{-4} m/s. It was concluded that the column experiment can be used as an efficient method to provide input data for numerical modeling of non-reactive tracers transport and fate in the soil-water system for the purpose of surface and groundwater protection.

Key Words: chlorides, migration, CXTFIT, advection, diffusion, dispersion

INTRODUCTION

Contaminants can be subdivided into three general groups: (a) aqueous contaminants dissolved in water, including inorganic major ions, nutrients, trace elements and dissolved organic compounds, (b) non-aqueous contaminants, including petroleum products or chlorinated solvents, and (c) particulate matter that may be inert or biologically active (Bear and Cheng 2010).

By the migration of contaminants is meant the transport of specific chemicals together with the transformations they may undergo during transport (Knox et al. 1993). The combination of physical, chemical and biological processes is the most often associated with the movement of pollutants in the soil-water environment. In the subsurface, the various contaminants undergo complex physical, chemical, and biological transformations (US.EPA 1987).

The issue of the transport and fate of contaminants in the soil-water environment is a subject of numerous researches worldwide and is strictly linked to the need of protection of environmental components from contamination. In recent years, the attention has been focused mainly on contamination of the soil-water system by landfill leachate, agricultural activities (the use of fertilizers, herbicides, pesticides), spills of oil and other toxic liquids or hazardous industrial wastes (Sieczka and Koda 2016a, Sieczka and Koda 2016b, Adamcová et al. 2017, Koda et al. 2017, Vaverková et al. 2017).

While many studies have been conducted to characterize the processes of pollutants migration for various types of sorbents (Khan et al. 1997, Xie et al. 2013), only few literature reports concern the description of migration processes of pollutants in natural soils. The studies on migration of contaminants through natural soils, generally through sands collected from areas of groundwater intakes, carried out in Polish scientific institutes, so far have been mainly concerned with conservative pollutants (e.g. chlorides) (Marciniak et al. 2006, Okońska 2010). Similarly, Ojuri and Ola (2010) expended considerable efforts to determine parameters of chlorides migration through sandy soils.

In order to provide complete overview of the migration of contaminants in the soil-water environment, it is required to have sufficient information about the medium in which the migration

takes place as well as the parameters occurring in mathematical equations describing the transport of pollutants (Koda 2012, Koda et al. 2013).

Due to the presence of numerous sources of contamination (e.g. agriculture, industry, communal infrastructure facilities, landfill sites), from which pollution can enter the soil-water environment, it is justified to undertake research activities aimed at recognizing basic processes responsible for contaminant migration as well as at developing procedures to control their spread for the purpose of the protection of the natural environment. Some studies have been conducted so far to identify the processes of transport of agricultural contaminants using tracer techniques. In these methods, chloride and bromide are being used commonly because they are not adsorbed by the soil and do not undergo biological transformations. Kanwar et al. (1997) showed in their work that chloride can be applied as a tracer of nitrate migration through soils.

Worth mentioning is also the application of the electrical resistivity methods which in the field conditions are being effectively used as a tool to interpret the migration paths of contaminants (Wychowaniak et al. 2015).

Although several methods can be used to determine the parameters of contaminant migration (Dowgiałło et al. 2002), this research focuses on the application of dynamic method in the assessment of the parameters of chlorides migration through natural soils.

MATERIAL AND METHODS

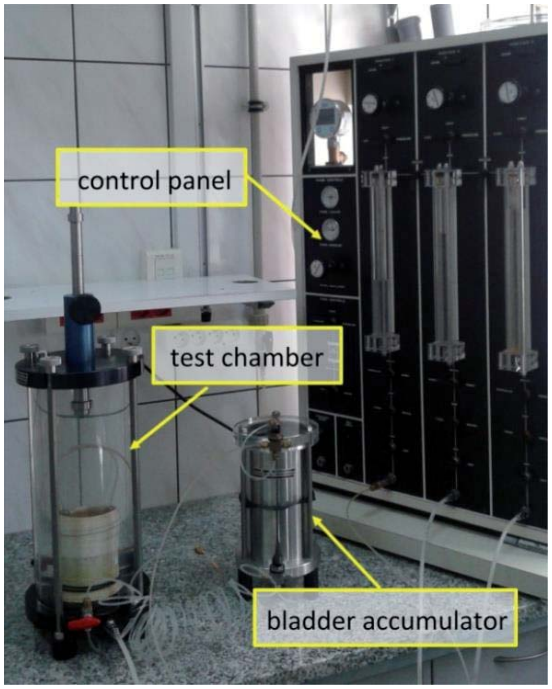
Tested materials

Soil samples collected from the agricultural areas were used as research materials. Identification of the particle size distribution was conducted using the sieve analysis and the Casagrande method in Prószyński modification according to the standard presented in PN-B-04481:1988. The textural names of the tested soils were determined based on the classification of the Polish Society of Soil Science (2009). Effective porosity (n_e) of the tested materials was calculated using the formula presented by Marciniak et al. (2006).

Column experiment

In this study the solution of chlorides imitated the contamination. Contaminant transport parameters were determined based on a column experiment using the Trautwein apparatus, a photograph of which is presented in Figure 1.

Figure 1 Experimental set-up for chlorides transport parameters testing



Dynamic tests were carried out in the saturated conditions using the constant head method with regard to the ASTM D5084-00 procedure. To imitate the stress conditions in the tested soils, the confining pressure equal to 40 kPa was applied. Effluent samples were collected at different time intervals regarding the flow rate and the volume of solution which passed through the soil sample during the experiment. The results of the column (dynamic) tests were presented in the form of breakthrough curves showing the concentration of contaminant versus time.

The results of a column experiment conducted in a Trautwein apparatus in the conditions of the constant head and full saturation were analysed using the CXTFIT - STANMOD package (Toride et al. 1999). Based on the breakthrough curves obtained in the laboratory studies, the parameters of advective-dispersion equation of contaminants migration were determined using a nonlinear least-square procedure of inversion parameter estimation. Basic equation taken into account during analysis was as follows:

$$R\frac{\partial C}{\partial t} = D\frac{\partial^2 C}{\partial z^2} - v\frac{\partial C}{\partial z}$$

where:

R - retardation factor (-), C – concentration (mg/l), t - time (s), z - distance (m) and D - dispersion coefficient (m^2/s).

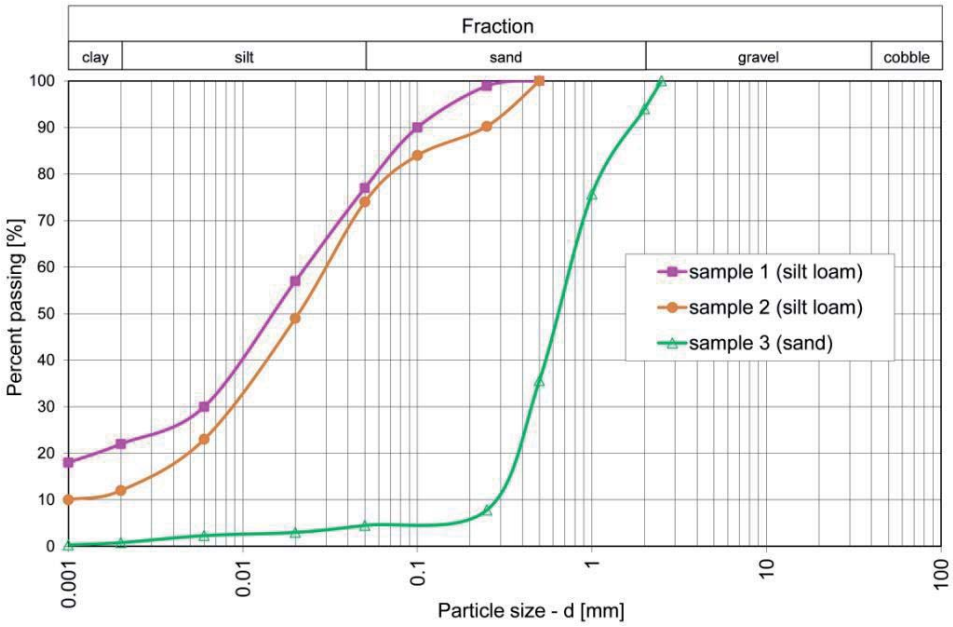
Prior to the column experiment, the soil samples were saturated with the distilled water and then, after the flow rate had become stable, the injection of chlorides solution started. The chlorides input concentration was of 100 mg/l. Samples were analysed for their chlorides content using Mohr method. All reagents were of analytical grade.

RESULTS AND DISCUSSION

Particle size distribution and effective porosity

Particle size distribution curves of the tested materials presenting the content of particular fractions are shown in Figure 2. According to the classification of the Polish Society of Soil Science, the tested soils were classified as silt loam (samples 1 and 2) and sand (sample 3), respectively. Effective porosity were equal to 0.40 for sample 1 and 0.38 for samples 2 and 3.

Figure 2 Particle size distribution of the tested materials



Parameters of chlorides transport

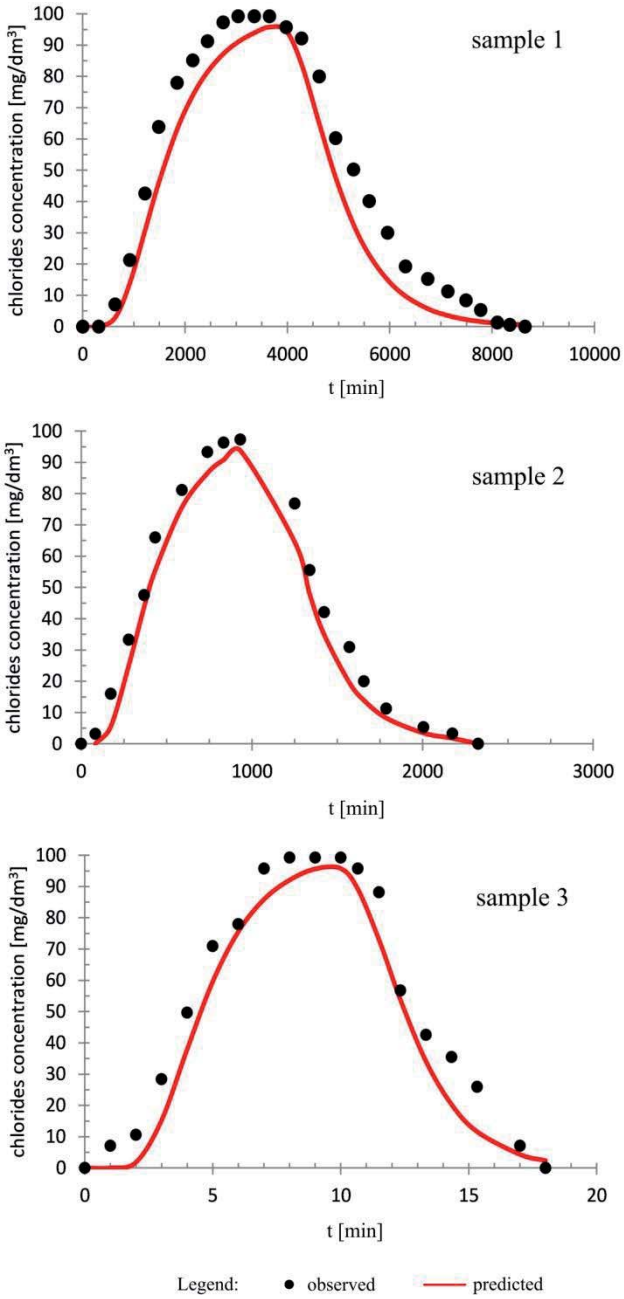
On the basis of the chlorides breakthrough curves, it was possible to calculate the velocity of flow *(v)*, dispersion coefficient *(D)*, longitudinal dispersivity *(α_L)* and Peclet number *(Pe)* (Table 1).

Average hydraulic conductivities obtained during column studies were equal to 1.58×10^{-8} m/s for sample 1, 6.00×10^{-8} m/s for sample 2 and 4.94×10^{-5} m/s for sample 3. Based on obtained breakthrough curves, it was stated that the maximum concentration of conservative markers were achieved after 3000 minutes for sample 1, after 830 minutes for sample 2 and after 9 minutes for sample 3 (Figure 3). Times period required to reach the maximum concentration in effluent were strictly dependent on the hydraulic permeabilities of the tested soils.

Table 1 Parameters of chlorides transport obtained using CXTFIT-STANMOD package

Parameter	Unit	Sample 1	Sample 2	Sample 3
Velocity of flow	m/s	4.8×10^{-7}	4.9×10^{-7}	3.1×10^{-4}
Dispersion coefficient	m^2/s	3.5×10^{-8}	1.2×10^{-7}	1.5×10^{-6}
Longitudinal dispersivity	m	6.0×10^{-3}	1.4×10^{-2}	9.2×10^{-3}
Peclet number	-	0.82	0.57	19.01
Retardation factor	-	1.0	1.0	1.0

Figure 3 Observed and predicted breakthrough curves for chlorides



Coefficients of determination calculated between observed and predicted chlorides concentrations were as follows: R^2=0.98 for sample 1, R^2=0.99 for sample 2 and R^2=0.98 for sample 3. These values indicate very strong correlation between concentrations measured in laboratory and those calculated using CXTFIT-STANMOD software.

The Peclet number is commonly used as a measure which determines the ratio of advective to diffusive transport (Berkowitz et al. 2008). According to Yong et al. (2012), the transition zone between dominantly diffusive transport and predominantly advective transport of the contaminant solution occurs in the range of Peclet numbers between 0.01 and 10, and when Pe is greater than 10, advection becomes the dominant process of contaminant transport. In reference to the results obtained in our study, it can be stated that the advection dominates during the chloride transport through sand. For silt loam, both dispersion and diffusion must be taken into account to describe the phenomena of chloride migration through cohesive soils.

CONCLUSION

Column experiments were performed to determine parameters of chloride migration through natural soils. The analysis of the breakthrough curves allowed us to estimate the parameters of advection-dispersion equation of contaminant transport. Results obtained indicate that the advection dominates when the chlorides flow through sandy soil. On contrary, considering the chlorides flow in cohesive soils with the significant content of clay fraction, the diffusive transport starts to dominate over the advection, which was strictly confirmed by calculated Peclet numbers. The outcomes obtained using CXTFIT-STANMOD package pointed out a significant correlation with results obtained under laboratory conditions. The values of velocity of flow clearly indicate that considering the hydrogeological conditions in which the aquifer is located beneath the layers composed of silt loam, the chlorides achieve the groundwater six hundred times slower than in the case when the upper part of the soil profile is composed of sandy materials. Low values of hydraulic conductivity measured for silt loam indicate that soil layers composed of this material can form a natural barrier against the spread of contaminants (e.g. chlorides) into groundwater. Moreover, it is worthy of note that the column experiment can be an efficient method to provide input data for numerical modeling of non-reactive tracers transport and fate in the soil-water system for the purpose of the aquatic environment protection.

ACKNOWLEDGEMENTS

This research was financially supported by the European Regional Development Fund under the Innovative Economy Operational Programme: BIOPRODUCTS, innovative production technologies of pro-healthy bakery products and pasta with reduced caloric value (POIG.01.03.01-14-041/12).

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SARCOSINE DEGRADATION PATHWAY IS INVOLVED IN THE EPIGENETICS OF PROSTATE CELLS

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Abstract: It has been shown that sarcosine suplementation stimulates the proliferation of prostate cells and also their invassiveness. In present study we show that enzymes conected with sarcosine conversion to glycine (sarcosine dehydrogenase, pipecolic acid oxidase) are stimulated due to sarcosine treatment. Further, sarcosine treatment increases *S*-adenosylmethioneine-to-*S*-adenosylhomocysteine ratio, which indicates a release and utilization of free methyl groups from sarcosine degradation pathway. We identified the highest induction of global methylation in non-malignant PNT1A cells, but global methylation profiles were altered also in malignant (22Rv1) and metastatic (LNCaP) cells. The influence on methylation changes was further verified using hypomethylating agent 5-azacytidine (5-aza). Co-treatment of prostate cells with 5-aza and sarcosine resulted in decrease in cells invassiveness when compared to treatment with sarcosine alone. This correlates with sarcosine-related hypermethylation of genes involved in cells growth and cell cycle.

Key Words: sarcosine, methylation, prostate, prostate cancer, human cells

INTRODUCTION

Sarcosine is an imino acid and a potential biomarker of prostate cancer (PCa). Concentration of sarcosine is substantially increased during PCa progression to its metastasis (Sreekumar et al. 2009). In its biochemical pathway, sarcosine is formed from dimethylglycine by dimethylglycine dehydrogenase (DMGHD, EC 1.5.8.4) (Metallo 2012) or from glycine by glycine-*N*-methyltransferase (GNMT, EC 2.1.1.20). On the other hand pipecolic acid oxidase (PIPOX, EC 1.5.3.1) or sarcosine dehydrogenase (SARDH, EC 1.5.8.32) can demethylate sarcosine to form glycine while providing free methyl group to methyl-donor *S*-adenosylmethionine (SAM), which is further demethylated into *S*-adenosylhomocysteine (SAH) (Dodt et al. 2000). This can be associated with a methylation of an acceptor (DNA, RNA, neurotransmitters or lipids). Methylation processes can impact a wide array of biological processes, including gene transcription, which could be connected with initiation and progression of PCa. For instance, methylation of cytosine-phosphate-guanine dinucleotides in promoter can inactivate the genes. Although the connection between epigenetics and cancer are well known, there is lack of data regarding the effect of increased amount of sarcosine on methylation status of prostate cells (Ianni et al. 2013).

MATERIAL AND METHODS

Prostatic cell lines

Three human prostatic cell lines were used for an experiment, representing benign and malignant cells: *i*) the PNT1A human cell line established by immortalization of normal adult prostatic epithelial cells by transfection with a plasmid containing SV40 genome with a defective replication origin, *ii*) 22Rv1 which is a human prostate carcinoma epithelial cell line derived from axenograft that was serially propagated in mice after castration-induced regression and relapse of the parental, androgen-dependent CWR22 xenograft, *iii*) LNCaP human cell line estabished from an androgen-sensitive metastasis located in the left supralavicular lymph node. All cell lines used for experiments were purchased from Health Protection Agency Culture Collections (Salisbury, UK).

Culture conditions and treatment protocols

All cell lines were cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS) supplemented by penicillin (100 U/ml) and streptomycin (0.1 mg/ml). The cells were maintained at 37 °C in humidified incubator with 5% CO_2. The exogenous supplementation with sarcosine (10 μM) and azacytidine (10 μM) was initiated after cells reached ~80 % confluence. The cells were harvested after 2; 6 ;12 and 24 h for HPLC-MS analysis and scratch test, after 24; 48 and 72 h for western blotting, and after 24 h for imunofluorescence. All experiments were designed as five biological replicates ($n = 5$) measured three times at each time point.

Imunofluorescence of sarcosine metabolsim-related enzymes

For imunofluorescence (IF) were culture cells seded into eight-well chamber slides and after 24 h of adherence were treated by sarcosine (10 μM) and azacytidine (10 μM). As a control were used cells without treating. Cells were fixated after 24 h incubation by 4% formaldehyde, permeabilizated by 0.25% Triton X-100, blocked in 5% bovine serum albumin in PBS and imunostained by primary antibody overnight in 4 °C. Detection was accomplished using fluorescein isothiocyanate (FITC)-conjugated or CruzFluor™ 645 (CFL 645) labeled secondary antibodys. DNA staining by Hoechst were used for counter. IF was evaulated by fluorescent microscope for GNMT, SARDH, DMGDH, and PIPOX as a enzymes involved in sarcosine metabolism.

Extraction and quantitation of SAM and SAH

SAM and SAH were extracted in MeOH and acetic acid (80 : 20; *v*/*v*). Solvent was added to the frozen cells followed by slow thawing on ice. After that, the cells wer snap-freezed in liquid nitrogen and thawed on ice again. After three times freez/thaw cycle were samples centrifuged at 9000 × g. The supernatant was transfered to 1.5 ml glass vial and washed with solvent. The quantitation of SAM and SAH was performed using high-performance liquid chromatography with electrospray ionization quadrupole-quadrupole-time-of-flight mass spectrometer (HPLC-ESI-QqTOF MS). The samples were separated on C18 reverse phase column. As mobil phases, water with 0.1% (*v*/*v*) formic acid and methanol with 0.1% (*v*/*v*) formic acid were used.

Global analysis of DNA methylation

The DNA was extracted by the ExtractNow™ DNA Mini Kit and quantified at λ = 260 nm. The global methylation was performed using a methylation DNA quantification kit, incubated with capture and detection antibodies and read by λ = 450 nm. Quantification of global DNA methylation was compared to positive control that had been previously fully methylated. The methylation level depends on global amount of methylated cytosines (5-mC) in samples relative to global cytidine (5-mC + dC).

Wound-healing assay (Scratch test)

The cells were seeded into 16-well plate to reach confluence ~80%. After seeding a pin was used to a scratch and remove cells from a discrete area of the confluent monolayer to form a cell-free zone. After that, cells were treated with sarcosine (10 μM), DNA hypomethylating agent 5-azacytidine (5-Aza, 10 μM). After 6; 12 and 24 h, the micrographs of cells were taken using EVOS FL Auto Cell Imaging System and compared with micrographs obtained in 0 h, using TScratch software.

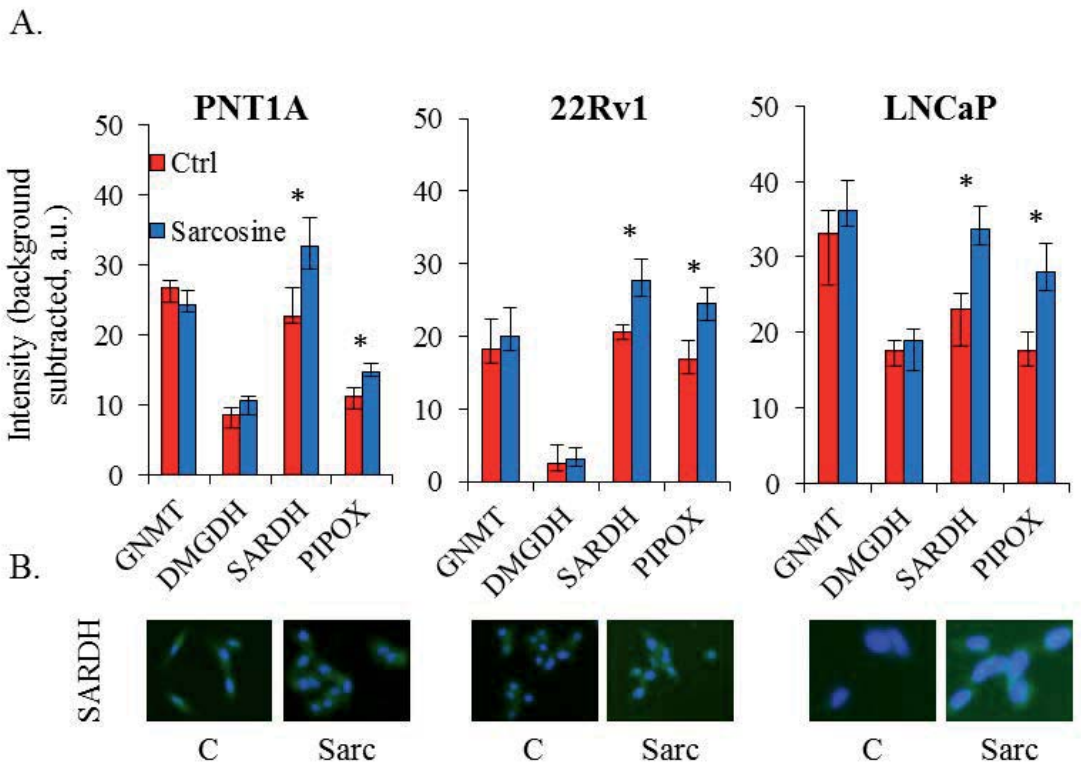
Descriptive statistics

For the statistical evaluation of the results, the mean was taken as the measurement of the main tendency, while standard deviation was taken as the dispersion measurement. Differences between groups were analyzed using paired t-test. Unless noted otherwise, the threshold for significance was $p < 0.05$. For analyses Software Statistica 12 (StatSoft, Tulsa, OK, USA) was employed.

RESULTS AND DISCUSSION

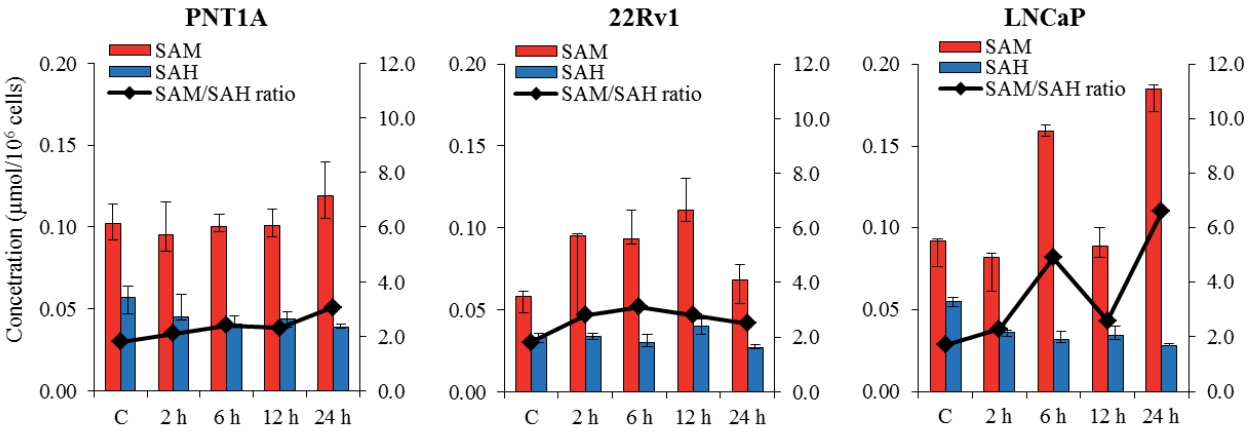
First, we focused on sarcosine stimulatory vs. inhibitory effects on enzymes involved in sarcosine metabolical pathway (GNMT, DMGDH, SARDH and PIPOX). Figure 1 illustrates representative immunofluorescence micrographs of sarcosine metabolism-related enzymes in prostate cells incubated without or with sarcosine (10 μM) together with quantitation of expression of certain enzymes. The stimulatory effects were found for SARDH and PIPOX, which are mainly connected with sarcosine degradation. Hence, it is obvious that an exogenous addition of sarcosine results in its demethylation through sarcosine degradation pathway. Methyl groups can be further used for methylation of various acceptors such as DNA, RNA, etc., which is one of the major hallmarks of cancer development and progression (Suh et al. 2011).

Figure 1 (A) Representative IF micrographs andquantitation of IF of analysed enzymes. (B) IF images of LNCaP cells for SARDH expression after sarcosine treatment compared to control



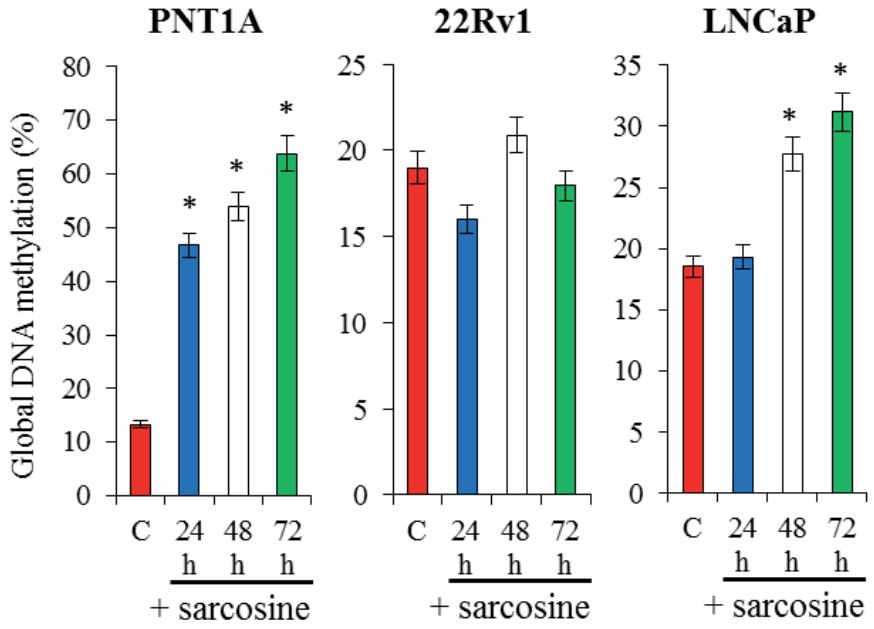
We further focused on estimation of ratio between SAM and SAH. Figure 2 clearly shows that SAM/SAH ratio changed when compared control cells and cells treated with sarcosine. The highest SAM/SAH ratio was determined at LNCaP cells treated with sarcosine after 24 h of incubation. These findings indicate that SARDH/PIPOX degradation of sarcosine had significant stimulatory effects on a formation of methyl-donor SAM, which delivers free methyl groups to the target site (Shukeir et al. 2006). Simultaneously, we identified slight decrease in SAH, which indicate higher needs of prostate cells to maintain SAM activity for transferring the methyl groups.

Figure 2 Values of SAM and SAH and their ratios in PNT1A, 22Rv1 and LNCaP cells non- and supplemented with sarcosine



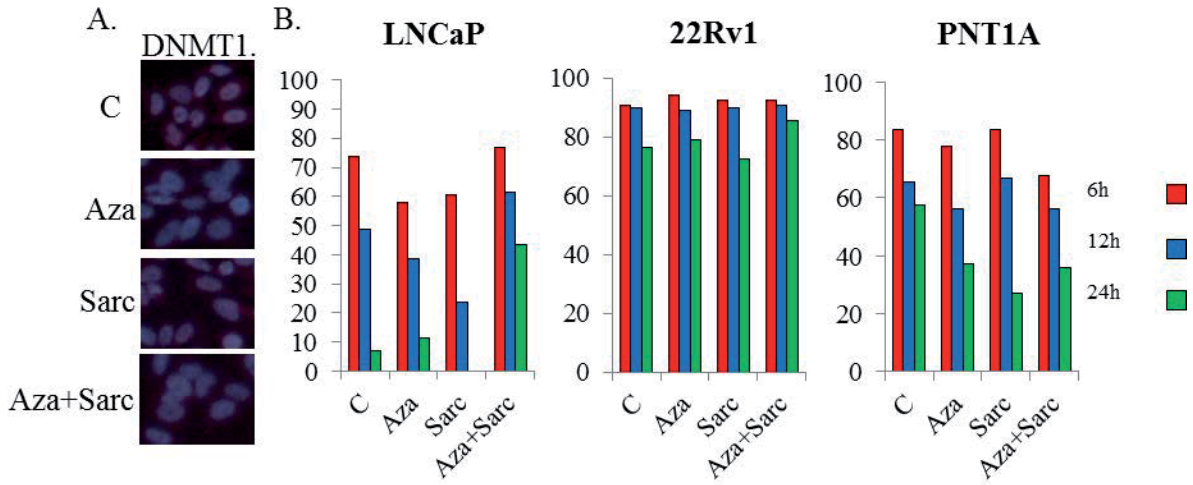
With respect to the obtained data we analyzed global methylation profiles in all tested cells. The highest level of global methylation was found in PNT1A after 72 h treatment (63.8%), which corresponds to the sarcosine-related induction of their proliferation and invassiveness investigated in our previous studies. Methyl groups coming from sarcosine can be used by DNA methyl transferase to methyation of DNA promotors. Overall, we show that sarcosine can efficiently provide free methyl group for DNA methylation processes.

Figure 3 Global methylation index of prostate cell lines treated with sarcosine



To determine whether sarcosine and 5-Aza treatment suppress cell migration was performed (Figure 4). The migration was stopped in the LNCaP by sarcosine treatment after 24 h. The migration was higher after sarcosine supplementation in all three cell lines. That indicate sarcosine as a potential donor of methy group, what can be used for methylation of regulatory genes. In non-malignant cell line PNT1A was not different in migration after 24 h supplementation by 5-Aza and combination of 5-Aza+Sarc. It highlights different metabolism of sarcosine in non-malignant cells and inhibition of methylation. In malignant cells (LNCaP and 22Rv1) after 24 h was proliferation slower in combinant treatment with 5-Aza+Sarc. Hypomethylation caused by 5-Aza probably inhibited methyl group transfer in malignant prostatic cells metaboslim connected with sarcosine metabolism.

Figure 4 (A) Representative IF images for DNMT1 ater treatment. (B) Sarcosine effect on migration of prostate cells



CONCLUSION

The mechanistically increased level of sarcosine stimulates expression of enzymes involved in its metabolism. Sarcosine can hence be utilized as efficient donor of methyl group. Aftervards the methyl groups can be transferred by DNMTs (DNA methl transferases) to promotor areas, which can be hypermethylated. This can result in abnormal transcription with subsequent alterations in cellular proliferation, cell cycle control, etc.

ACKNOWLEDGEMENTS

The research was financially supported by the Czech Science Foundation (GA CR 16-18917S), League Against Cancer Prague and CEITEC 2020 (LQ1601).

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THE COMPARISON OF EFFECT OF ZINC SULPHATE AND ZINC OXIDE NANOPARTICLES ON PLANTS

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Abstract: Zinc oxide nanoparticles are one of the most versatile materials, due to their diverse properties, functionalities, and applications. Their potential in agriculture is also not negligible. The zinc in form of nanoparticles has better ability to penetrate into the plant roots. This makes this form of zinc more available for plants. However, there is still lack of information about their toxicity. In this work we focused on the evaluation and comparison of the effect of common zinc source ($ZnSO_4.7H_2O$), and zinc in form of nanoparticles (nanoZnO) in *Helianthus annuus* L. Our pilot results show that that nanoZnO have significantly negative impact on the growth parameters of the sunflower. Growth retardation increased with increasing applied concentration of excess zinc. Our results also indicated that as opposed to control, zinc ions with a prolonged experimental time significantly inhibited protein production in plants in the roots, stems, and in the leaves.

Key Words: zinc nanoparticles, phytotoxicity, *Helianthus annuus*, plant nutrition, stress biomarkers

INTRODUCTION

Nanotechnology is one of the revolutionary technology of this century. It deals with nanoparticles that are atomic or molecular aggregates characterized by size less than 100 nm. These are modified forms of basic elements derived by altering their atomic and molecular properties of elements (Wang 2004). This technology has a wide range of uses, as optics, electronics, and biomedical and material sciences. Among other applications, nanotechnology has a great potential to modify conventional agricultural practises. Nanoparticles could be used to minimize losses of nutrients, reduce the applied amounts of plant protection products and increase yields through optimized nutrient management (Das et al. 2015, Rizwan et al. 2017). Full understanding of the interaction mechanism between nanoparticles/nanomaterials and biological systems, however, is still out of sight. In this respect, significant progress has been made in research regarding the use nanotechnologies in medicine; however, the study of the nanoparticles and plant interaction that could find use in the future agriculture is only in its beginnings.

Zinc is an essential mineral element for plants. Zinc deficiency in plants, common in alkaline soils, results in growth arrest and sterility, but on the other hand, zinc can also become toxic at elevated concentrations. Zinc sulfate is ordinarily used as a main component of common zinc fertilizers, but there is growing interest in the use of zinc oxide nanoparticles (ZnO NPs) in agriculture. The normal ZnO and its nanoparticles are commonly added to plastic, glass, ceramics, cement, and rubber materials, as well as pigments, paints, food supplements, batteries and non-flammable materials. The reason for this is their wide range of suitable properties, which is also linked with the easy availability and low price of the chemical. These properties include relatively high electrical and thermal conductivity, stability in high temperatures, ability to absorb UV radiation and, with a neutral pH, mild antimicrobial effects (Moezzi et al. 2012).

On the one hand ZnO NPs have potential to boost yield and growth of crops (Prasad et al. 2012, A El-Kereti et al. 2013), but on the other hand, toxicological effects of these NPs should be also taken into consideration. In this case, one of the most important toxicity factors is concentration of ZnO NPs treatment, which should not reach too high levels (García-Gómez et al. 2017, Lin and Xing 2007).

In this study, we chose to compare the effect of $ZnSO_4.7H_2O$ and ZnO nanoparticles on sunflower plants and evaluate both positive and negative effects caused by these treatments.

MATERIAL AND METHODS

Plant cultivation and experimental design

We used sunflower (*Helianthus annuus* L.) Kongo hybrid as an experimental plant. The achenes were sterilized 20 minutes in 20% SAVO solution and planted in perlite substrate, then germinated for seven days at 22 °C with photoperiod day/night 16/8 hours. After this period, we transplanted the grown seedling plants into the hydroponic container containing a Richter nutrient solution (Laštůvka and Minář 1967). Extra boric acid solution was added due to the sunflower sensitivity to boron deficiency (Blamey et al. 1979). We also adjusted pH to 5.6 with KOH (1M).

Plants (2 weeks old) were treated for a month with $ZnSO_4.7H_2O$ or ZnO nanoparticles at zinc ions concentration 0, 0.6, and 6 mg/l. The samples (six individual plants) were harvested every week (for 3 weeks totally). First, we evaluated growth parameters (root and shoot length, fresh weight, and dry weight), and secondly, we determined the content of stress biomarkers (described below) spectroscopically. For spectroscopic measurements, plants were powdered in liquid nitrogen and aliquots were taken for analysis. The determination was done for roots, shoots, and leaves separately.

Spectroscopic measurement

Spectrophotometric measurements were carried out using an automated chemical analyser BS-400 (Mindray, Shenzhen, China). Reagents and samples were placed at cooled sample holder (4 °C) and automatically pipetted directly into plastic cuvettes. All incubations proceeded at 37 °C. All measurements were done in triplicate. Methods were calibrated using these standard compounds (from Sigma Aldrich, St. Luis, MO, USA): Bovine Serum Albumin for Bradford reaction, acetylcysteine for Ellman reaction, L-ascorbic acid for DPPH test, and gallic acid for phenolic compounds.

Determination of antioxidant activity by the DPPH test

This test is based on the ability of the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical to react with hydrogen donors. A solution of radical is decolourized after reduction with an antioxidant or a radical. A 200 µl volume of DPPH reagent was incubated with 20 µl of sample and absorbance was measured after 15 minutes at wavelength 510 nm.

Determination of total protein – Bradford reaction

Reagent Coomassie Brilliant blue G-250 (0.01% Coomassie Briliant Blue G-250, 4.7% CH_3CH_2OH, 8.5% H_3PO_4) in volume of 190 µl was pipetted. The sample in volume 10 µl was added. Mixture was incubated for 10 min and absorbance was measured at wavelength 595 nm.

Statistical analysis

Samples were analysed by one-way analysis of variance (ANOVA) with significant differences between means (n=3) ($p<0.05$). The data were analysed by using software STATISTICA version 12.

RESULTS AND DISCUSSION

In our experiment we exposed the 14-day old sunflower plants to excess zinc in form of $ZnSO_4.7H_2O$ and ZnO nanoparticles (nZnO), separately. In both cases, we used these concentrations of excess zinc: 0 (control); 0.6; 6 mg/l. The experimental treatment lasted for 3 weeks. Six plants were evaluated each week for the effect of zinc ions on growth and stress markers. The results are summarized in Table 1. In the case of $ZnSO_4$, it has been observed that plants exhibit mild growth stimulation throughout the experiment compared with the control. However, this increase

was 22% higher in the first week in comparison to control, 7% higher in the second week and only 4.4% higher in the third week. However, when we applied the same concentration of ZnO nanoparticles to the plants, we observed the opposite trend. These plants with prolonged experimental period and increasing zinc concentration exhibited increasing growth depression, ranging from 8.2% (after the first week) to 15.6% (after three weeks).

In addition to growth, we were also interested in the influence of applied forms of zinc ions on the growth of biomass. We determined both fresh weight and dry matter of plants. In the case of fresh weight, in the variant when the plants were exposed to zinc ions in the form of $ZnSO_4$, we observed that in the first weeks, compared with the control, the biomass increased significantly by up to 44.7%. With a prolonged experiment time, however, this trend was slowing down, and a moderate growth depression of up to 3.4% was observed in all the $ZnSO_4$ zinc concentrations in the last week. We observed a similar trend in this variant even in the case of dry matter, except that no statistically significant increase in dry matter was observed in any of the applied concentrations in the last week (compared to the control).

When ZnO nanoparticles were applied to the plants, we observed a decrease in biomass by 21.5% in the first week, and we determined similar values in the second week. However, in the last week of the experiment, the decrease in biomass was again reduced by 21.2% compared to the control. In the case of dry matter, we found that the plants exposed to 0.6 mg/l at all times showed a slight decrease in the dry matter content of 6.2% over the whole time, while at the applied concentration of 6 mg/l this decrease was much more pronounced (by 47.9%).

Table 1 Overview of treatments in comparison to control (% difference). The asterisk (*) indicates significant differences ($p < 0.05$) between control samples and individual treatment samples.

Treatment	Week 1	Week 2	Week 3	Growth parameter
$ZnSO_4$ (both concentrations)	22% ± 3.21% *	7% ± 0.89% *	4.4% ± 1.08%*	plant length
nZnO (both concentrations)	-8.2% ± 3.01%	-12.8 % ± 2.65%*	-15.6% ± 2.47%*	
$ZnSO_4$ (both concentrations)	44.7% ± 10.58%*	40.6% ± 2.78%*	37.2% ± 6.91%*	fresh weight
nZnO (both concentrations)	-21.5% ± 4.78%*	-21.2% ± 3.55%*	-19.8% ± 6.73%*	
$ZnSO_4$ (both concentrations)	40.2% ± 6.79%*	36.7% ± 4.34%*	no data	dry matter
nZnO (0.6 mg/l)	-6.2% ± 3.28%			
nZnO (6 mg/l)	-47.9% ± 5.84%*			

From these basic data, we can state that zinc nanoparticles have significantly negative impact on the growth parameters of the sunflower. Growth retardation increased with increasing applied concentration of excess zinc.

In addition to the growth parameters, we have also explored the effects of nanoparticles on stress markers in our experiments. In particular, we were interested in the effect on protein content and antioxidant activity.

Our results show, that as opposed to control, zinc ions with a prolonged experimental time significantly inhibited protein production in plants in the roots, stems, and in the leaves. In the case of antioxidant activity, it was found that defensive mechanisms of plants were triggered in first week.

Plants treated with excess zinc exhibited higher antioxidant activity in comparison to control plants. Surprisingly, their antioxidant activity decreased in the second week. In third week, it regained the level comparable to the activity level in the first week.

These results (in general) slightly differ from majority of other studies targeting different plant species. One example is study about comparison of effect of $ZnSO_4$ and ZnO nanoparticles on tomato plants (Singh et al. 2016). Concentration of treatment was similar to our experiment. Nanoparticles had (in this case) positive effect on the observed plant parameters (seedlings vitality, germination, protein content).

The reason for this reverse effect may not be only the different plant species, but also the youth of plants. The ZnO nanoparticles in low concentration have positive effect (in general) on germination and growth of young seedlings. As example can be used study done on these plant species: *Vigna radiate* and *Cicer arietinum* (Mahajan et al. 2011). Authors applied ZnO nanoparticles in different concentrations. Lowest concentration (1 mg/l) promoted the germination and vitality of *Cicer arietinum*. *Vigna radiate* plants had the best vitality at 10 and 20 mg/l.

The studies concerning impact of ZnO nanoparticles on plant in the long term experiments are still sparse. However, in these rare studies a toxicity trend emerges. As example can be used greenhouse experiment made on tomato plants and bean plants (García-Gómez et al. 2017). Authors observed plants for total 90 days and compared effect of ZnO nanoparticles and $ZnSO_4$ in various concentrations. Both treatments caused similar toxicity associated with reduction of chlorophyll content and increase of antioxidant activity. These results show similarity to results of our experiment. The ZnO nanoparticles seem to have harmful effects on plants in prolonged period of time.

CONCLUSION

Zinc belongs to the micronutrients and conventional fertilizers (not only) of zinc are faced with the problem of poor bioavailability, due to the fixation of this element to insoluble compounds in the soil. Improving the knowledge about individual forms of zinc and their up-take and assimilation within higher plants may be the first step towards a wider involvement of zinc nanoparticles into agriculture in the field of plant nutrition (nanofertilizers) and protection (nanopesticides). We have touched the issue by our pilot experiments in which we compared the difference of effect on the plants between zinc in form of nanoparticles and zinc in form of simple inorganic salts.

ACKNOWLEDGEMENTS

This research has been financially supported by the Ministry of Education, Youth and Sports of the Czech Republic under the project CEITEC 2020 (LQ1601) and Internal Grant Agency of Mendel University in Brno (IP 28/2017).

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SURFACE PEGYLATION AND PASYLATION TO REGULATE NANOPARTICLE INTERACTIONS WITH BIOLOGICAL ENVIRONMENT

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Abstract: Many researchers are developing nanocarriers in order to minimalize side effects of cytotoxic drugs during cancer treatment *via* chemotherapy. Nanocarriers can serve as a suitable platform for targeted drug delivery. To overcome their failure in *in vivo* use, the effects of surface modifications (PEGylation and PASylation) of natural nanocarriers based on apoferritin were tested in this work. Various properties of these modified apoferitin nanoparticles were studied, such as their size or degree of hemolysis. TEM characterization was also performed. The formation of hard coronas on these particles in plasma environment was evaluated using SDS-PAGE electrophoresis. The best biocompatibility results were obtained using apoferritin nanoparticles with PEG surface modification.

Key Words: apoferritin; nanomedicine; protein coronas; surface modifications

INTRODUCTION

Nowadays there are over 200 different types of cancer affecting humans (Broto et al. 2017) Chemotherapy is a method for treating cancer using cytotoxic agents, whose main disadvantage is their non-specify for cancer cells. This means that they are also strongly affecting healthy cells. Thus, the actual dose of cytostatic received by affected tissue is hard to control (Broto et al. 2017). By targeting of cytostatics directly to diseased tissue, it is possible to minimize the serious side effects of chemotherapy, such as the cardiotoxicity of doxorubicin or mutagenicity of ellipticine (Bazak et al. 2015). The number of nanocarriers that could be used for this purpose is growing exponentially, but only a few of these nanocarriers are currently tested in clinical trials and even lower number is used in clinical practice. To improve the success of their therapeutic use, a better understanding of their biological identity is needed. Many nanoconstructs cannot fulfil their role after exposition to body environment; they can even lead to hemolysis or aggregation of blood platelets. Or, upon entering blood stream, nanoparticles are often coated by protein corona, changing their surface, and hampering their internalization into diseased cells. Protein corona forms after exposure of nanocarrier to plasma proteins that occur at 60-80 g/l (Broto et al. 2017). We can define protein corona as a natural interface between nanomaterials and living matter in biological milieu (Monopoli et al. 2013).

In order to control and reduce the binding of additional biomolecules and formation of protein corona, surface modifications with various polymers can be used. In this contribution, we studied the effects of modification with poly-ethylene glycol (PEGylation) and peptides rich in proline, alanine and serine residues (PASylation). PEGylation represents chemical conjugation with synthetic hydrophilic and uncharged synthetic polymer PEG (Binder andSkerra 2017). This usually happens *via* the ε-amino group of lysine residues or the thiol group of cysteine. PEGylation is the most widely established method of prolonging the half-life of drug in bodily fluids (Pasut andVeronese 2012).

To this date, there are up to 15 approved PEGylated drugs (Gabizon et al. 2016). The main drawbacks of using PEG for surface modification are its high cost, non-biodegradability and possible cellular vacuolation caused by PEG (Ivens et al. 2015). PASylation is a biological alternative to PEGylation based on genetic fusion or chemical coupling of nanoparticles with polypeptides (Chow et al. 2008). PASylation bypasses disadvantages of PEGylation, such as its high cost and polydispersity. Moreover, PAS has lower viscosity and is more hydrophilic than PEG, which enables intravenous administration of PASylated drugs or nanoparticles (Binder and Skerra 2017). Their most important features are their non-immunogenicity (Harari et al. 2014), stability in plasma (Kuhn et al. 2016) and quick degradation by intracellular/lysosomal proteases after cellular uptake, which does not cause cellular vacuolization (Binder and Skerra 2017).

The effects of PEGylation and PASylation on *in vivo* behaviour of nanoparticles were studied using ubiquitous protein cage apoferritin (Apo) with encapsulated ellipticine (Elli). Apo self-assembles into hollow rhombic dodecahedral cage of 12 nm in diameter, which stores and transports iron and iron ions in organism (Bulvik et al. 2012). Ellipticine is a natural pyridocarbazole type alkaloid showing cytotoxic activity against many cancer cell lines but it is highly mutagenic, which can be risky for healthy cells (Kizek et al. 2012). We encapsulated Elli into Apo and modified its surface with PEG or PAS sequences. The characterization of these nanoformulations was performed *via* dynamic light scattering (DLS) and transmission electron microscopy (TEM). The behaviour of these nanoparticles in human blood was evaluated using SDS-PAGE electrophoresis and hemolytic assay.

MATERIAL AND METHODS

Chemicals

All chemicals of ACS purity were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Encapsulation of Elli into Apo

The stock solution of ellipticine with concentration of 1 mg/ml was prepared by dissolving Elli in 1 M HCl and deionized water in ratio 1 : 150. For each sample, 200 µl of 1 mg/ml Elli was added to 100 µl of deionized water and 20 µl of 50 mg/ml horse spleen Apo and gently mixed for 15 min. To reassemble the Apo structure disassembled by acidic Elli and encapsulate Elli in Apo cavity, 0.66 µl of 1 M sodium hydroxide solution was added and the samples were mixed for further 15 min. To filter out non-encapsulated Elli, solution exchange was performed three times (centrifugation at 6000 g for 15 min).

Surface modification with PEG

50 µl of 10 mM PEG maleimide in PBS (phosphate buffered saline, pH 7.4: 0.137 M NaCl + 0.0027 M KCl + 0.0014 M KH_2PO_4 + 0.0043 M Na_2HPO_4) and 629 µl of PBS was added to ApoElli and mixed for 1 h. To filter unbound PEG, the sample was 5 times diafiltrated using Amicon® Ultra 0-5 ml 50K Merck Millipore (Billerica, MA, USA) at 6000 g for 15 min.

Surface modification with PAS

25 µl of 1.3 nm gold nanoparticles was added to ApoElli and the samples were mixed for 14 h to allow binding of Au nanoparticles on the surface of ApoElli nanoparticles (creating ApoElli-Au). Solution exchange was performed two times to remove unbound Au nanoparticles. 3 µl of 1.25 mg/ml PAS-10 (ASPAAPAPASC) and PAS-20 (ASPAAPAPASPAAPAPSAPAC) was added to ApoElli-Au and the samples were incubated for 1 h at 45 °C to allow binding of cysteine to gold. Then, solution exchange was performed to remove unbound molecules of PAS peptides.

Characterization of prepared samples

Concentration of encapsulated Elli

The concentration of encapsulated Elli was evaluated by absorbance measurement at 420 nm using Tecan Infinite 200 PRO (Männedorf, Switzerland).

Size characterization by DLS

The average size of prepared nanoparticles was determined by quasielastic dynamic light scattering using Zetasizer Nano ZS instrument, Malvern Instruments Ltd. (Worcestershire, UK). Polystyrene latex cells ZEN0040 were used for measurements and the conditions of measurements were as follows: detector angle of 173°, wavelength of 633 nm and temperature of 25 °C, equilibration time 120 s. The measurements were performed in hexaplicates.

TEM characterization

Visualization of nanocarriers and their surface modification was performed using transmission electron microscopy (TEM) with negative staining technique. An organotungsten compound Nano-W Nanoprobes (Yaphank, NY, USA) was used. 4 µ of samples were deposited onto 400-mesh copper grids coated with a continuous carbon layer. Dried grids were imaged by TEM Tecnai F20; FEI, (Hillsboro, OR, USA).

In vitro evaluation of behaviour in human blood

Blood samples were collected into EDTA coated Vacutainer tubes and centrifuged at 3 000 rpm and 25 °C for 10 min. Red blood cells (RBC) were used for hemolytic assay while pooled plasma was further centrifuged at 22 000 rcf and 4 °C and for 30 min and used to evaluate formation of protein corona.

Hard corona formation

100 µl of plasma was mixed with 100 µl of 200 µg/ml Elli, either free or encapsulated in modified and unmodified Apo nanocarrier. The samples were incubated at 600 rpm and 37 °C for 35 min. To remove unbound plasma proteins from nanoparticles, 4 times solution exchange was performed. To determine the degree of protein corona formed on tested nanoformulations, SDS-PAGE electrophoresis was used. Proteins were separated at 200 V for 35 min using 12.5% polyacrylamide gel.

Hemolysis assay

RBC were centrifuged at 3 000 rpm for 10 min multiple times and resuspended in 150 mM NaCl until the supernatants were transparent. Then, RBCs were diluted with PBS and 150 µl was mixed with 150 µl of decreasing concentrations of Elli (100; 50; 25; 12.5 and 6.3 µg/ml), either free or encapsulated in Apo nanoformulations; negative control (PBS) or positive control (0.2% Triton X-100). The samples were incubated at 37 °C and 600 rpm for 1 h. Incubation was followed by centrifugation at 3 000 rpm for 10 min. Absorbance at 540 nm was measured and the percentage of hemolysis was calculated *via* following formula:

$$\%\text{hemolysis} = [(A_t - A_c)/(A_{100\%} - A_c)] \cdot 100 \quad (1)$$

where A_t stands for absorbance of sample's supernatant, A_c represents absorbance of negative control's supernatant, $A_{100\%}$ stands for absorbance of positive control's supernatant.

RESULTS AND DISCUSSION

Characterization of prepared samples

Elli encapsulation efficiency, size characterization by DLS

The highest encapsulation efficiency (9%) was achieved in sample with PEG modification (Table 1). The size measurements (Table 1) showed that the size of Apo increased from 12 nm to 18 nm after encapsulation of Elli. The various surface modifications further influenced the size of ApoElli, where PAS-10 modification caused aggregation of multiple ApoElli molecules while PAS-20 and PEG modifications did not cause any aggregation.

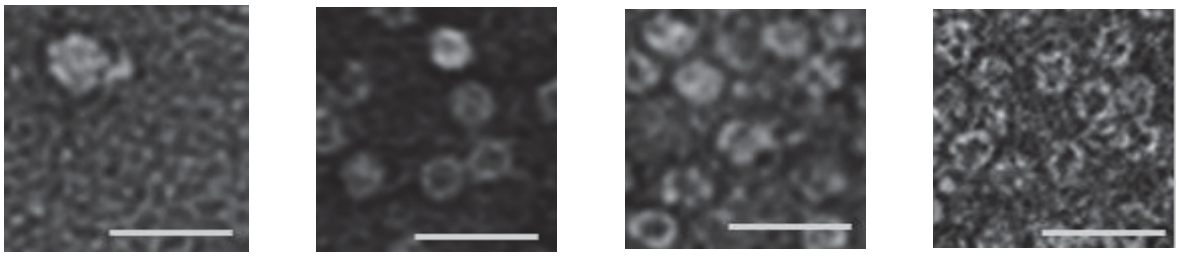
Table 1 Characterization of prepared nanoparticles

Sample		Encapsulation efficiency [%]	Size of prepared nanoparticles [nm]
Number	Surface modification		
1	PEG	91.20	13.545
2	PAS-10	52.83	79.671
3	PAS-20	43.96	13.545
4	No surface modification	69.00	18.166

TEM characterization

TEM characterization was performed to detect changes in apoferritin structure after surface modifications (Figure 1). The results showed that the tested surface modifications did not cause disassembly of Apo quaternary structure, as typical icosahedral particles were observed in all samples. The presence of polymer on ApoElli surface can be seen in PEGylated samples. PASylation was also proven by thicker protein shell of ApoElli.

Figure 1 TEM images, scale corresponds to 25 nm (from left to right: apoferritin modified with PEG; apoferritin modified with PAS-10; apoferritin modified with PAS-20; control without any surface modification)



Hemolysis assay

Table 2 Degree of hemolysis caused by all tested formulations

Concentration of Elli [µg/ml]	PEG	PAS-10	PAS-20	No surface modification	Elli
100.00	0.05	0.00	0.00	0.06	5.41
50.00	0.00	0.00	0.00	0.00	3.74
25.00	0.00	0.00	0.00	0.00	1.67
12.50	0.00	0.00	0.00	0.00	0.84
6.25	0.00	0.00	0.00	0.00	0.15

To evaluate hemotoxicity of the tested nanoparticles, RBC hemolytic assay was performed. The results of hemolytic assay are summarized in Table 2. The lysed RBC caused red coloring of the supernatant, whereas transparent supernatant showed no occurring hemolysis. Free Elli caused hemolysis at all tested concentrations (see Figure 2) and the degree of hemolysis was dependent on Elli concentration (5% at 100 µg/ml). The encapsulation of Elli in Apo nanocarrier favorably influenced this hemolysis caused by Elli. Very low degree of hemolysis was observed only at highest Elli concentration in samples containing PEGylated ApoElli and sample without any surface modification (0.05% for PEGylated ApoElli and 0.06% for ApoElli). Samples with surface modified with PAS-10 (see Figure 3) and PAS-20 did not show any degree of hemolysis. The obtained results clearly show that all ApoElli nanoformulations, both unmodified and surface modified, are highly biocompatible with human RBC.

Figure 2 Samples without using apoferritin nanocarrier (from left to right: negative control; 6.25; 12.5; 25; 50 and 100 µg/ml Elli; positive control)

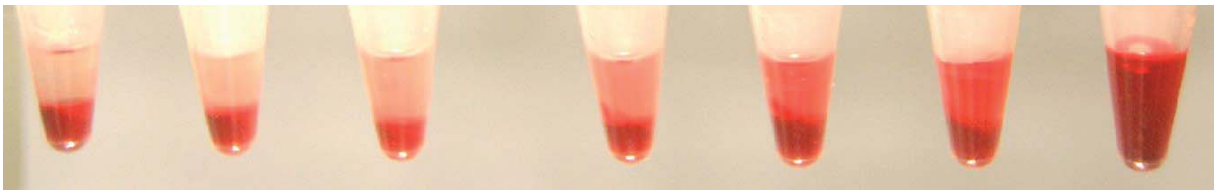
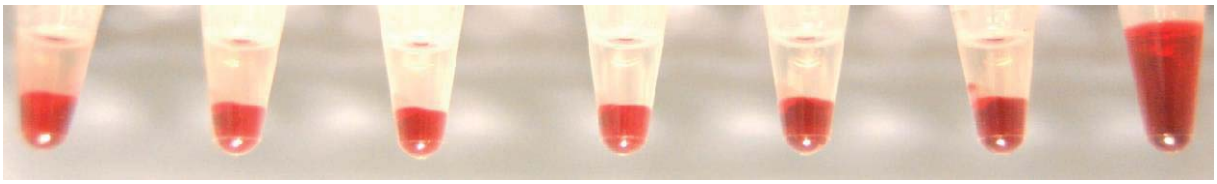


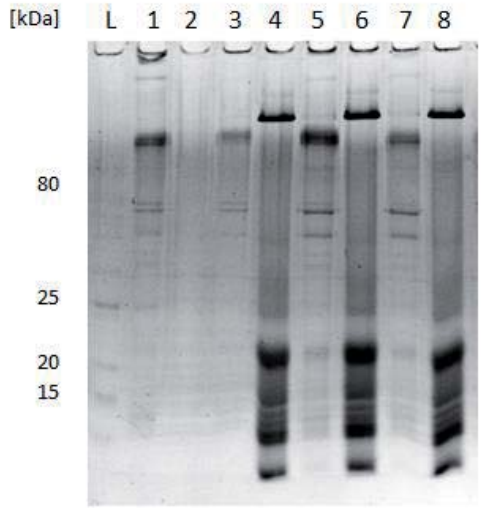
Figure 3 Samples modified with PAS-10 (from left to right: negative control; 6.25; 12.5; 25; 50 and 100 µg/ml Elli encapsulated in Apo modified with PAS-10; positive control)



Evaluation of protein coronas

We further performed SDS-PAGE in order to evaluate the influence of PEGylation and PASylation on the formation of protein corona on ApoElli surface (see Figure 4). The ideal surface modification of nanocarrier should minimalize interactions between the surface of nanocarrier and plasma proteins, which naturally occur in human plasma. Efficient surface modification would reduce binding of proteins on the surface of nanocarrier, which leads to reduced protein corona formation or even inhibits protein corona formation all together. The surface modification with PAS-10 and PAS-20 did not decrease the interactions with plasma proteins and the protein corona was formed in a very similar way to that formed around unmodified ApoElli. Thus it can be concluded, that these modifications were not suitable for surface modifications of nanocarriers in order to avoid protein-nanocarrier interactions in bodily fluids. On the other hand, PEGylated ApoElli showed presence of no proteins from human plasma. Surface modification with PEG proved more suitable for future *in vivo* use as it was able to significantly lower protein-nanocarrier interactions.

Figure 4 SDS-PAGE (L - NEB Protein Ladder 10-250 kDa; 1 - PEGylated ApoElli; 2 - protein corona on PEGylated ApoElli; 3 - PAS-10ylated ApoElli; 4 - protein corona on PAS-10ylated ApoElli; 5 - PAS-20ylated ApoElli, 6 - protein corona on PAS-20ylated ApoElli; 7 - ApoElli; 8 - protein corona on ApoElli)



CONCLUSION

The experiment presented in this work dealt with the prediction of *in vivo* behaviour of apoferritin nanocarrier, based on *in vitro* tests of their hemotoxicity and formation of protein corona around these particles in human plasma environment. The surface of this apoferritin nanocarrier was modified with polymer (PEG) and peptides (PAS) in order to decrease these negative interactions. Overall, the results showed that while all tested modifications favourably influenced unwanted hemotoxicity of ellipticine, only PEGylation was able to lower interactions between nanocarrier surface and proteins in bodily fluids.

ACKNOWLEDGEMENTS

The research was financially supported by the Grant Agency of the Czech Republic (GA CR 17-12816S) and CEITEC 2020 (LQ1601).

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MULTIFUNCTIONAL PIPETEING PLATFORM FOR MOLECULAR BIOLOGY AND BIOCHEMISTRY

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Abstract: The classical hand pipetting approach can be partially substituted in several cases by sophisticated devices that are taking over the necessity of sample manipulation. The automatic pipetting method can facilitate routine laboratory work, but the created method has to meet several criteria including – minimizing the possibility of contamination. In this work is briefly summarized four years experiences with the liquid handling system BioNex Nanodrop II. This device is able to pipette (using eight pipetting tips) small volumes in the range of 0.100 µl up to 500 µl into the three common microplate formats – MTP 96, MTP 384 and MTP 1536. The possibility of sample (bacteria, bacterial plasmids and DNA templates for PCR) cross-contamination was estimated in three experiments. The cross-contamination was not presented or it was detected at very low level. The main advantages – speed and accuracy – of the automatic pipetting are described in the last experiment where the enzyme activity was measured.

Key Words: liquid handling system, pipetting, cross-contamination, *E. coli*, enzyme kinetic

INTRODUCTION

In the field of molecular biology and biochemistry where pipetting samples and chemical reagents is daily necessity any help is useful. Each researcher is responsible for precision, accuracy and reproducibility of obtained results. In several experimental procedures and methods numbers of pipetting samples is enormous and therefore arise the chance of mistakes caused by manual pipetting. A programmable pipetting equipment (the liquid handling system) offers in such particular situation a solution. The correct choice of appropriate devices is always challenging task because each device has several limitations. To facilitate this problematic decision we present here our four years experiences with the BioNex Nanodrop II pipetting station. This device is able to aspirate liquids (max. volume 500 µl for each tip) and eight pipetting tips allow to dispense volume (min. 0.100 µl) mainly into MTP96, MTP384 and MTP1536 plates. Tips are fixed on a robotic arm that moves in X, Y and Z dimensions above the plate(s). The inner and outer surface of pipetting needles (tips) have to be washed after each pipetting cycle in detergents or water to clean residual liquids (samples) (Bionex 2017).

Each researcher is concerned about the possibility of contamination (that may occur during the sample pipetting) that complicates the interpretation of experimental result. For that purpose four examples here are presented where the most important and sensitive problem is tested – the cross-contamination.

MATERIAL AND METHODS

Used materials and methods

All experimental details are partially described in the main text or can be found in full details in particular publications cited in text.

Used equipment

A Nanodrop II liquid handling system (BioNex Solutions, USA) was used for all pipetting steps. Absorbance and fluorescence were measured using an Infinite M1000Pro plate reader (Tecan, CH).

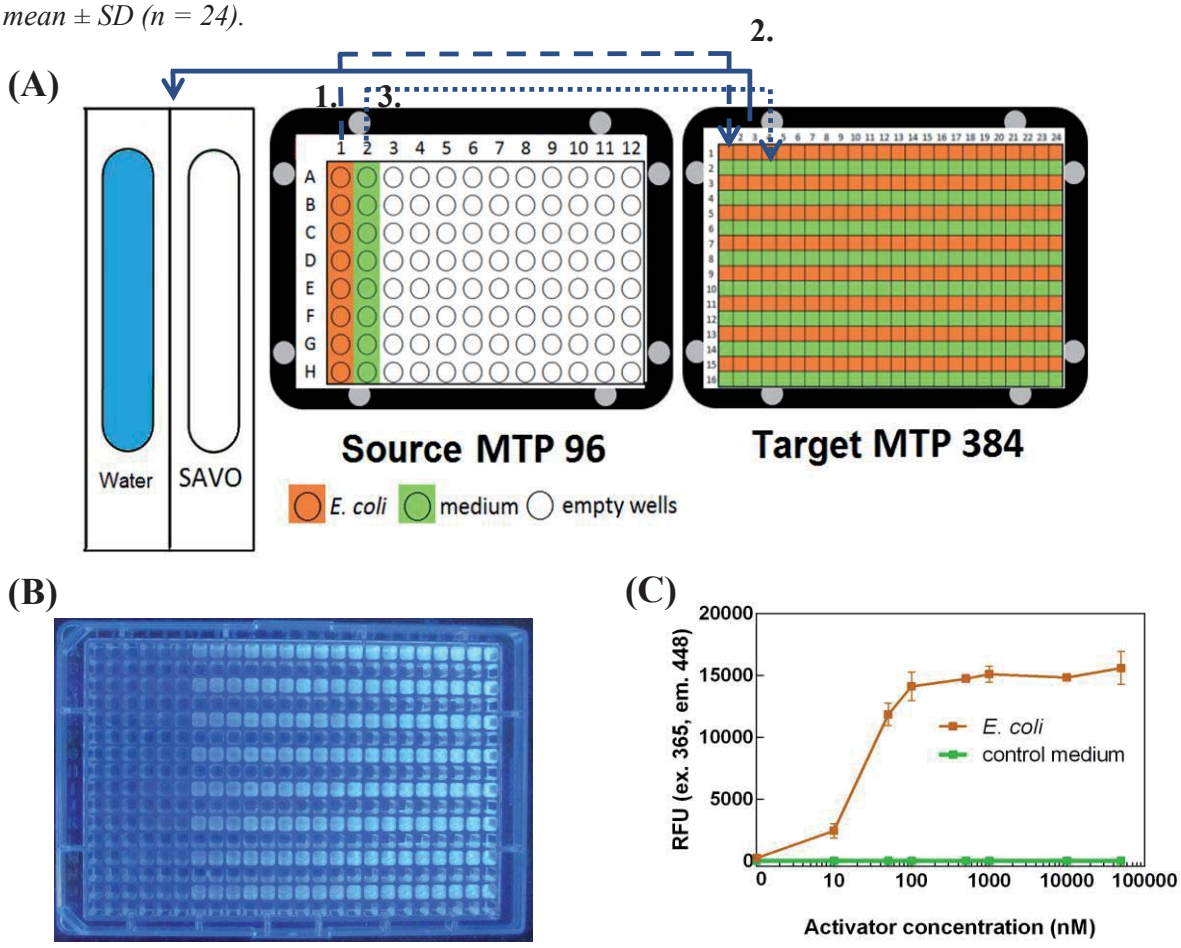
Quantitative PCR was performed using the LightCycler® 480 SYBR Green I Master kit (Roche Applied Science) and PCR was performed with LightCycler® 480 System Technology (ROCHE, CH), as described in (Skalák 2016).

RESULTS AND DISCUSSION

The Nanodrop II (the liquid handling system) was used in experiments where the high precision of pipetting is required. In this article we demonstrate the reliability of pipetting of the Nanodrop II in four examples where different experimental conditions were tested.

Test of *E. coli* cross-contamination

Figure 1. ***(A)*** *Arrangement of E. coli and control medium in the source MTP96 plate. Blue arrows represent the sequence of movement. The sequence of pipetting samples (1 µL) into the target MTP384 plate is: 1. dashed arrow - E. coli pipetting, 2. solid arrow - the pipetting needles were washed in 50% bleach (SAVO) and 2x in distilled water container, 3. dotted arrow - and then control medium was pipetted.* ***(B)*** *Picture of the target MTP384 plate. Fluorescent product is visualized by UV light. As expected, the fluorescence is visible only in those parts where E. coli were growing (odd lines). Other parts (even lines) are not contaminated from previous pipetting of E. coli and therefore the fluorescence is not detected. There is a typical correlation between increasing concentration of the activator and final fluorescent intensity.* ***(C)*** *Fluorescence measurement of the target MTP384 plate. Fluoresce was measured for all wells of the MTP384 plate and the signal appeared in those where E. coli were present. There are three replicates for each concentration of the activator in every row of the MTP384 and the test was performed by eight independent needles. Each point then represents mean ± SD (n = 24).*



E. coli is very often used in many biological assays and therefore it is necessary to take care about the cross-contamination of biological sample(s) during manipulation. The Nanodrop II is normally used in our experiments where *E. coli* is transferred between two plates. To test the possibility of bacterial cross-contamination, elegant test was performed where two parameters

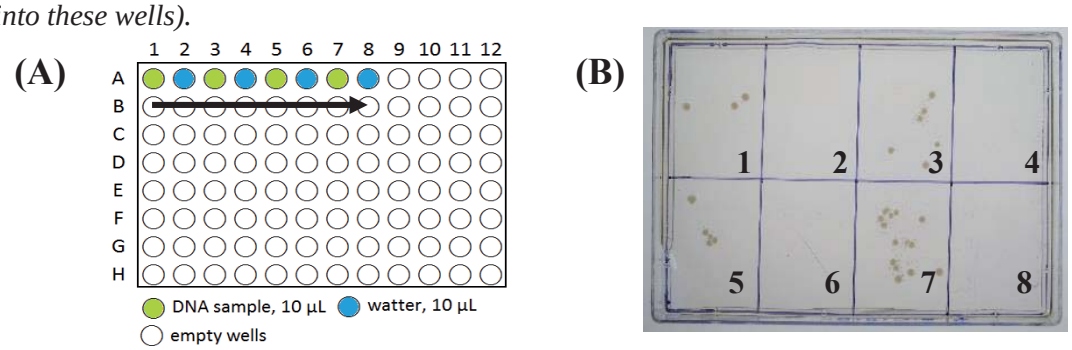
are measured: a) optical density and b) activity of the signalling pathway (measured in fluorescence assay) of transferred *E. coli*. For experimental details see (Klimeš 2017b) and principle of signalling pathway is described in (Yamada 2001, Suzuki 2001). From a technical point of view 30 µl of *E. coli* (over-night culture) was aspirated from the wells of column A of the MTP96 source plate (filled with 200 µl of bacterial suspension) and 1 µl of this bacterial culture was dispensed 24-times into odd rows of the MTP384 target plate (prefilled with 29 µl of growth medium containing concentration gradients of activators for the *E. coli* signalling pathway). All needles were washed in 50% bleach (SAVO) and 2x in distilled water containers. Then 30 µl of control medium was aspirated from the column B of the identical MTP96 source plate (filled only with 200 µl of sterile medium) and 1 µl was dispensed 24-times into the even rows of the MTP384 target plate (also prefilled with 29 µl of growth medium containing concentration gradients of activators for the *E. coli* signalling pathway). The pipetting sequence is indicated in Figure 1 A. Such prepared plate was cultivated according to (Klimeš 2017b). During that time *E. coli* were growing and the signalling pathway was responding to external activators presented in growth medium. Results of the test are presented in Figure 1 B, C.

Optical density was measured in all wells. The MTP384 plate without medium has optical density (0.045 ± 0.006) (n = 384). Optical density of odd lines (lines with *E. coli*) was (0.242 ± 0.016) (n = 192) and for even lines (with tested medium) was (0.050 ± 0.008) (n = 192) at the end of the experiment. Comparison of these values between each other provides clear evidence that contamination has not occurred in even lines of the MTP384 plate.

In this experiment it was confirmed that *E. coli* is not transferred from column A to column B during pipetting and so the cross-contamination has not occurred. The washing step of pipetting needles in bleach (50% SAVO) and distilled water (to remove traces of bleach) is sufficient to keep pipetting needles clean. Moreover all eight needles were used in pipetting so eight biological replications are presented at the same time and all of them are consistent.

Test of plasmids cross-contamination in procedure of *E. coli* transformation

Figure 2. ***(A)*** *Scheme of samples arrangement in source MTP96 plate. Black arrow represents the direction and order of pipetting samples (2 µL) into target MTP96 plate (not shown). After each sample pipetting, the pipetting needle was washed in 50% bleach (SAVO) and 2x in distilled water containers. After the pipetting of the last sample (from well no. 8) into target MTP96 plate, the competent E. coli were added into wells of that plate, plate was cultivated for 1 hour at 37 °C and cell suspension was spread with the same order on the agar plate.* ***(B)*** *The agar plate with E. coli colonies after the transformation. The numbers represent order of samples pipetting. If DNA was presented in particular wells, E. coli colonies appear in corresponding regions. Empty regions corresponds with wells where DNA was not present (was not transferred during pipetting DNA samples into these wells).*



E. coli transformation is one of the classical laboratory procedure that can be parallelized when multiple transformations are needed. Protocol itself consist of several follow-up steps and begins mixing the bacterial plasmid (DNA) with competent *E. coli* together in a test tube. In standard manual procedure one tip for each DNA sample is used and after use the tip is discarded. So the contamination is in principle very unlikely. In the case of parallelization where the MTP96 plate is used as storage container of bacterial plasmids, one needle must be used in pipetting of several DNA samples and the chance of contamination increase. The following test was designed to reflect the real experimental needs and also possibility of cross-contamination caused

by transferring DNA samples. The order of DNA samples in source MTP96 plate, pipetting pattern for one needle and results of the experiment are presented in Figure 2.

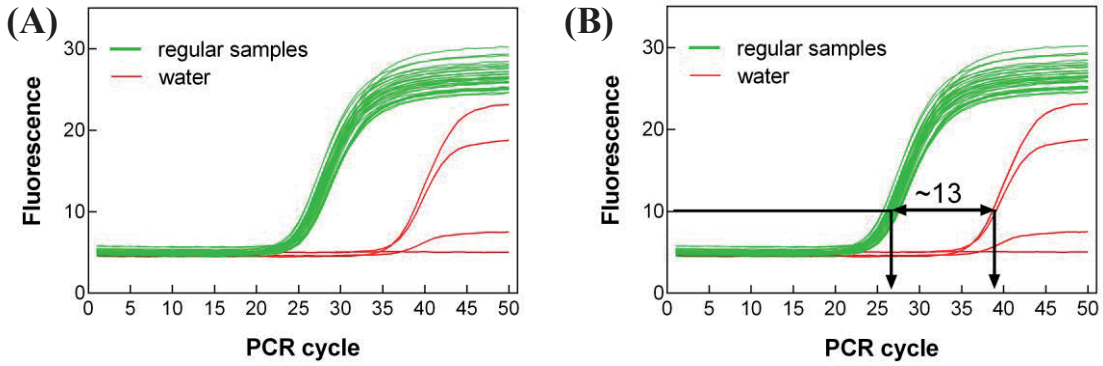
Result presented in Figure 2 B practically demonstrates three conclusions:

1.) Washing procedures (used during pipetting procedure) are sufficient to avoid the transferring of bacterial plasmids (DNA) into the water samples (absence of colonies in 2, 4, 6 and 8 segments).
2.) Washing procedures designed for pipetting needles are sufficient to keep them sterile after *E. coli* pipetting and *E. coli* are presented only in expected segments 1, 3, 5 and 7 of the agar plate.
3.) The last and most important one - with the Nanodrop II it is possible to perform (and so parallelize) the transformation procedure in the MTP96 plate format. So in one experimental setup up to 96 different bacterial plasmids are transformed into competent *E. coli*.

Test of sample cross-contamination in Real Time-qPCR

RT-qPCR is very sensitive technique where small amount of DNA is detected. Sample preparation and DNA isolation depends on the type of biological material provided as a source. In final step samples (DNA template) have to be transferred into detection plate where master mix (with PCR reagents) is added subsequently. During PCR is the fluorescence intensity measured after each cycle (in "real time") in special transparent plates and if sample contains targeted gene sequence, fluorescence slowly increases after each PCR cycle. The final "curve shape" is used for DNA quantification and obtained results are then relatively compared in relation to the control.

Figure 3 ***(A)*** *Graph bellow represents record from RT-qPCR where fluorescence intensity is measured for each sample at the end of each PCR cycle. Time points are transformed into individual smooth lines. Regular samples represent green lines and four red lines represent water (non-template control). Red lines rise up in later cycles compared to regular samples. That is, original source wells (with water) indicate possible contamination from the previous sample pipetting.* ***(B)*** *The estimation of contamination level in water is based on the measurement of the cycle shift between the regular samples and water. Black lines and arrows helps to follow the explanation indicated in main text under the graph.*



Sample pipetting can be handled still manually in the MTP96 detection plate but in case of the MTP384 detection plate the automatic pipetting procedure is needed. Also in this situation the special care is taken for sample (DNA template) cross-contamination. One typical sample arrangement is described in the following example. The pipetting sequence is straight: 1.) Sample one (of four) was transferred from the source MTP96 plate to the target MTP384 detection, 2.) all pipetting needles were intensively washed in distilled water and then next sample was pipetted into other position into the MTP384 detection plate. Previous two steps were repeated for all four samples. Based on this arrangement one pipetting needle was transferring four samples: three real samples and one negative control (water sample was pipetted as the last one). Then the RT-qPCR procedure was performed. The RT-qPCR of the MTP384 detection plate provides a substantial amount of results and therefore only the representative data set is presented in the Figure 3.

As mentioned before, only the order of samples pipetting was described. Results were intentionally chosen to show that sample cross-contamination can occur but on the other hand,

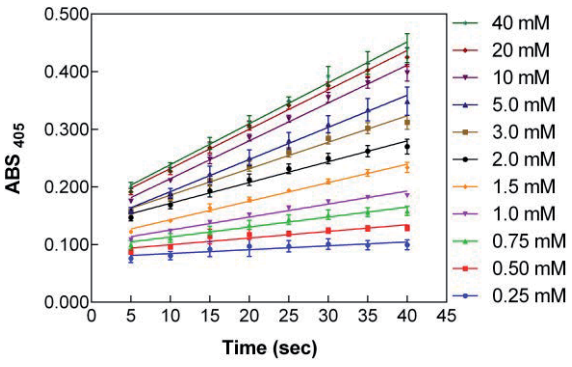
this contamination can be easily distinguished from the real sample (containing the targeted genome sequence). Based on the principle of the PCR it is possible to estimate the level of contamination by simple comparison of the number of cycles where the contamination was detected. The regular and water samples had the same fluorescence intensity (10 fluorescence units) approximately in the 26 and 39 PCR cycle. The difference is 13 cycles (Figure 3 B). Because PCR products in approximation arise exponentially, the initial amount of contamination presented in water was $2^{13} = 8\ 192$ times lower compared to regular samples. For other red lines is the contamination even lower or not present. Based on this estimation, the level of contaminations do not significantly change the RT-qPCR interpretation and is in accepted range.

Measurement of the rate of enzymatic reaction

Rate of enzymatic reactions can be also investigated using the Nanodrop II. In this part is omitted the sample and chemicals preparation, only direct pipetting and measurement is considered. All other experimental details were published in (Klimeš 2017). The following steps are described for one pipetting needle, in reality 8 needles were used simultaneously. Whole target MTP384 was prefilled with the stop solution (0.2 M Na_2CO_3). 1 µl of enzyme was dispensed into one well (MTP96 plate). Into the same well 60 µl of substrate was pipetted and the enzymatic reaction has started. 50 µl of this "running" reaction mixture was aspirated and then 5 µl was dispensed eight times in 5 second interval into eight different wells of the MTP384 plate with the stop solution (0.2 M Na_2CO_3). The yellow product of enzymatic reaction was measured at 405 nm and the dependence of absorbance and time was plotted. Measured absorbance and time points were interpolated by linear regression. Rate of enzymatic reaction was estimated for 11 different substrate concentrations and results are summarized in Figure 4.

Figure 4 ***(A)*** *The rate of product formation measured as an increase in absorbance over time is presented for 11 different substrate concentrations. Each concentration was measured at least three times and liner regression represents the best fit for particular substrate concentration. Naturally, the increasing absorbance corresponds with the product concentration formed during the enzymatic reaction.* ***(B)*** *Detailed information about linear regressions.*

(A)



(B)

Substrate concentration	Slope ($Abs_{405} \cdot s^{-1} \cdot 10000$) Average slope	SD	Rel. error (%)	Pearson corr. coefficient Average R^2	SD	n
40 mM	71.0	3.7	5.2	0.992	0.004	3
20 mM	68.3	1.8	2.6	0.992	0.003	3
10 mM	65.7	2.1	3.2	0.991	0.003	3
5.0 mM	55.8	3.8	6.8	0.993	0.003	3
3.0 mM	45.9	1.4	2.9	0.987	0.001	3
2.0 mM	36.1	1.1	3.1	0.981	0.001	3
1.5 mM	32.1	0.8	2.6	0.989	0.001	3
1.0 mM	22.6	0.4	1.8	0.972	0.004	6
0.75 mM	17.3	0.5	2.9	0.961	0.010	6
0.50 mM	12.4	0.2	1.7	0.959	0.007	6
0.25 mM	7.1	0.7	10.4	0.882	0.136	6

In experiment presented above were measured enzymatic reaction rates using 11 substrate concentrations. In total there were 45 individual measurements (Figure 4 B, the sum of the *n* parameter). Hand pipetting of the one enzymatic reaction takes 2.5 minute because 20 second is needed to get one point from eight while robotic arm is able to pipette eight reactions simultaneously in 40 seconds. When the "reaction time" is taken into account, a hand pipetting needs $8 \cdot 20 \cdot 45 = 2$ hours (to get complete data set) and an automatic pipetting needs $8 \cdot 5 \cdot (45/8) = 3.75$ minute (but robotic arm have to pipette 45 reactions 6 times, so the correct time is 4 minute) to get complete data set. The time saving is not so enormous in reality because the time needed to experiment preparation have be added in both calculations. Depending on the experimental conditions, the automatic procedure is still approximately eight times faster than manual pipetting (Klimeš 2017).

All measurements have reproducibility highly visible (Figure 4 A) which is reflected also in lower relative error (less than 10%). Moreover, calculated Pearson correlation coefficients are very close

to 1 that clearly show the linearity of individual measurement is very high (Figure 4 B). All these results indicate that automatic measurement of enzyme kinetic is possible with great accuracy.

In general, lower substrate concentration concentrations is always challenging to measure. The substrate is consumed very fast and prolonged time of reaction does not facilitate (rather complicates the absorbance measurement). The amount of final product is presented in very low concentration (Cornish-Bowden 2014) that can be difficult to detect, especially for chromogenic substrates. One possible solution can be using the fluorescent substrate or/and measure the enzymatic reaction in very short time (Mazura 2006).

CONCLUSION

Molecular biology and biochemistry in principle require manipulation with liquid samples. Any automation can be useful but on the other hand the researcher have to consider limitations that are connected with the chosen solution. Here was briefly described 4 years of experiences with the liquid handling system BioNex Nanodrop II that was used in different experiments to show one of the possible solution for the parallel sample manipulation. The main emphasis was put on the technical implementation of samples pipetting with regard to the possibility of cross-contamination. In four examples were explained testing conditions and obtained results. The cross-contamination was not detected or it was in acceptable range. In summary, the liquid handling system opens new possibilities of automation and miniaturization of lab-routine as well as advanced procedures.

ACKNOWLEDGEMENTS

This research has been financially supported by the Czech Science Foundation under project GA15-19266S and the Ministry of Education, Youth and Sports of the Czech Republic under project CEITEC 2020 (LQ1601).

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FLUORESCENCE IMAGING FOR EVALUATION OF WATER AVAILABILITY TO PLANTS

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Abstract: The aim of this study is to investigate the applicability of fluorescence imaging for dynamic aspects of soil-water availability to plants. Rhodamine B solution was used as a fluorophore and its transport through the plants was monitored. Mainly the fluorophore optimal concentration and image acquisition parameters were investigated.

Key Words: fluorophores, plants, imaging

INTRODUCTION

The proper circumstances are necessary for plant growth, which enhances the slopes stability by mechanical support of plant roots, and improve the aboveground biomass by the presence of water in soil. Therefore, it is essential to identify the appropriate materials improving the water-holding capacity of soil (Yang et al. 2014).

Water shortage is commonly related to increased level of reactive oxygen species, which affects nearly all plant functions. High temperatures and irradiation and low water accessibility during the growing season result in stress perception (Farzi et al. 2017).

Silica-based granules as well as hydrogels are two types of the most commonly used water-retention additives. Hydrogels are conventionally polyacrylamide gels in a crystalline form, which are capable of absorbing water up to several hundred-times of their own weight. SiO_2-based additives, on the other hand, are usually enriched with carbon components or cellulose and therefore they are able to adhere to soil particles. This effect leads to increase of the surface area followed by adsorption of water or other nutrients (Farrell et al. 2013).

In this study, for the first time, the applicability of real-time monitoring of solution transport by fluorescence *in vivo* imaging system was investigated. The aim of this study was to determine if the fluorescence imaging technique may serve for characterization of water-retention additives and for observation of the water availability.

MATERIAL AND METHODS

Preparation of experimental plant model

Sunflower (*Helianthus annuus*) used as an experimental plant was cultivated by procedure as described in previous work (Vaneckova et al. 2016). In first experiments, leaves of 2 weeks old plant were cut, washed with water and immersed in 2 ml tube containing water solution of rhodamine B.

Similarly, whole plants (with roots) were first washed and then placed in the 5 ml tube containing the fluorophore. All the plant models were fixed in the tube with parafilm.

Fluorescence imaging

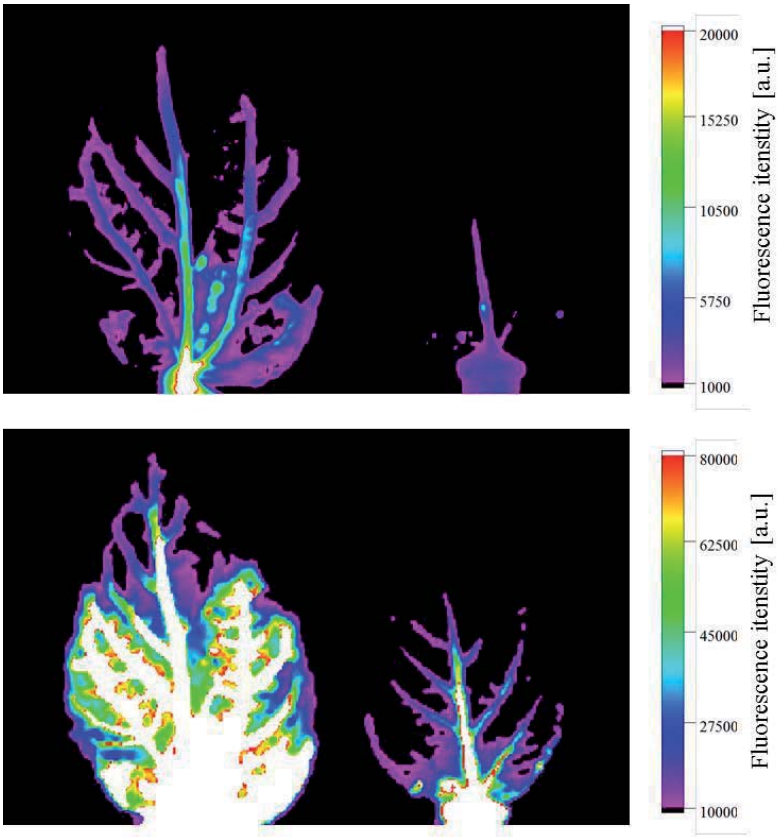
The fluorescent images were captured by *In Vivo* Xtreme Imaging System (Bruker, MA, USA). Rhodamine B in plants was examined at excitation wavelength λ_{ex} 550 nm and emission wavelength λ_{em} 600 nm. The parameters of imaging were set as follows: f-stop 1.1, field of view 19 × 19 cm; exposure time and binning were manually optimized to maximize detection efficiency. Fluorescence intensity in the regions of interest was quantified by Bruker Molecular Imaging Software.

RESULTS AND DISCUSSION

Imaging parameters optimization

As a first step, imaging parameters were optimized. Under the exposure time 2 s and binning 1×1 (Figure 1, top), we were able to visualize rhodamine B in concentration of 1×10^{-7} M (Figure 1, Top left), whereas transport of rhodamine in concentration of 1×10^{-7} M (Figure 1, Top right) cannot be clearly seen in the plant. However, increased exposure time and binning enabled to expand the detection capability. As shown in Figure 1 Bottom, concentration as low as 1×10^{-8} M can be observed under acquisition parameters: exposure time 10 s and binning 4 × 4.

Figure 1 Fluorescence images of sunflower leaves immersed in rhodamine B solution (1×10^{-7} M – left, 1×10^{-8} M – right), excitation wavelength 550 nm, emission wavelength 600 nm. Top – exposition time 2 seconds, binning 1×1, Bottom – exposition time 10 seconds, binning 4×4



Time dependence

Based on the results obtained using the leaves, the whole-plant experiments were carried out. As shown in Figure 2, the sunflower plant was immersed in the rhodamine B solution and images were taken at specific time-intervals (0, 4, and 8 hours). In comparison with control experiment (immersion in pure water), significant increase in fluorescence intensity was observed in dependence on time of treatment. The proposed method is enabling not only to monitor the extremely low amounts of fluid being transported into the plant, but also real-time visualization of the distribution within the plant (Figure 3). Moreover, it is possible to quantify the fluorescent signal in the particular point of the plant and therefore to determine the time required to deliver the solution to the specific plant part (Figure 4).

Figure 2 Photograph of sunflower plant immersed in rhodamine solution

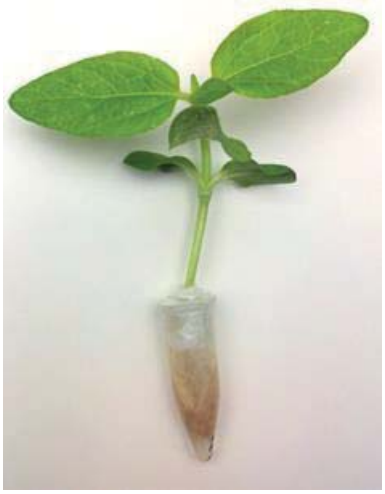


Figure 3 Whole plant imaging in 4-hour time intervals. Top row – rhodamine 1×10^{-7}M (in water), bottom row – control (water).

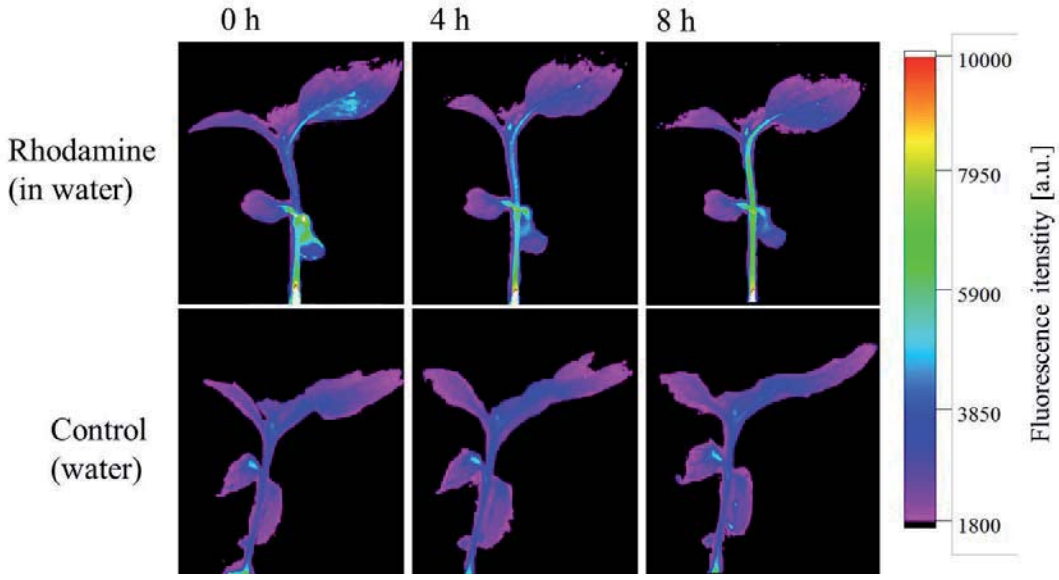
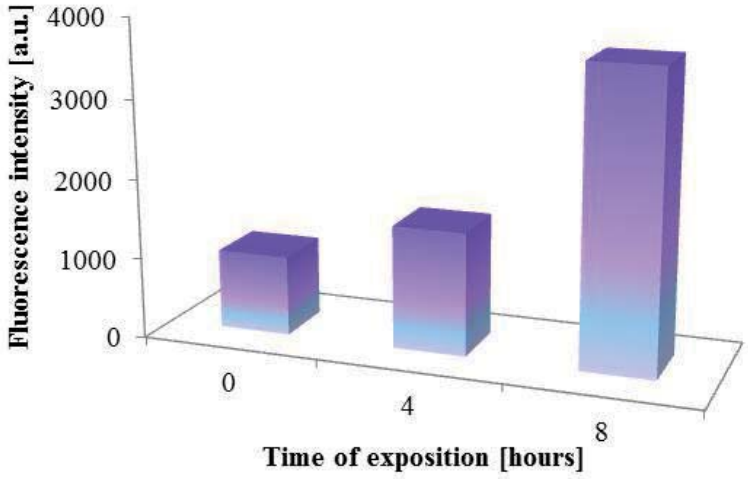


Figure 4 Quantification of fluorescence in the sunflower stem (mean intensity, control signal subtracted)



Even though, the presence of the rhodamine B might potentially influence the uptake of the fluid from the water-retention compound due to the toxicity to the plant cells, the sensitivity of the fluorescence imaging enables the use of extremely low concentrations (submicromalar). As shown elsewhere (Tan et al. 2014), the toxic effect due to the reactive oxygen species was observed in thousand-time higher concentrations (submilimolar). Therefore, it is believed that the influence of root cell damage on the fluid transport is eliminated.

CONCLUSION

Based on the results, the fluorescence imaging is demonstrated to be capable of monitoring and evaluate the ability of the water-retention additive to deliver water to the plant. Using the submicromolar concentrations of fluorophores (e.g., rhodamine B), it is possible to quantify the effectivity of water-supply systems in long-term real-time experiments.

In future, the water-retention additives will be soaked by the solution of rhodamine B and their ability of water supplementation will be investigated.

ACKNOWLEDGEMENTS

This research was carried out under the project CEITEC 2020 (LQ1601) with financial support from the Ministry of Education, Youth and Sports of the Czech Republic under the National Sustainability Programme II and by the Internal Grant Agency of Mendel University in Brno IGA TP 1/2017. Tereza Vaneckova is a Brno Ph.D. Talent Scholarship holder – funded by the Brno City Municipality.

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CHARACTERIZATION OF UPCONVERSION NANOPARTICLES BY FLUORESCENCE SPECTROMETRY AND CAPILLARY ELECTROPHORESIS

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Abstract: Upconversion nanoparticles (UCNPs) are a novel class of luminescent tags for applications in life and material sciences. Unlike traditional fluorophores, UCNPs exhibit emission of shorter wavelength under near-infrared excitation (typically 980 nm). In this work, we have examined these unique photophysical properties by fluorescence spectrometry and capillary electrophoresis. UCNPs co-doped with Yb(III) and Er(III) were characterized using laboratory-made fluorescence spectrometer. We have exploited and evaluated two excitation sources and the dependence of the fluorescence of UCNPs on the relative excitation power. Moreover, capillary electrophoresis with laser-induced fluorescence (CE-LIF) detection was for the first time used for characterization of the nanoparticles. It was proved that CE-LIF is a valuable method to be used for investigation of upconversion luminiscence and monitoring of the interactions of UCNPs with other molecules of interest.

Key Words: upconversion nanoparticles, UCNP, fluorescence spectrometry, capillary electrophoresis

INTRODUCTION

Lanthanide-doped photon upconversion nanoparticles (UCNPs) have been in the focus of research interest due to their unique photophysical properties. The anti-Stokes shifted luminescence is a result of a sequential photon absorption (Nadort et al. 2016, Zhu et al. 2017). Most upconversion materials rely on a crystalline host such as metal fluoride (most commonly $NaYF_4$, CaF_2), oxide (Y_2O_3), or phosphate (YPO_4), and are co-doped with a single Ln^{3+} ion or a combination of Ln^{3+} such as Er^{3+}, Yb^{3+}, Tm^{3+} Ho^{3+}, and Gd^{3+} (Gai et al. 2014).

Advantageous features of UCNPs are basically no background due to anti-Stokes shifted emission (Zhu et al. 2017), low toxicity (Zhou et al. 2015), no photobleaching or photobrightening (Zheng et al. 2015, Zhou et al. 2015), applicability for long-term imaging (Wu et al. 2015), and particularly well suitability for deep tissue imaging (Wu et al. 2015, Xu et al. 2013, Yang 2014, Zhou et al. 2012). Therefore, UCNPs can in many ways overcome limitations of traditional fluorescent reporters, such as organic dyes or semiconductor nanocrystals (quantum dots).

Due to their superior properties, a broad field of applications of UCNPs can be found. Recent progress enabled an increasing number of (bio)analytical (Hlavacek et al. 2016, Hlavacek et al. 2017, Chatterjee et al. 2010), diagnostic (Yang 2014), and sensing applications (Hao et al. 2013, Shi et al. 2015), as well as photovoltaic (Ramasamy et al. 2014) and security applications (Meruga et al. 2014).

Despite above mentioned advantages, there is no commercially available system with ability to measure upconversion properties, which limits further expansion of the applications of UCNPs. To measure fluorescence emission properties, one needs to utilize custom-made spectrometers. This analytical tool, however, needs to be standardized. As mentioned in literature, different applications have specific requirements regarding laser power density (Kaiser et al. 2017).

In addition to the fluorescent spectrometry and gel electrophoresis (Hlavacek et al. 2014, Sedlmeier et al. 2016), capillary electrophoresis is a useful tool to characterize nanoparticles. As reviewed in work of (Stanisavljevic et al. 2014), CE is an indispensable method for examination of bioconjugation to targeting ligands (e.g., folic acid, RGD peptide) or monitoring of their interactions with other molecules of interest.

In this study, we have characterized carboxyl-silica-coated UCNPs by laboratory-made spectrometer. We have examined two excitation sources and the dependence of the fluorescence of the UCNPs on the excitation power. In addition, CE was involved to characterize the proposed nanoparticles. To the best of our knowledge, this is the first time that CE was used for characterization of UCNPs.

MATERIAL AND METHODS

Synthesis of the upconversion nanoparticles

UCNPs were synthesized by high-temperature coprecipitation method according to a protocol described elsewhere (Hlavacek et al. 2014, Wang et al. 2010). Subsequently, carboxyl-silica-coated UCNPs (COOH-UCNPs) were prepared by a reverse microemulsion method: UCNPs (418 mg) were diluted in cyclohexane to a final volume of 36.8 ml. This dispersion was mixed with 4600 mg of Igepal CO-520 and 251 µl of tetraethyl orthosilicate (TEOS) and stirred intensively for 10 min. A mixture of 279 µl 25% (w/v) of aqueous ammonium hydroxide and 279 µl of water was added to form a microemulsion that was slowly stirred overnight. Then, 126 µl of TEOS were added and the microemulsion was again stirred for 240 min. After adding 251 µl of 25% (w/v) sodium carboxyethylsilanetriol in water, the microemulsion was first sonicated for 15 min and then stirred for 60 min. The COOH-UCNPs were extracted with 5000 µl of dimethylformamide and washed four times with 20 ml of acetone, and five times with 6000 µl of water.

Laboratory-made spectrometer

A laboratory-made detection device for optical characterization of the nanoparticles was built using modular spectrometer components purchased by Ocean Optics (Dunedin, FL, USA) and external light sources, laser diode (980 nm, 300 mW) or LED diode (980 nm, 3 mW), obtained from Roithner Lasertechnik (Vienna, Austria). Spectra of UCNPs under excitation with different relative power of the diode were acquired (scans to average 2, integration time 1000 ms) with use of OCEANview 1.6.3 software and postprocessed in MATLAB.

Capillary electrophoresis with IR light source

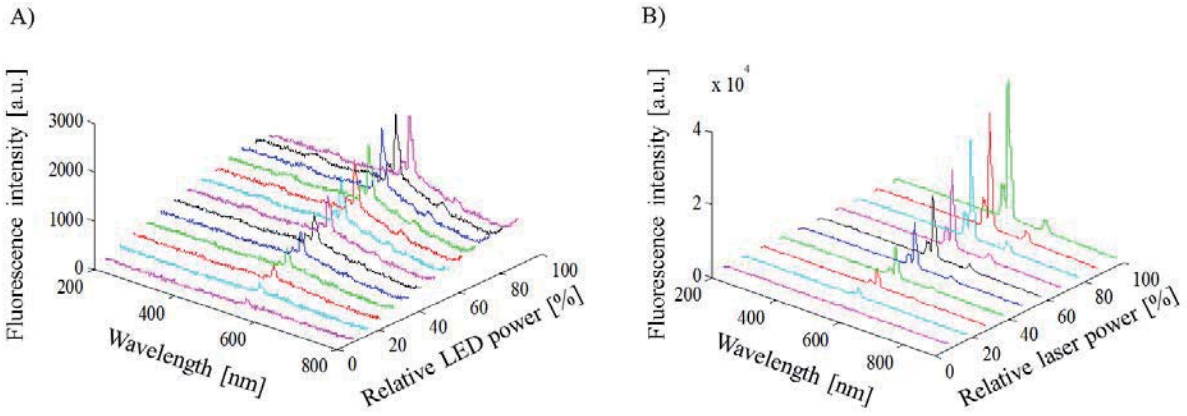
Capillary electrophoresis Beckman Coulter PACE/MDQ was coupled with the external 980 nm laser diode connected via optical fibre. The separation was performed in uncoated fused silica capillary with internal dimeter of 75 µm, total length of 64 cm and effective length of 54 cm. 20 mM borate buffer (pH 9) was used as a separation electrolyte and separation voltage of 25 kV was applied.

RESULTS AND DISCUSSION

Comparison of excitation sources

UCNPs spectra were measured using LED light source set to 100–700 mA (Figure 1A). Laser diode was set to 10 values of power from 200 mA (1.75 V) to 670 mA (3 V) (Figure 1B). As expected, increasing excitation power leads to higher fluorescence yields. Based on the results obtained, the laser diode was used in subsequent CE experiments taking into account the extremely low sample volume injected into CE (nl).

Figure 1 Emission spectra of UCNPs – comparison of the excitation sources and their settings. A) LED diode, B) laser diode

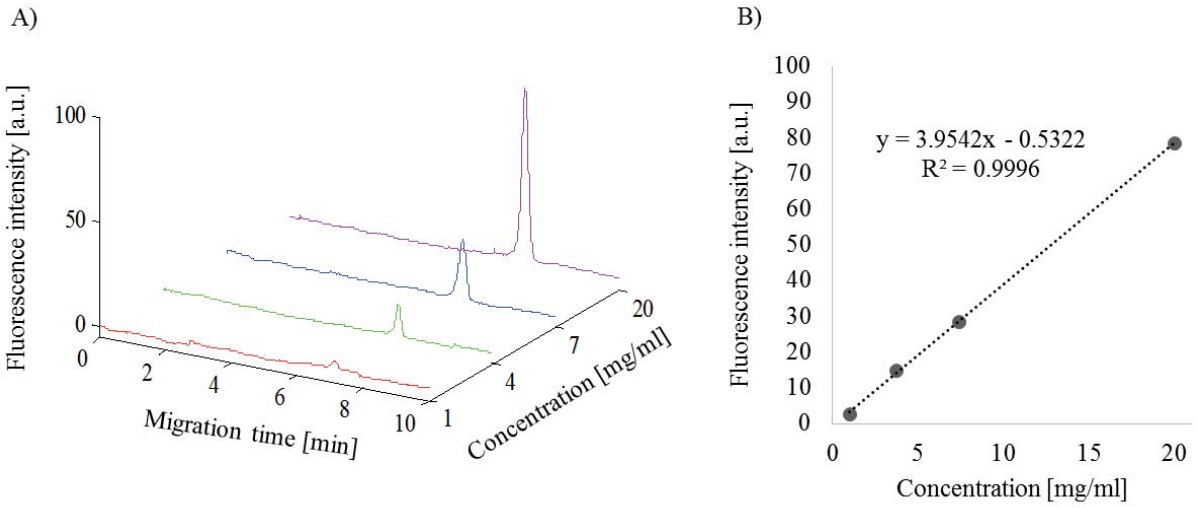


CE-LIF characterization

According to the literature, CE-LIF is an excellent tool for characterization of nanoparticles as well as for monitoring of their interaction with other biomolecules (e.g. antibodies).

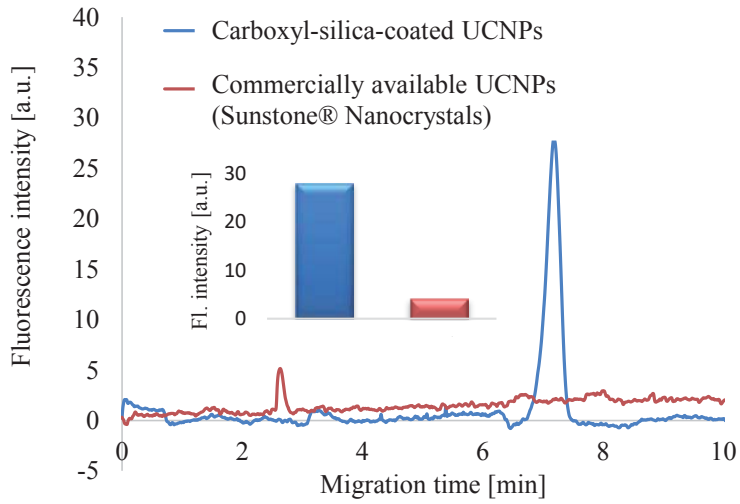
As shown in Figure 2A, UCNPs exhibited under given CE conditions a single peak with migration time of 6.7 minutes. The peak height was linearly dependent on nanoparticle concentration. The coefficient of determination $R^2 = 0.9996$ (Figure 2B) was reached and obtained limit of detection (LOD = 3 × SD/slope) was 0.26 mg/ml. The absolute amount of the injected nanoparticles was calculated to be 9.1 ng (injected volume was 35 nl).

Figure 2 CE characterization of UCNPs@Yb,Er. A) electropherograms of different concentrations of UCNPs, B) calibration curve obtained from the peak heights



The potential improvement of LOD would involve increasing the excitation power used, however the main limitations are in the imperfect coupling of the laser diode light into the optical fibre used in the CE instrument. Despite these limitations, it was possible to compare the behaviour of the prepared UCNPs and commercially obtained nanoparticles. As shown in Figure 3, the fluorescence intensity of the prepared nanoparticles was significantly higher (5.6×). Both particle types were carboxylated and therefore negatively charged in pH of the separation electrolyte (pH 9). The fact that migration time of the commercial nanoparticles was shorter (2.4 minutes) corresponds to the smaller size declared by the supplier (60 nm) in comparison to the size of prepared nanoparticles (105 nm).

Figure 3 CE characterization. Carboxyl-silica-coated UCNPS@Yb,Er (blue) and commercially available UCNPs (red), both in concentration 7 mg/ml (diluted by water)



CONCLUSION

Upconversion nanoparticles belong to a group of novel nanomaterials exhibiting fluorescence with large anti-Stokes shift. In this work, laboratory-made spectrometer was built. Different excitation sources and power were used for spectra acquisition. Additionally, UCNPs were characterized by capillary electrophoresis. In comparison to commercially available UCNPs, prepared nanoparticles exhibited 5.6-times higher fluorescence intensity.

ACKNOWLEDGEMENTS

This research was carried out under the project CEITEC 2020 (LQ1601) with financial support from the Ministry of Education, Youth and Sports of the Czech Republic under the National Sustainability Programme II, Grant Agency of the Czech Republic (P20612G014 GACR) and by the Internal Grant Agency of Mendel University in Brno, IGA no. IP 6/2017. Tereza Vaneckova is a Brno Ph.D. Talent Scholarship holder – funded by the Brno City Municipality.

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COMPARISON OF INTERACTION OF TWO ISOFORMS OF METALLOTHIONEIN (POTENTIAL SOURCE OF THE ANTITUMOR DRUG RESISTANCE) WITH PLATINUM-BASED CYTOSTATICS AND PLATINUM NANOPARTICLES

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Abstract: Platinum-based cytostatics are the metal-containing anticancer cytostatic drugs that have found application in clinical practice. Antitumor activity of platinum-based drugs is caused by the crosslinking of DNA and formation of DNA adducts with subsequent triggering the apoptosis leading to cell death. Disadvantage of this type of cytostatics is that some kind of cancer is resistant against them. This resistance can be potentially caused by metalloproteins such as metallothioneins (MTs) that bind platinum to their structure and make the interaction with DNA of cell impossible. MTs are low molecular mass, intracellular cysteine-rich, metal-binding proteins and ensure a number of functions in body for example detoxification of heavy metals or maintenance cellular zinc homeostasis. In this work, the interaction between two isoforms of MTs (MT3 and MT2) and several types of platinum cytostatics (oxaliplatin, carboplatin and cisplatin) as well as platinum nanoparticles (size of 10 and 40 nm) were examined by fluorimetric analysis using a fluorescence zinc indicator (Fluozin-3). Fluorescence spectrometry with laser-induced fluorescence detection (ex–488 nm, em–520 nm) was used in the study.

Key Words: platinum, cytostatics, nanoparticles, metallothionein, fluorescence

INTRODUCTION

Platinum cytostatics have been commonly used for treatment of cancer for a long time but mechanism of their action is still not completely clear. It is assumed that platinum-based cytostatics destroy cancer cells by binding to DNA and interfering with the cell's repair mechanisms, which eventually leads to cell death (Knipp 2009). The cytotoxic effects of the platinum drugs are directly related to the quantity of drug that enters the cell. However, large number of platinum cytostatics may be inactivated by creation of stable Pt-thiol adducts with metallothionein in cytosol and only 1% or less of the intravenously administered platinum binds to DNA (Nejdl et al. 2015a).

Metallothioneins (MTs) are a family of small cysteine-rich metal-binding proteins, which consist from two domains (α, β). These proteins are present in almost all forms of life. Mammals express at least four isoforms of MT (MT1–MT4) and 13 MT-like human proteins were identified (Palumaa et al. 2005). Mammalian MTs contain 61–68 amino acids, and among them, 20 are cysteines. The differences between the isoforms come mainly from post-translational modifications, small changes in primary structure, or speed of degradation. Differences are also in their presence in individual organs. MT1 and MT2 are present almost in all types of soft tissues, MT3 is expressed mostly in brain tissue, but also in heart, kidneys and reproductive organs

and the MT4 isoform was detected in some epithelial cells. MTs participate in the regulation of cellular metabolism of zinc and copper, and in protection of cells against reactive oxygen species. But their most studied function is detoxification of heavy metals and maintaining of essential metal ion homeostasis given by their high affinity to these metals (Ruttkay-Nedecky et al. 2013).

One of the possible solutions currently being investigated is to prevent the interaction between MTs and platinum cytostatics by use of platinum nanoparticles (PtNPs) for treatment of cancer. There is a hypothesis that PtNPs are not interacting directly with the DNA in their particulate form, but instead release soluble Pt species (Pt^{2+} ions) that form complexes with DNA (Gehrke et al. 2011). Use of PtNPs seems to be an appropriate solution for the problem of resistance of some cells against platinum-based cytostatics.

MATERIAL AND METHODS

Materials

$PtCl_4$, polyvinylpyrrolidone (PVP, 40 k), HCl (37%, *w*/*w*), $NaBH_4$ were purchased from Sigma-Aldrich (USA) in ACS purity. All chemicals used for electrochemical detection were purchased also from Sigma-Aldrich (USA) in ACS purity. N-[2-(2-{2-[bis(carboxymethyl)amino]-5-methoxy phenoxy}ethoxy)-4-(2,7-difluoro-6-hydroxy-3-oxo-3H xanthen-9-yl)phenyl]glycine (FluoZin-3) was purchased from Labeling and detection (USA). HEPES was obtained from Merck KGaA (Germany). MT2 and MT3 were supplied from Karolinska Institute in Stockholm (Sweden). Zinc, platinum and lead was used in AAS standard from Sigma-Aldrich (St. Louis, MO, USA). Carboplatin and Oxalplatin were purchased from Teva (CZ). Cisplatin was purchased from Sigma-Aldrich (USA). Chelex 100 Resin was from BIO-RAD (USA).

Preparation of platinum nanoparticles

Platinum nanoparticles were prepared by dissolving $PtCl_4$ (0.034 g) in acidic water (5 mL) with 16 µL of 37% HCl. The solution of $PtCl_4$ (5 mL) was added, with stirring, to another solution of 0.135 g PVP in water (45 mL). The mixture was stirred for 1 hour at temperature 25 °C. After the addition of $NaBH_4$ (50 mg) the final colour of the solution became black. The mixture was stirred overnight (Buchtelova et al. 2017).

Electrochemical detection

MT was determined by differential pulse voltammetry (DPV). The measurement was performed with 663 VA Computrace instrument (Metrohm, Switzerland), using a standard cell with three electrodes (working electrode – hanging mercury drop electrode with a drop area of 0.4 mm^2, the reference electrode – Ag/AgCl/3M KCl electrode and auxiliary electrode – glassy carbon electrode). For data processing 663 VA Computrace software from Metrohm CH was employed. For de-oxygenation of analysed samples was used purging with argon (99.999%). The Brdicka supporting electrolyte containing 1 mM $[Co(NH_3)_6]Cl_3$ and 1 M ammonia buffer (NH_3(aq) + NH_4Cl, pH = 9.6) was used. The supporting electrolyte was exchanged after each analysis. The parameters of the measurement by differential pulse voltammetry were as follows: initial potential of -0.7 V, end potential -1.8 V, deoxygenating with argon 90 s, deposition 120 s, time interval 0.2 s, step potential 1.95 mV, modulation amplitude 25 mV, and modulation time 0.57 s. For electrochemical measurement, the volume of injected sample was 10 µL and volume of measurement cell was 2 mL (10 µL of sample + 1990 µL ammonium buffer). All measurements were carried out at temperature 6 ± 1 °C (Nejdl et al. 2015b).

Samples preparation

For comparison of interaction between both MT isoforms 5 µl 60 nM protein in MiliQ water was used. 3 µM Fluozin-3 (Fluozin-3 was prepared in 100 mM HEPES, pH 7.4) was added followed by 5 µl of 800 nM metal ion in MiliQ water. Reaction of MT with metal (e.g. Pt^{2+}, Pb^{2+} and other) led to release of zinc. Released zinc was detected by Fluozin-3 (Zn^{2+} selective fluorescent indicator). Fluorescence of FluoZin-3 and Zn^{2+} was measured at 25 °C by an Plate reader Infinite® 200

PRO-Tecan (Switzerland) using a NanoQuantPlate (Tecan). All measurements were repeated three times. The evaluated data are the average of three repeated measurements.

RESULTS AND DISCUSSION

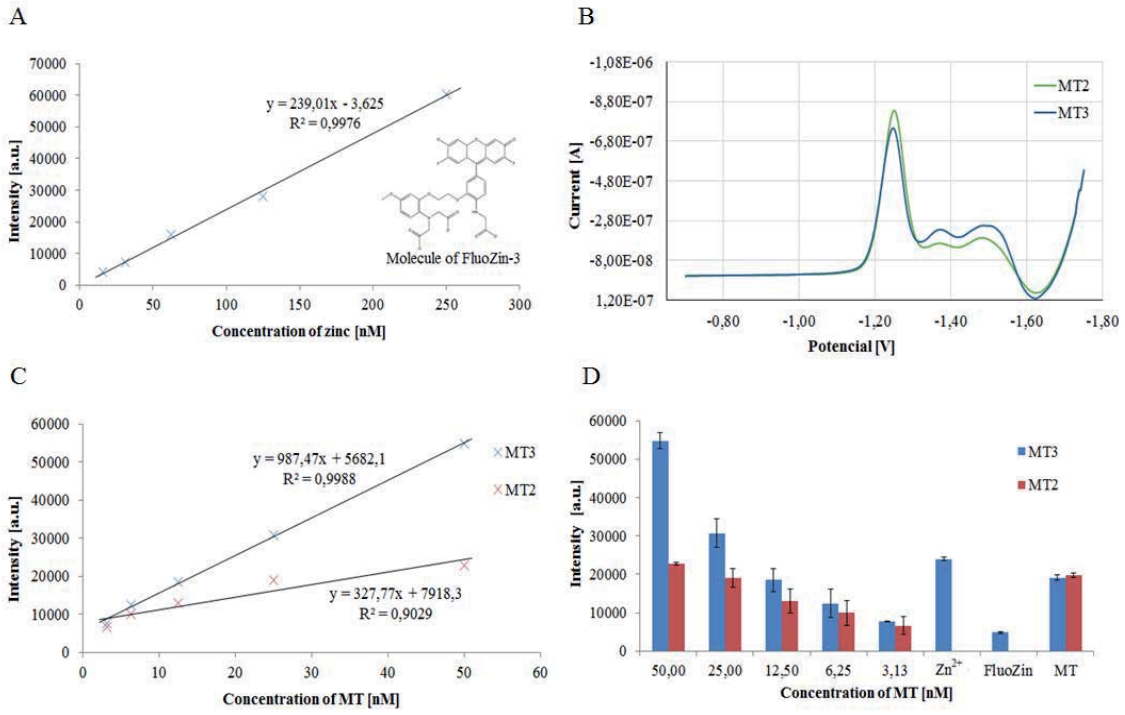
Verification of actual protein concentrations

This work aims at finding differences in binding affinity of two isoforms of MT to various platinum species (ions, Pt-based cytostatics, as well as Pt nanoparticles). The experiment is based on interaction of FluoZin-3 (selective fluorescent Zn^{2+} indicator) and zinc released from the protein. By this method is possible to prepare calibration curve of zinc (Figure 1A). Linear dependence in Zn concentration on fluorescent signal of FluoZin-3 was observed exhibiting the coefficient of determination $R^2 = 0.9976$.

Subsequently, this method was applied to detection of zinc released from MT. Comparison of concentrations of both isoforms of MT was carried out by DPV. As can be seen in the Figure 1B, the concentrations of both isoforms detected in the stock solutions were equal (6.5 µM). Each MT contains in his structure seven atoms of zinc that can be released by interaction with another metal ion with higher affinity (e.g. Pt^{2+}, Pb^{2+} etc.). In this experiment, Pb^{2+} was used for release of zinc from MT. Released zinc was detected by interaction with Fluozin-3. By this interaction is possible to define calibration curves of both MT isoforms.

From calibration curves (Figure 1C) is noticeable that MT3 is providing higher zinc signal compared to MT2. MT3 has distinct primary structure and also is produced in other part of body compared to MT2. As already mentioned above, MT2 is present almost in all types of soft tissues. However MT3 is expressed mostly in brain tissue. MT3 plays a special role in brain zinc metabolism and therefore it is expected that it has to be able to release zinc flexibly upon certain stimulus. There is the theory that MT3 has lower affinity for zinc than MT2 because his role is balancing of concentration of zinc in brain. These results are in agreement with previous study of Sweden scientists (Palumaa et al. 2005). Figure 1D also confirms that concentrations of both isoforms of MT are same.

Figure 1 In the picture A there is calibration curve of zinc. Verification of protein concentration was performed by electrochemistry (picture B). In the picture C there are calibration curves of MT2A and MT3. Picture D shows the same data like picture C plus conformation that concentrations of both proteins are same.

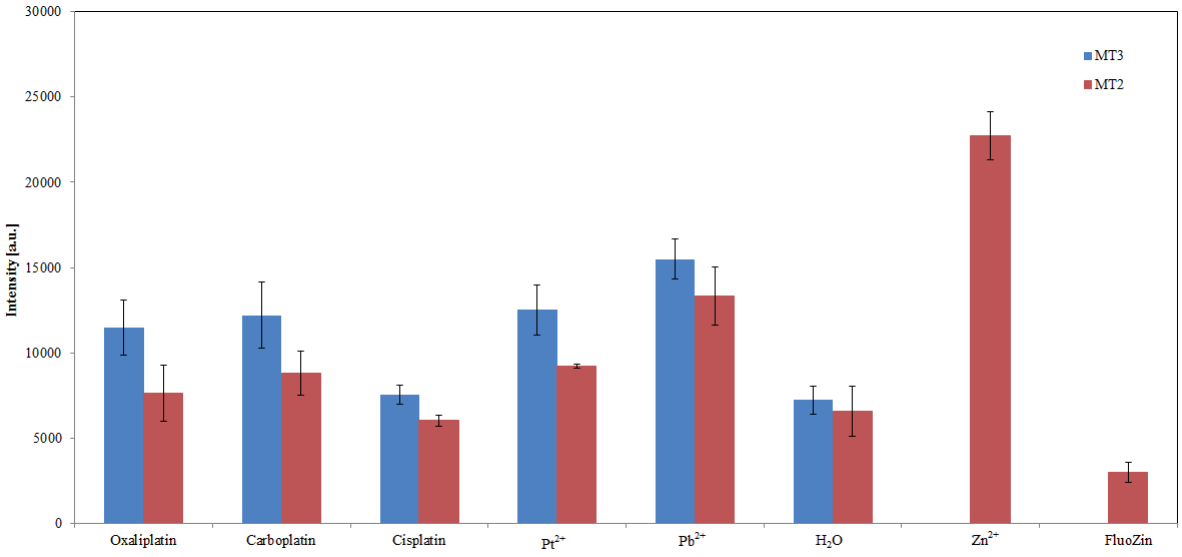


Study of reaction between MTs and platinum-based cytostatics

In second part of this study, the reaction between both isoforms of MTs and different types of platinum-based cytostatics (cisplatin, carboplatin, oxaliplatin) was examined. The reaction of platinum-based cytostatics with both isoforms led to release of zinc that was detected by FluoZin-3.

As can be observed from Figure 2, MT3 again showed lower affinity to zinc (higher ability to release zinc) than MT2. Also was established that difference platinum-based cytostatics show different reaction with MTs. Lead exhibited higher affinity to MTs compared to platinum ions so it was used such as control in this case. From acquired data can be seen that carboplatin had the highest intensity of interaction from studied platinum-based cytostatics with MT reports. On the other hand, the lowest intensity of interaction had cisplatin.

Figure 2 Interaction between platinum-based cytostatic and lead with both isoforms of metallo-thionein (MT2, MT3)



Study of reaction between MTs and PtNPs

This experiment is focused on investigation of use of platinum nanoparticles as an alternative to antitumor drugs. Two types of platinum nanoparticles of different size (40 and 10 nm) were studied and compared. As shown in Figure 3, the nanoparticles with smaller size interact (10 nm) with both isoforms of MTs with higher intensity than the bigger ones (40 nm). Higher reactivity of smaller nanoparticles may be connected with their higher ratio of surface to volume.

There is also a hypothesis that PtNPs are not interacting directly with the DNA in their particulate form, but instead they release soluble Pt^{2+} ions that forms DNA complexes (Asharani et al. 2010). This hypothesis was verified also for the interactions with MT. Particles of size of 10 nm were divided to two aliquots. Chelex resin particles, which are able to bind Pt^{2+} ions released from PtNPs, were added to one of the aliquots for 48 hours. It was observed that PtNPs kept in presence of chelex resin interacted with metallothionein with lower ability to replace zinc ions in comparison to PtNPs kept in absence of chelex resin. So, this means that ions released from PtNPs were removed from solution by the resin and therefore they were not able to interact with MT replacing the zinc ions. The results are shown in Figure 4.

There is a possibility that smaller PtNPs have higher tendency to decay and release bigger amount of platinum ions than the bigger ones because they have higher ratio of surface area to volume. Therefore, it is probable that PtNPs would create in the cell a source of ions which will be constantly attacking DNA of cells and cause of apoptosis in a long time. Maybe it is the advantages in compare with conventional platinum-based cytostatics. Confirmation of this theory would require further research.

Figure 3 Comparison of interaction between MT isoforms with PtNPs of different size (10 and 40 nm)

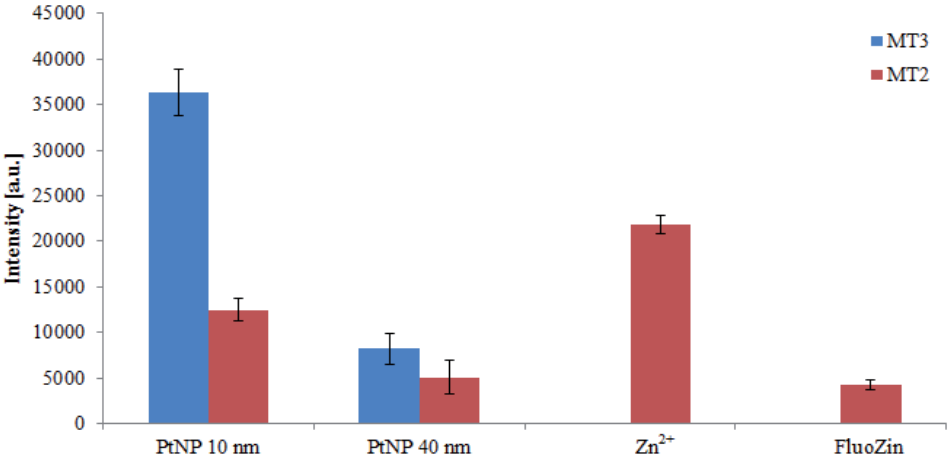
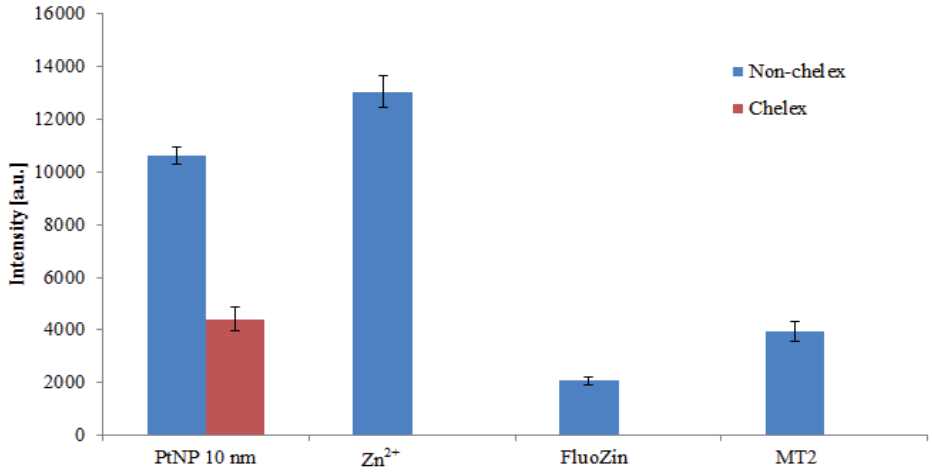


Figure 4 Differences between interactions of MT2A with PtNPs (10 nm) in presence of chelex resin and in absence of chelex resin



CONCLUSION

It was demonstrated that the interaction of MT3 isoform with either platinum-based cytostatics or PtNPs caused the release of higher amount of zinc ions in comparison to isoform MT2. The difference of affinity is probably due to the different role of both isoforms of MTs in organisms.

Also differences between the particular cytostatics (cisplatin, carboplatin, and oxaliplatin) in the ability of zinc release were observed. From acquired data is seen that the highest ability to release zinc ions was observed in case of carboplatin. On the contrary, cisplatin exhibited the lowest replacing ability.

Next, the interactions of metallothionein with two nanoparticles with different size (10 and 40 nm) were investigated. It was found that smaller PtNPs react with both isoforms of MT with higher intensity than the bigger ones. This phenomenon is probably caused by high ratio of surface area to volume of smaller particles.

ACKNOWLEDGEMENTS

Research described in this paper was financed by Czech Ministry of Education, Youth and Sports of the Czech Republic in frame of National Sustainability Program under grant LO1401. For research, infrastructure of the SIX Center was used.

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Authors Index

Name of publication:	MendelNet 2017 *Proceedings of 24th International PhD Students Conference*
Editors:	Assoc. Prof. Ing. Radim Cerkal, Ph.D. Ing. Natálie Březinová Belcredi, Ph.D. Ing. Lenka Prokešová Mgr. Patrik Vacek
Publisher:	Mendel University in Brno Zemědělská 1665/1 613 00 Brno Czech Republic
Year of publication:	2017
Number of pages:	976
ISBN:	978-80-7509-529-9