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Effect of plant essential oils on the mortality of *Ditylenchus dipsaci* (Kühn, 1857) nematode under *in vitro* conditions

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Abstract: With the need to obtain new methods to protect seed material from *Ditylenchus dipsaci* (Kühn, 1857) nematodes, a study was conducted to evaluate the nematicidal effects of several plants' essential oils on the mortality of *D. dipsaci*. Tests were performed under *in vitro* conditions; a concentration of 2 000 ppm was tested, nematodes were added into diluted oils, and numbers of living/dead nematodes were scored after 4 and 24 h. The results show a significant effect of several plant essential oils on *D. dipsaci* mortality, with the highest efficacy found for oil from *Cinnamomum cassia* ((L.) J. Presl), with 100% mortality observed even after 4 h.

Keywords: stem nematode; plant extract; pest management; crop protection

The restriction of pesticide usage in recent years has led to a lack of chemicals available for crop protection against plant parasitic nematodes. In the case of stem and bulb nematodes *Ditylenchus dipsaci* (Kühn, 1857), preventive treatment of seed cloves of garlic, onion and bulbous ornamental plants is of crucial importance.

Ditylenchus dipsaci is a key pest of onion vegetables, ornamental flowers and fodder crops in the north as well as south temperate zone (Aftalion and Cohn 1990). This is the consequence of the extremely broad host range of the species, its rapid reproduction, ability to survive long periods in anabiosis in the absence of its host plant and also the existence of so-called host races – the nematode populations differing by its host plant status (Janssen 1994). It is a migratory free, living endoparasitic nematode; both sexes are of worm shape; it enters the host plant *via* stomata moving in water film on the surface of plant tissue. It lives in plant tissue afterwards for several generations and stays in drying plant material after the death of the host plant, where it can survive in the anabiotic stage for as long as 20 years (Seinhorst 1956, Wharton and Barrett 1985, Subbotin et al. 2005).

Ditylenchus dipsaci is listed as a regulated harmful species in several countries (A2 category according to European and Mediterranean Plant Protection Organisation; EPPO 2021). Spreading of this pest into unaffected areas often happens by infected seed material (Mouttet et al. 2014). In the case of onion vegetables, vegetable seeds (chives, leek), as well as seed cloves or bulbs (garlic, leek), could be infested. Therefore, treatment of seed material before sowing is of key importance (Hanks and Linfield 1999).

As methyl bromide is no longer available in the European Union, seed treatment against *D. dipsaci* is conducted almost exclusively by hot water (EPPO 2000). However, as the effectiveness of this technique was questioned in the past (Roberts and Greathead 1986) and the large-scale application of hot water treatment has been difficult, new methods of addressing this problem are being tested. Promising results were obtained after the fumigation of garlic cloves using hydrogen cyanide (Zouhar et al. 2016); however,

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this method is more appropriate for industrial-scale treatment and cannot be employed directly by farmers because of the high acute toxicity of hydrogen cyanide and related legislative limitations.

Much attention has been dedicated to alternatives to chemical substances, mainly plant extracts and essences, as these substances are already frequently used in the food industry or cosmetics. When considering *D. dipsaci* nematodes, the nematocidal properties of *Syzygium aromaticum* ((L.) Merr. & L. M. Perry), *Origanum compactum* Benth, *Origanum vulgare* L., *Thymus mastichina* (L.) and *Thymus vulgaris* L. have been described (Zouhar et al. 2009). Extracts from fresh leaves of *Plantago major* L. and *Ruta graveolens* L. also effectively protected garlic against *D. dipsaci* infestation (Insunza and Valenzuela 1995). However, the number of studies conducted on the alternative management of *D. dipsaci* remains rather low; more attention has been dedicated to root-knot nematodes (*Meloidogyne* sp.) and other plant parasitic nematode genera.

Therefore, the main aim of this work was to assess the influence of several plant extracts on the viability of *D. dipsaci* nematodes under *in vitro* conditions to obtain basic data for the further development of practical treatment against this important pest.

MATERIAL AND METHODS

Nematode inoculum was obtained from fresh-infested garlic plants in the locality Drásov (South Moravian Region, Czech Republic). Nematodes (mainly 4th development stage juveniles) were extracted from infested tissue using the modified Baermann funnel method (Baermann 1917). The obtained nematode suspension was washed on a nylon sieve (mesh size 25 μm) and stored at 5 °C for 7–10 days prior to testing.

All essential oils used for testing were obtained from Saloos, Czech Republic, except for one essential oil from *Thymus vulgaris,* which was purchased from Sigma (St. Louis, USA); the essential oils were isolated *via* steam distillation from fresh plant material. Two other cultivars of *Thymus vulgaris* ("red" and "white") and pure Thymol were also tested. Essential oils were diluted using the 1:1 solution of Tween 20 (Sigma, St. Louis, USA) and distilled water; the choice of Tween 20 was conducted about the work of Oka et al. (2000). The control contained only Tween 20 and distilled water.

Testing was conducted in plastic multiwell plates (Asahi Glass, Tokyo, Japan) with a suitable diameter of 20 mm. A drop of nematode suspension (25 μL) containing, on average, 40 specimens was pipetted into each well, and 1 mL of essential oil solution with a concentration of 2 000 ppm was added. After the experiment establishment, the plates were covered by plastic lids and kept at room temperature, and natural illumination, shaking, or additional saturation with oxygen were not used. Nematodes were counted under a stereomicroscope immediately after experiment establishment and then after 4 and 24 h. Nematodes were considered dead when laying straight and not moving even after being touched with an entomological micropin to rule out that nematodes were only immobilised by the essential oil. Each treatment was replicated five times in separate wells. For capacity reasons, the experiment was performed in 4 days using four separate batches with equivalent conditions; a control (water with a detergent) was used with each batch. Mortality was established according to the Abbott formula (Abbott 1925). The percentage of mortality was transformed using arcsine transformation and subjected to statistical analysis. Statistical evaluation was conducted separately for each batch using a one-way ANOVA followed by post hoc comparisons using the Tukey honestly significant difference (*HSD*) method. Separate analyses for 4 and 24 h treatments were conducted. All calculations were performed in Statistica 13.3 (TIBCO Software Inc., 2017, Palo Alto, USA).

RESULTS AND DISCUSSION

All results are summarised in Table 1. By far, the strongest effect was observed after applying the essential oil from *Cinnamomum cassia* (L.) J. Presl, and 100% mortality was observed 4 h after application. Relatively strong effects (75% of dead nematodes after 24 h) were also observed in the cases of essential oils from Frankincense (*Boswellia sacra* Flueck.) and dill (*Anethum graveolens* L.). Statistically significant numbers of dead nematodes were also seen after 24 h of application of essential oils from conifers (*Abies alba* Mill., *Pinus mugo* Turra); in the case of the essential oil from *Pinus sylvestris* L., although mortality was observed, the result was not statistically significant (P = 0.065583). However, no increased mortality when compared to control was achieved after the application of the essential oil from European spruce (*Picea abies* (L.) H. Karst.). Statistically significant numbers of dead nematodes were also found for treatments with essential oils from *Thymus vulgaris*

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Table 1. Mortality of nematodes after 4 and 24 h of treatment with essential oils dissolved in Tween 20: distilled water (1:1), which also served as a control; values in the same line followed by a different letter(s) are significantly different at $P \leq 0.05$ based on Tukey's multiple range test, ($n = 5$). Experiments were conducted in four separate batches with respective controls

%	Peppermint *Mentha piperita* L.	European spruce *Picea abies* (L.) H. Karst.	Basil *Ocimum basilicum* L.	Common sage *Salvia officinalis* L.	Rose geranium *Pelargonium graveolens* L'Hér.	Wormwood *Artemisia absinthium* L.	Lemon *Citrus limon* (L.) Osbeck	Control
Mortality 4 h	8.83 ± 4.02^{a}	6.01 ± 7.91^{a}	12.7 ± 7.99^{a}	9.79 ± 7.35^{a}	5.96 ± 4.03^{a}	11.86 ± 9.69^{a}	10.27 ± 4.65^{a}	6.59 ± 5.6^{a}
Mortality 24 h	12.05 ± 4.79^{a}	9.61 ± 6.94^{a}	16.2 ± 2.57^{a}	12.48 ± 9.65^{a}	11.86 ± 8.92^{a}	12.52 ± 9.56^{a}	11.64 ± 5.49^{a}	6.98 ± 5.2^{a}

%	Bergamot orange *Citrus bergamia* Risso	Marjoram *Origanum majorana* L.	Southern blue gum *Eucalyptus globulus* Labill.	Mediterranean cypress *Cupressus sempervirens* L.	English lavender *Lavandula angustifolia* Mill.	Fennel *Foeniculum vulgare* Mill.	Lemon-scented teatree *Leptospermum petersonii* F. M. Bailey	control
Mortality 4 h	17.16 ± 3.69^{a}	14.00 ± 5.90^{a}	20.33 ± 4.06^{a}	17.75 ± 8.41^{a}	20.09 ± 9.36^{a}	20.30 ± 7.53^{a}	19.21 ± 22.14^{a}	12.34 ± 2.07^{a}
Mortality 24 h	18.85 ± 3.14^{a}	16.14 ± 4.77^{a}	20.57 ± 3.54^{a}	19.16 ± 7.31^{a}	23.79 ± 9.93^{a}	27.12 ± 9.16^{a}	23.57 ± 18.21^{a}	14.36 ± 2.11^{a}

%	Frankincense *Boswellia sacra* Flueck.	African myrrh *Commiphora myrrha* (Nees) Engl.	Dill *Anethum graveolens* L.	European silver fir *Abies alba* Mill.	Chinese cinnamon *Cinnamomum cassia* (L.) J. Presl	Mountain pine *Pinus mugo* Turra	Scots pine *Pinus sylvestris* L.	control
Mortality 4 h	19.06 ± 3.40^{a}	7.62 ± 6.64^{b}	21.36 ± 8.40^{a}	11.44 ± 5.75^{b}	100.00 ± 0.00^{c}	10.19 ± 2.23^{b}	11.69 ± 3.86^{b}	4.60 ± 3.40^{b}
Mortality 24 h	75.28 ± 6.82^{a}	22.52 ± 11.95^{b}	60.42 ± 11.11^{ad}	48.43 ± 19.27^{de}	100.00 ± 0.00^{c}	66.35 ± 4.76^{ade}	45.56 ± 22.24^{be}	19.67 ± 3.49^{b}

%	Thymol	Broad-leaved thyme *Thymus pulegioides* L.	Common thyme *Thymus vulgaris* L. (Sigma)	Common thyme *Thymus vulgaris* L. white	Common thyme *Thymus vulgaris* L. red	control
Mortality 4 h	13.66 ± 12.49^{a}	30.11 ± 10.49^{ac}	39.82 ± 5.79^{bc}	58.10 ± 7.97^{b}	49.67 ± 11.44^{b}	14.78 ± 11.83^{a}
Mortality 24 h	62.45 ± 11.51^{a}	58.01 ± 9.41^{a}	48.87 ± 11.4^{a}	66.33 ± 9.25^{a}	51.11 ± 9.24^{a}	19.92 ± 12.39^{b}

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L. and pure thymol; in this case, 24 h of treatment resulted in considerably higher mortality when compared to control. Considering the remaining plant essential oils tested, no statistically significant effects were detected, but certain trends suggesting increased mortality could be observed, especially in the cases of essential oils from *Foeniculum vulgare* Mill., *Mentha piperita* L., *Ocimum basilicum* L., *Salvia officinalis* L., *Pelargonium graveolens* L'Hér. and *Artemisia absinthium* L.; these trends were apparent even after 4 h treatment.

These results suggest that there is significant potential in the use of plant essential oils as a tool for seed material protection against *D. dipsaci* nematodes, as the nematocidal effects of several essential oils were clearly shown. Compared with the currently used hot water treatment technique (EPPO 2000), which regards a treatment period of 1 h to be sufficient for nematode destruction, most essential oils need a longer time period to increase nematode mortality. An increase in nematode mortality was observed for most essential oils after a longer period (24 h), except for the essential oil from *Cinnamomum cassia* (L.) J. Presl. A negative effect of thymol on *D. dipsaci* viability was noted by Stavropoulou et al. (2021); however, in that case, although significant under *in vitro* conditions, the nematode mortality was lower (25.3% and 11.3% of dead nematodes after 24 h; Stavropoulou et al. 2021) than that presented here (13.66% and 62.45% of dead nematodes). Better results were also obtained in our previous study (Zouhar et al. 2009), where approximately 90% mortality was observed at 5 000 ppm *Thymus vulgaris* L. oil concentrations. Differences between the results obtained in this study and those from other studies could be a consequence of the different compositions of these oils; thus, the amounts of different compounds in certain essential oils could vary even among oils from the same plant species obtained from different companies, localities or different growing seasons (Pavela and Benelli 2016). The technique used to obtain plant extracts or essential oils is also of crucial importance. Highly efficient mortality of *D. dipsaci* juveniles present in garlic was detected with extracts from *Plantago major* L. and *Ruta graveolens* (Insunza and Valenzuela 1995); this only occurred when extracts from fresh leaves were used, as extracts from dry tissue were substantially less effective.

Hassan et al. (2015) reported high nematocidal effects of water and ethanol extracts from *Dittrichia viscosa* (L.) Greuter and *Melia azedarach* L. on *D. dipsaci* species. Stavropoulou et al. (2021) evaluated the effects of four terpenes on the mortality of *D. dipsaci,* with 100% nematode mortality observed after applying carvacrol at a concentration of 2 000 ppm under *in vitro* conditions. Moreover, nematocidal effects of the essential oil extracted from *Elsholtzia fruticosa* (D. Don) Rehder on *Ditylenchus destructor* Thorne were reported (Liang et al. 2020), and antagonistic activity of the essential oils from *Ajania fruticulosa* (Ledeb.) Poljakov and *Ajania potaninii* (Krasch.) Poljakov on the same species was also detected (Liang et al. 2018) and so proving the negative effect of plant essential oils on the viability of *Ditylenchus* sp. nematodes. *D. destructor* and *D. dipsaci* differ in their biology, which complicates the transfer of these results to the management of the target species. Regarding the essential oil from *Cinnamomum cassia,* promising results were obtained when testing its effect on *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle nematodes (Kong et al. 2007), but no nematocidal effects against *D. dipsaci* have been reported thus far.

There are many studies describing the positive effects of plant substances on plant parasitic nematode mortality; however, the number of registered and practically used nematicides based on these results remains limited. This lack of products may be due to the expensive and demanding registration process that is needed for commercial applications. Within the European Union territory, the registration process could be simplified when the applied substance is already used in the food or cosmetic industry (European Commission 2009), but credible proof of the harmlessness of the substance to human health and the environment must still be presented. Additionally, the possible phytotoxic effects of plant substances, as well as the abovementioned differences in the composition of plant essential oils or extracts across different suppliers or growing seasons, could present another issue. This problem could be solved by biological tests for possible phytotoxicity and quantification of the content of all important plant compounds in tested plant substances using chemical analysis. Strong and fast effect of essential oil from *Cinnamomum cassia* (L.) J. Presl., as well as its low price, makes this plant species an ideal candidate for more research of its nematocidal properties. Future research will focus on the treatment of infested seed garlic under *in vivo* conditions, with special attention given to the possible phytotoxic effects of essential oil application and its effects on the resting stage of *D. dipsaci* nematode.

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