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Activity of *Satureja montana* Allelochemical Volatiles Against the Pinewood Nematode †

Gonçalo Pereira [1] and Jorge M. S. Faria [1,2,*]

[1] INIAV, I.P., National Institute for Agrarian and Veterinary Research, Quinta do Marquês, 2780-159 Oeiras, Portugal; goncalo.pereira@iniav.pt
[2] GREEN-IT Bioresources for Sustainability, Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa (ITQB NOVA), Av. da República, 2780-157 Oeiras, Portugal
* Correspondence: fariajms@gmail.com
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Abstract: Essential oils (EOs) are complex mixtures of mainly volatile terpenes and phenylpropanoids with strong biological activities. Screening their nematicidal activity against plant parasitic nematodes can yield important information on anti-nematodal chemical structures. In previous studies, the EO of winter savory, *Satureja montana*, revealed a high nematicidal activity against the pinewood nematode (PWN), a dangerous phytoparasite that attacks pine trees and causes pine wilt disease (PWD). Its activity was solely attributed to the oxygen-containing molecules, however, interactions between EO compounds were not fully ascertained. In the present study, the main compounds of winter savory EO were tested solely and in combination to understand which were responsible for the nematicidal strength of the EO. The main EO compound, carvacrol, induced the strongest activities, however, γ-terpinene and *p*-cymene appear to influence its activity, even though they promote a low PWN mortality. Uncovering the interactions between the components of nematicidal EOs can provide clues to better formulate sustainable alternatives to traditional pesticides.

Keywords: *Bursaphelenchus xylophilus*; carvacrol; essential oil; nematicide; toxicity; volatiles; winter savory

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1. Introduction

Plant parasitic nematodes (PPN) have been recognized as some of the most widespread and damaging global pests, with an estimated 12% loss in yield due to PPN-caused diseases, twice as high as the loss caused by phytophagous insects [1]. The PWN, *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickel (PWN), is the pathogenic agent responsible for PWD. Infected pines display a yellow and wilted canopy, and a reduction in defences that leads to secondary infections with opportunistic pathogens. PWD imposes significant economic and ecological damages affecting pine wood dependent industries [2,3].

Currently, pest management strategies rely on restrictions to wood transportation, the elimination of infected wood material, wood treatment with heat or pesticides, and chemical or cultural control of the insect vector [4]. The application of (hemi)synthetic pesticides through trunk injection is one of the most direct PWD control strategies [5]. However, the use of pesticides has been increasingly restricted owing to environmental and human health concerns, due to the adverse effects of these pesticides on non-target organisms [6,7]. This has led to an increase in demand for the development of more ecological biopesticides, which tend to be more environmentally friendly when compared to traditional chemical pesticides. Several approaches are being conducted for the development of new pesticides, particularly the development of novel microbial and biochemical biopesticides. Biochemical pesticides can be based on cost-effective natural compounds

which exhibit significant anti-nematode properties [6,8]. Essential oils (EOs) can be valuable alternatives to synthetic pesticides, given they are complex mixtures of naturally occurring bioactive compounds, which do not accumulate in the environment, and present a broad range of activity thereby reducing the risk of developing resistant pathogenic strains [9,10]. The EOs extracted from plants of the Lamiaceae family have been widely screened against the PWN [4]. The Lamiaceae with the highest activities reported so far are *Nepeta tenuifolia*, *Perilla frutescens*, *Satureja montana*, *Thymbra capitata* and *Thymus caespititius*, with *S. montana* reaching the lowest EC_{50} values (0.26–0.38 μL/mL) [4]. In previous studies, the nematicidal strength of *S. montana* EO was linked to the oxygen-containing compounds in its composition, with little to no influence of its hydrocarbon molecules [11]. However, antagonistic and synergistic interactions are known to occur between EOs and/or EO compounds [10,12].

The present study aimed at determining the specific activity of the main compounds of *S. montana* EO, by screening the mortality induced by pure standards on the PWN, at the proportions they are present in the EO. Also, the specific synergistic and antagonistic interactions were ascertained by screening combinations of EO compounds.

We intend to contribute to the current state of research by mapping the bioactive properties of active EOs against the PWN and leverage the development of novel sustainable pest management strategies.

2. Materials and Methods

2.1. Chemicals

To determine the nematicidal activities of the major (>5%) *S. montana* EO volatiles, pure chemical standards of carvacrol (98% purity), *p*-cymene (99% purity) and γ-terpinene (97% purity) were acquired from Sigma-Aldrich (St. Louis, MO, USA) and diluted in HPLC-grade methanol (Fischer Chemicals, Hampton, NH, USA) to an initial concentration of 20 mg/mL. Winter savory (*Satureja montana*) EO was acquired from certified local retail sellers and diluted as described above. Stock solutions were stored at −20 °C until used.

2.2. In Vitro Cultures of the Pinewood Nematode

Large quantities of the PWN were grown in vitro to be used in direct-contact bioassays. The reference isolate Bx0.13.003, kept at the Plant Nematology Lab of the National Institute for Agrarian and Veterinary Research (INIAV, I.P) in Oeiras, Portugal, was used for experiments [12]. PWNs were cultured in aseptic conditions by feeding on axenic cultures of a non-sporulating strain of *Botrytis cinerea* (de Bary) Whetzel. Certified organic barley grains (*Hordeum vulgare* L.) were hydrated and steam-sterilized (approximately 15 g of barley with 15 mL of ultrapure water in 250 mL Erlenmeyer flasks), inoculated with a fungal culture plug and maintained for 7 to 10 days at 25 ± 1 °C. After the cereal was covered by fungal culture, 1 mL of a mixed life stage suspension of PWNs, containing about 1000 nematodes per mL was added and the culture and kept in darkness at 25 ± 1 °C for 7 to 10 days, or until the fungus was completely consumed. To avoid microbial contamination, the nematodes were surface sterilized with a hydrogen peroxide (H_2O_2) solution (20%, *v*/*v*) for 20 min, and washed 3× with sterilized water, in the flow hood, before being introduced into the axenic mycelial cultures. The modified Baermann funnel technique was used to isolate PWNs [13]. The PWN suspensions were used immediately for the direct contact bioassays or stored at 11 °C for up to 1 week. PWN numbers and mortality were assessed using an Olympus SX12 stereomicroscope (40×).

2.3. Nematicidal Activity of Volatiles

The volatiles were tested at the proportions presented in the essential oil. Profiling of volatiles was performed through gas chromatography coupled to mass spectrometry (GC-MS), as detailed before [12]. The stock solutions of the dominant compounds (>5%)

carvacrol, *p*-cymene and γ-terpinene were used to make dilutions according to their proportions in the EO (Table 1). The influence of each compound and their potential synergistic or antagonistic interactions were evaluated by screening combinations of compounds at their respective proportions in the EO (Table 1). The reconstituted EO was tested by combining the dominant compounds at their respective proportions in the EO (Table 1).

Table 1. Concentration of volatiles and combinations of volatiles (mg/mL) at the proportion (%) presented in the essential oil of *Satureja montana*. Stock solutions were made by diluting the pure compound or combinations of compounds in methanol.

Compound/Combination	**Concentration in 1 mg/mL of EO (mg/mL)** [1]		
	carvacrol (64 [2])	γ-terpinene (18 [2])	*p*-cymene (8 [2])
carvacrol	0.64		
γ-terpinene		0.18	
p-cymene			0.08
carvacrol + γ-terpinene	0.64	0.18	
carvacrol + *p*-cymene	0.64		0.08
γ-terpinene + *p*-cymene		0.18	0.08
carvacrol + γ-terpinene + *p*-cymene [3]	0.64	0.18	0.08

[1] for lower concentrations (0.5, 0.25 and 0.125 mg / mL), serial dilutions were performed with methanol, at a dilution factor of two; [2] amounts in the essential oil (%); [3] reconstituted essential oil.

The direct-contact bioassays were conducted using a flat-bottom 96-well microtiter plates (Carl Roth GmbH & Co. KG, Karlsruhe, Germany). Each well was filled with a 95 µL suspension containing approximately 60 mixed life-stage PWNs, followed by 5 µL of a volatile or combination of volatiles stock solution prepared in HPLC-grade methanol. Controls included blank wells containing 5 µL of ultrapure water to measure natural PWN mortality, and wells with 5 µL of methanol to assess mortality caused by the solvent. The microtiter plates were then sealed with plastic film to prevent excessive compound volatilization and shaken on an orbital shaker (IKA labortechnik, Staufen, Germany) at 800 r.p.m. for 1 min. Afterwards, the plates were covered with aluminum foil to maintain darkness and incubated for 24 h in an orbital shaker set to 60 r.p.m. at 25 ± 1 °C. The bioassays were performed in triplicate for each sample, with a total of 9 bioassays conducted. Volatiles and their combinations that resulted in complete mortality were further tested at lower concentrations (0.5, 0.25, 0.125 mg/mL) using serial dilutions with methanol, at a dilution factor of 2, to determine toxicity thresholds.

2.4. Data Treatment and Statistical Analysis

Nematode mortality percentages were calculated using Formula (1):

$$\text{Mortality \%} = (\text{dead PWNs/total no. of PWNs}) \times 100 \quad (1)$$

To obtain corrected mortality percentages for each volatile or combination of volatiles, Formula (2) was applied:

$$\text{Corrected mortality \%} = [(\text{mortality \% in treatment} - \text{mortality \% in control})/(100 - \text{mortality \% in control})] \times 100 \quad (2)$$

The toxicological strength at various concentrations was classified using previously established criteria, where mortality was deemed complete at 100%, strong above 80%, moderate between 80 and 61%, weak between 60 and 40%, and low or inactive below 40%.

The half-maximal effective concentration (EC_{50}) values were calculated using Origin Graphing and Analysis software Version 2019 (OriginLab, Northampton, MA, USA). This involved a nonlinear regression analysis, where corrected mortality values were plotted

against concentrations of volatiles or combinations of volatiles, fitting a dose-response log-logistic model detailed in Formula (3):

$$y = A1 + (A2 - A1)/1 + \exp\{[\log(x) - \log(EC_{50})]p\} \quad (3)$$

where A1 and A2 are the lower and upper limits of the sigmoidal dose–response curve, respectively; p is the slope and EC_{50} is the EO concentration that induces a response halfway between the lower and upper limits. The lower (A1) and upper (A2) limits were set to 0 and 100%, respectively. The evaluation of synergistic or antagonistic effects involved comparing the activity of compounds or mixtures to the additive effects of individual compounds. The lowest maximal effective concentration (EC_{100}) was determined by resolving the curve equation to find the first instance of 100% mortality.

3. Results and Discussion

Nematicidal Activity of Essential Oils and Mixtures

The nematicidal activity of *S. montana* EO was compared to the activity of its three main volatiles, carvacrol (64%), *p*-cymene (18%) and γ-terpinene (8%), their binomial mixtures and the reconstituted EO (mixture of the three main volatiles at their proportions in the EO). Complete mortality (100 ± 0.0%) was found for *S. montana* EO, the monoterpenoid phenol carvacrol, and any mixture where carvacrol was included (Figure 2). Significantly lower mortality percentages were observed for the monoterpene hydrocarbons, *p*-cymene (16.4 ± 0.4%) and γ-terpinene (23.5 ± 0.3%), that can be classified as compounds with low to no toxicity, at the proportions present in the EO. The binary mixture of the hydrocarbons resulted in a mortality rate of 63.7 ± 1.2%, which was higher than the expected addition of the mortalities of the sole compounds (approx. 40%), suggesting the occurrence of a possible synergistic interaction between these two compounds. For the volatiles and mixtures that induced complete mortality, lower concentrations were assayed to determine their toxicological strength, namely the half-maximal concentration (EC_{50}) values (Table 2).

Table 2. Activity of *Satureja montana* essential oil, its respective dominant volatiles (>5%), their binary combinations, and the reconstituted essential oil (combination of the dominant volatiles) against the pinewood nematode, expressed by their corrected mortality percentage at 1 mg/mL, half-maximal effective concentration (EC_{50}) and lowest maximal effective concentration (EC_{100}). Slope (p) and goodness of fit (R^2) are shown for comparison purposes.

EO/Compounds	Mortality % at 1 mg/mL	$EC_{50\ 24h}$ (mg/mL)	$EC_{100\ 24h}$ (mg/mL)	*p*	R^2
Satureja montana EO	100 ± 0.0	0.151 ± 0.002	0.59–0.78	10.1 ± 0.5	0.98
carvacrol	100 ± 0.0	0.292 ± 0.004	0.84–0.99	6.1 ± 0.3	0.99
γ-terpinene	23.5 ± 0.3				
p-cymene	16.4 ± 0.4				
carvacrol + γ-terpinene	100 ± 0.0	0.292 ± 0.006	0.71–0.81	4.2 ± 0.2	0.97
carvacrol + *p*-cymene	100 ± 0.0	0.152 ± 0.003	0.66–0.79	6.7 ± 0.3	0.97
γ-terpinene + *p*-cymene	63.7 ± 1.2				
γ-terpinene + *p*-cymene + carvacrol [1]	100 ± 0.0	0.117 ± 0.001	0.38–0.62	14.9 ± 0.8	0.99

[1] Reconstituted essential oil is composed by its dominant volatiles at their respective proportions on the original EO.

The lowest activities were obtained for carvacrol (0.292 ± 0.004 mg/mL) and carvacrol + γ-terpinene (0.292 ± 0.006 mg/mL) (Figure 1), indicating that the hydrocarbon shows no interaction on carvacrol activity. However, the mixture of carvacrol and *p*-cymene reached a nematicidal activity (0.152 ± 0.003 mg/mL) similar to that of the EO of *S. montana* (0.151 ± 0.002 mg/mL), suggesting a synergistic interaction between these two components (carvacrol and *p*-cymene). Interestingly, the reconstituted EO of *S. montana*, which consisted of the dominant volatiles (<5%) at their respective proportions in the original EO, yielded the highest activity (0.117 ± 0.001 mg/mL), pointing towards a possible strong synergistic interaction between these dominant volatiles and/or an antagonistic interaction between these and the volatile components with proportion lower than 5%. Nevertheless, the reconstituted EO appears to be a promising candidate for the development of a biopesticide, exhibiting the lowest maximal effective concentration (EC_{100}) (0.38–0.62 mg/mL) when compared to the remaining experimentally tested mixtures (Table 2 and Figure 1).

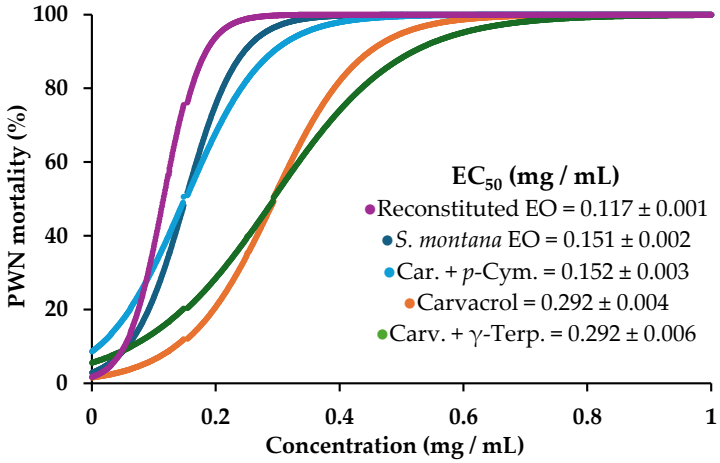

Figure 1. Graphical representation of the dose–response sigmoidal curves fitted to the corrected mortality values obtained for the pinewood nematode with decreasing concentrations of of *Satureja montana* essential oil (teal), carvacrol (orange), binary combinations of carvacrol with *p*-cymene (blue) or γ-terpinene (green), and the reconstituted EO (purple). Half-maximal effective concentration (EC_{50}) values are provided for comparison.

4. Conclusions

Essential oils are being tested as possible alternatives for the development of more sustainable biopesticides. The essential oil of *S. montana* is known to exhibit strong nematicidal activity against the pinewood nematode, which appears to be associated with the presence of the monoterpenoid phenol carvacrol and the hydrocarbon *p*-cymene. However, the best activity was obtained when carvacrol and *p*-cymene were added to γ-terpinene. This study contributed to the development of more effective and sustainable biopesticides to replace traditional pesticides that are responsible for environmental and human health concerns.

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