Botanical pesticides and their human health safety on the example of *Citrus sinensis* essential oil and *Oulema melanopus* under laboratory conditions

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Abstract: Bio-pesticides derived from plants have recently received increasing interest as an alternative to conventional chemicals suitable for integrated pest management and organic farming. In this study, *Citrus sinensis* (Osbeck) essential oil was tested for its potential for wheat protection against *Oulema melanopus* (L.). The chemical composition of the essential oil was analysed by gas chromatography-mass spectrometry (GC-MS). Twenty-five compounds were identified in the oil among which d-limonene was predominant component (89.49%), followed by myrcene, α-pinene, linalool, sabinene, decanal. and other minor components. Direct contact toxicity assay showed the effectiveness of the essential oil against *O. melanopus* larvae causing the mortality of 85% during 48 hours. The persistence study revealed high dissipation rate of the oil from treated plants whereas concentrations lower than 0.01 g/kg were detected by GC-MS 5 min after the application of the oil, suggesting its environmental and food safety. These results, together with previous literature reports reviewed in this study, indicate the great potential of the *C. sinensis* essential oil for future use in crop protection against insect pests.

Keywords: crop protection; bio-pesticides; d–limonene; larvicidal; organic agriculture; *Triticum aestivum*

Introduction

Synthetic pesticides are widely used for decades in agriculture for plant pest and disease control and their overuse has caused serious health and environmental hazards in the past. Although the new EU and USA legislation led to a dramatic decrease in approved products and to significant safety improvement the application of synthetic pesticides is still considered as a potential threat to human health, food safety and the environment [6]. In this respect botanicals pose little risk. Due to their natural origin they are an ideal eco-friendly alternative to chemical control [32] suitable for implementation into the integrated crop protection systems [30], particularly in organic farming where the use of synthetic chemicals is banned [15].

Considerably large group of potential biopesticides is represented by essential oils known to exert a wide spectrum of biological effects including various pests controlling and repellent properties [15]. Some of the oils exhibit certain degree of phytotoxicity, usually inhibiting seed germination, root and seedling growth, which indicates their possible use for weed management. On the other hand many other oils with none or very low phytotoxicity

[32] suggesting their potential for direct application against pests and diseases.

Probably the most conspicuous pests in green grain crops are small cereal leaf beetles (CLB) *Oulema melanopus* L. and *O. galleciana* Heyden (Coleoptera: Chrysomelidae). Both are found in many species of cultural and weed grasses whereas *Triticum aestivum* L. is their preferable host plant. They can cause economically important damages especially on wheat and barley (*Hordeum vulgare* L.). Beetles arrive in late April and early May. After feeding on grasses they are moving in the fields of grain, where the beetles damaged the leaves. After mating, the female deposits from mid-May its bright yellow oval eggs individually or in short rows on top of the highest ranking leaves. Extraordinarily warm and dry spring and summer weather encourages reproduction of the pest whereas each larva destroys 2.5 to 3.5 cm^2 leaf area, which in wheat accounts for about 10 % of the flag leaf. The damages caused by CLB adults are not economically important but pesticides are commonly applied against CLB larvae [4]. Conventional pesticides play a very important role in the plant protection, but their negative impact on the environment and human health leads to a re-evaluation of plant protection systems. One of the reasons is the incidence of resistant pest populations to widely used active substances as in case of the beetle *O. oryzae* Kuwayama, an important pest of rice, that has developed fipronil resistance in Japan [23]. In this regard, it is advisable to look for new procedures that will reduce the emergence of this phenomenon. Extending the range of applicable plant protection methods is also an essential prerequisite for the application of integrated pest management principles. Several alternatives for *O. melanopus* control have been described previously such as the use of enthomopathogenic nematodes [19], selection of appropriate varieties according to natural resistance parameters [26] or the application of resistance inducers [7].

So far there are just few essential oils allowed for plant protection in the EU: citronella, clove, spear mint, tea tree [9] and most recently orange oil derived from *Citrus sinensis* (L.) Osbeck, the first insecticidal essential oil approved [10] authorized in France for the control of whiteflies on pumpkins and tomatoes [26].

Essential oil from *C. sinensis* was found as an effective pesticide on different plant and storage products pests. Its effect has previously been described against *Sternechus subsignatus* Fabricius and *Rhyssomatus subtilis* Fiedler [32], *Tribolium castaneum* Herbst, *Sitophilus granarius* L. and *Callosobruchus maculatus* Fabricius [21] *Bemisia tabaci* Gennadius [30] and *Tetranychus urticae* Koch [4] Moreover, *C. sinensis* has been reported to exert activity against common house fly [18, 25] and several mosquito species [38, 27, 3, 12, 22].

The main components of *C. sinensis* essential oil reported in literature are d-limonene, aplha-pinene, myrcene and linalool [34, 33, 2, 18, 40] d-limonene (Fig. 1), as the most abundant component of the oil, is considered to be responsible for the majority of the anti-insect activity. As well as *C. sinensis* essential oil, d-limonene has also been reported to be effective against various crop pests [5,13,14,16,17,37,40] stored product pests [98,11,20] and some other insects [36,12,1].

Although high concentrations of d-limonene can be phytotoxic to sugar beet, cabbage, carrot and strawberry seedlings [13] however, it should be otherwise relatively harmless to the environment because it is biodegradable and highly volatile and thus probably low-persistent compound. d-limonene is relatively safe also for humans since it is used as food additive whereas its ADI was established up to 1.5 mg/kg bw [39].

The aim of this work was to monitor the effect of essential oil from *C. sinensis* on *O. melanopus* in correlation with its evaporation from leaves of wheat (var. Bohemia) which enable us to obtain the information about the food and feed safety of plants treated with these bio-pesticides and focused on level of safety application. In addition to residuals analysis of *C. sinensis* and its decrease during the time the effect of essential oil on larvae and adults *O. melanopus* was also tested.

Material and Methods

Chemicals

C. sinensis essential oil, Limonene and Tween 20% were purchased from Sigma-Aldrich (Prague, CZ). Hexane (Merck, Prague, CZ) was used as extraction solvent.

Contact toxicity assay

Specimens of *O. melanopus* used for experiments were obtained from field cultures of wheat from the locality Semice (N 50.157709, W 14.871727). The *C. sinensis* essential oil was diluted in water to the concentration of 1% with 0.5% Tween 20 and subsequently used for the experiments. Specimens of *O. melanopus* larvae and adults were placed individually into Petri dishes (90 mm) and 1 µl of diluted plant essence was pipetted topically on insect body. Mortality of larvae and adults was evaluated visually in the time intervals of 1, 24, and 48 hours in four independent experiments whereas 10 replicates were included within the evaluation. Larvae and adults treated with 0.5% solution of Tween 20 were included as a negative control groups.

Persistence study

Plants of wheat (var. Bohemia) were cultivated under controlled conditions till the essential oil application. The mixture of 1% *C. sinensis* essential oil and 0.5% Tween 20% in H_2O was prepared and the leaves of each plant were sprayed until runoff to achieve maximum coverage of the leaves. The leave samples were then taken in the time intervals of 1, 5, 10, 20, 30 and 60 min. 0.5 g of leaves was frozen by liquid nitrogen, powdered and extracted to 0.5 mL of hexane for 5 min shaking. Samples were subsequently centrifuged; the supernatants were transferred to glass vials and stored in a freezer till the chemical analysis.

GC-MS analysis

Samples were analysed by GC/MS using Agilent 7890A GC coupled to Agilent 5975C single-quadrupole mass detector with a HP-5MS column (30 m × 0.25 mm, 0.25 µm film) from Agilent (Santa Clara, CA, USA). The sample volume of 1 µL was injected in splitless mode, the injector temperature was 250 °C and the electron ionization energy set at 70eV. The oven temperature started at 60 °C for 3 min. and was programmed to 250 °C at a rate of 3 °C/min, and then kept constant for 10 min. The flow rate was 1 mL/min. and helium was used as carrier gas. The analysis of the essential oil composition was carried out in full scan mode. In case of residue detection the concentration of limonene, the prevailing constituent of the oil (89.49%), was examined in the selected ion-monitoring mode (m/z 68, 93, 136). The identification of constituents was based on the comparison of their mass spectra and relative retention indices with the National Institute of Standards and Technology Library (NIST, USA).

Results

Essential oil composition

The volatile components of fruit peel essential oil of *C. sinensis* (obtained by cold-press extraction and analyzed by GC-MS) are listed in Table 1. A total of 25 different compounds with 99.19% of total areas were isolated and identified using spectroscopic (mass spectra) criteria. d-limonene was the most prevailing component of the oil (89.49%) followed by myrcene (3.55%), *a*-pinene (1.26%), linalool (1.04%), sabinene (0.62%) and decanal (0.62%).

Table 1 Chemical composition of *Citrus sinensis* essential oil

Peak	Compounds	Area (%)	RT[a] (min)
1	*a*-pinene	1.26	6.713
2	sabinene	0.62	8.079
3	myrcene	3.55	8.738
4	octanal	0.49	9.186
5	*d*-3-carene	0.23	9.461
6	d-limonene	89.49	10.455
7	1-octanol	0.08	12.062
8	linalool	1.04	13.343
9	nonanal	0.09	13.453
10	*cis*-limonene oxide	0.11	14.705
11	*trans*-limonene oxide	0.12	14.908
12	citronellal	0.33	15.631
13	a-terpineol	0.15	17.394
14	decanal	0.62	17.990
15	citronellol	0.07	19.077
16	neral	0.08	19.568
17	carvone	0.31	19.682
18	citral	0.18	20.900
19	perillal	0.06	21.018
20	anethole	0.28	21.593
21	copaene	0.07	25.395
22	dodecanal	0.11	26.841
23	germacrene D	0.09	27.619
24	valencene	0.11	30.249
25	cadinene	0.08	31.471

RT: retention time

Persistence of Citrus sinensis essential oil on wheat leaves

The concentrations of *C. sinensis* essential oil were examined in time intervals (Table 2) after the application of its 1% (v/v) suspension in water whereas d-limonene, as the most abundant compound (89.49%), was used for the residue detection. The GC-MS analysis revealed decreasing concentrations of the oil in time whereas the concentration of 0.01038 μL/g was detected 5 min after application (Fig. 2) which is below the minimum residue limit (0.01 mg/kg) established for substances that are not included in any of the annexes in EU regulations.

Table 2 Decreasing essential oil concentration on wheat leaves in time

Time (min)	**Concentration (μL/g)**
1	0.01803
5	0.01038
10	0.00979
20	0.00474
30	0.00292
60	0.00287

The effect of essential oil on Oulema melanopus

In our toxicity assay, the *C. sinensis* essential oil showed to be highly effective against *O. melanopus* larvae but no insecticidal effect was observed on the adults during 48 hours (Table 3).

Table 3 Mortality of *Oulema melanopus* larvae and adults after apical application of *Citrus sinensis* essential oil

Exposition time	Mortality (%)[a]	
	larvae	adults
1	12.5 ± 4.3	0
24	42.5 ± 8.3	0
48	85.0 ± 5.0	0

a mortality in % ± standard deviation of 4 repetitions

The larvae mortality of 10% was observed even after only one hour of exposure. The mortality further increased to 42.5% and 85% during 24 and 48 hours of exposure, respectively. No mortality was observed for the control group during the 48 hour experiment.

Discussion

With the aim to follow the new strategy of integrated pest management focussed on agricultural greening together with food quality and safety we examined the *C. sinensis* essential oil potential for the use against one of the most serious insect pest of wheat, *O. melanopus,* under laboratory conditions. Our direct contact toxicity assay showed no effect of the oil on the *O. melanopus* adults. On the other hand, it revealed its remarkable larvicidal potential with the larvae mortality of 42.5% and 85% observed 24 and 48 hours after topical application, respectively (Table 3). Similar mortality rates have been reported by [21, 40] for some coleopteran beetles, nevertheless the active concentrations are incomparable due to different experimental designs used. The dose applied in this study (1uL of 1% solution) corresponds to approx. 8.45 μg/specimen (the density of 0.845 g/mL, given by the supplier (Sigma, 2014), was used for the calculation).

The essential oil composition obtained by GC/SMS (Table 1) is in accordance with literature data reporting d-limonene, myrcene, alpha pinene, linalool and sabinene amongst the main constituents. Also the d-limonene content of 89.49% is within the range previously reported (73.24 - 94.8%) [34, 33, 2, 18, 40].

The detailed examination of the *C. sinensis* essential oil effect on *O. melanopus* specimens was not the subject of this study. However, we can assume that the larvicidal activity might be partially caused by the larvae dehydration and surface distortion as has been observed by scanning electron microscopy on housefly larvae [18]. Although there was no direct insecticidal effect observed in our contact toxicity assay against *O. melanopus* adults, some previous studies indicate that the *C. sinensis* oil can have other anti-isect properties such as repellent or anti-oviposition activities as described e.g. in case of *Costelotrycha zealandica* White [24] and *Bemisia tabaci* Gennadius [30] respectively. Moreover, the oilmight perhaps be active against adults in vapour phase as in the fumigant bioassay of [21] against three coleopteran beetles, of which *Callosobruchus maculatus* belongs to the same family as *O. melanopus*.

An important factor, both in terms of food safety and the efficiency against crop pests, is the persistence of a pesticide in plants. The results of our persistence assay using d-limonene for the residue detection showed that the *C. sinensis* essential oil evaporates very quickly from the treated plants (Table 2). The concentration of 0.01038 µL/g was detected by GC-MS 5 min after application (Fig. 2) which is below the minimum residue limit (0.01 mg/kg) established for substances that are not included in any of the annexes in EU regulations [8]. Thus it indicates *C. sinensis* essential oil presents minimum health risk and there is no need for protection period after application. Due to its low persistence it is also harmless to the environment and it is safe for non-target organisms if not directly exposed to the oil.

Although the low persistence can constitute certain disadvantage regarding to the pesticide efficiency, the results of our toxicity assay indicate that the larvicidal effect is irreversible once the specimen is exposed to the oil. Moreover, the oil might have also some pupicidal effect as in case of *Musca domestica* pupae [18] or some other anti-insect activity as already discussed above.

As was already mentioned the occurrence of this pest is subject to a suitable year. The harmfulness of adults is usually not significant and treatment against adults is not recommended. Significant yield losses are caused by the larvae of the last stage of development (75 % of the total quantity of damage throughout the larvae development). The larval density of 22-26 larvae per 100 stems of winter wheat can cause yield losses up to 4%. [31] whreas the density of 1 larva per stem can cause up to 12.65% yield loss which gives the economic threshold of 0.4 larvae per stem during the spike emergence to anthesis stages [4].

In conclusion, this is the first report on the use of essential oil against the serious cereal crop pest *O. melanopus*. Although the *C. sinensis* essential oil was not active against *O. melanopus* adults it exerted interesting larvicidal effect. Considering its very low persistence in treated plants and low toxicity and thus low negative impact on the environment, *C. sinensis* is very promising alternative to synthetic pesticides, suitable for implementation to integrated pest management and organic farming.

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