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Synergistic Activity of *Cymbopogon citratus* and *Mentha piperita* Essential Oils against the Pinewood Nematode †

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Abstract: The pinewood nematode (PWN) *Bursaphelenchus xylophilus* is a major threat to pine forests. Research on sustainable pest management strategies is now a priority. Essential oils (EOs) are biodegradable, complex mixtures of volatiles that can show low toxicity to mammals and powerful nematicidal activities depending on their chemical composition. In the present work, the EOs of four plants were screened against the PWN, solely and in combination to identify possible synergistic interactions. The combination of *Cymbopogon citratus* and *Mentha piperita* EOs (1:1) resulted in higher activities than those of each tested solely, suggesting the occurrence of synergistic interactions between the compounds of these EOs. Research on the combination of synergistic EOs may lead to the development of plant based biopesticides with optimized activities against the PWN.

Keywords: biopesticides; *Bursaphelenchus xylophilus*; essential oils; nematicides; synergistic interactions

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1. Introduction

The health of forest ecosystems is of pivotal importance to the improvement of global biodiversity and its impact on environmental resilience to climate change. However, in the last few years, forest management has been increasingly challenged by a rising number of pathogenic agents, e.g., invasive insects, microbes and fungi, undoubtedly linked to a growing trade industry and the establishment of new trading routes [1]. These numerous passageways for pathogenic entrance are mostly unchecked or only minimally regulated, given the extremely high resource- and time-consuming techniques used for monitoring [2]. *Bursaphelenchus xylophilus* (Steiner & Buhrer 1934), the pinewood nematode (PWN), is a highly pathogenic forest parasitic nematode that causes Pine Wilt Disease (PWD) in pine trees, and is responsible for the devastation of large stretches of pine stands in Asia and Europe. Given its extreme pathogenicity and the abundance of susceptible pines in European countries (e.g., *Pinus pinaster* and *Pinus sylvestris*), the European Plant Protection Organization (EPPO) has considered the PWN as an A2 type quarantine pest in the EU, even though its presence in Europe is currently limited to Portugal and Spain [3]. However, it is believed

that future environmental conditions in northern European countries, as a result of climate change, may create a highly susceptible environment that endangers the extensive northern pine forests [4,5]. Currently, pest management of PWD is focused on containing the spread of the PWN, or its insect vectors of the *Monochamus* genus. Direct control of the PWN can be successfully performed through the trunk injection of strong nematicides. However, the use of many pesticides—including insecticides and nematicides—has been linked to environmental pollution and negative effects on public health [6]. Recent research efforts have been directed at the development of sustainable pest management methods able to reduce PWN populations and safeguard the biodiversity of natural environments. Essential oils (EOs) have shown many advantages toward the development of bionematicides, e.g., they can be easily obtained using accessible technology, are biodegradable, and are potentially non-toxic to mammals [7]. Additionally, they are composed of a variety of volatile compounds, mainly mono- and sesquiterpenes and phenylpropanoids in high amounts, with several biological activities able to regulate not just the targeted pest but also opportunistic species and resistant strains [8]. Many volatile compounds that characteristically make up EOs have often displayed synergistic or antagonistic interactions [9,10]. However, the synergistic potential of EOs remains largely unexplored.

This study aimed to screen the activity of four EOs against the PWN, and analyze the possible synergistic interactions of combinations of two EOs towards anti-PWN activity. Researching the synergistic interactions of EO combinations can be an important contribution to support the establishment of sustainable pest management practices for maintaining the integrity and safety of pine forests.

2. Material and Methods

2.1. Essential Oils

The EOs of *Cymbopogon citratus* (lemongrass), *Eucalyptus globulus* (eucalypt), *Mentha piperita* (peppermint) and *Satureja montana* (winter savory) were acquired from commercial sources. To profile the volatiles, samples of the EOs were diluted (1:1, *v*/*v*) in *n*-hexane (95%, Optima grade for HPLC and GC-MS, Fisher Chemicals, Hampton, NH, USA), and 0.1 µL was analyzed in a Shimadzu GC2010 gas chromatographer coupled to a GCMS-QP2010 Plus Mass Spectrometer (Shimadzu, Kyoto, Japan). The gas chromatograph was equipped with a Zebron column ZB-5HT (30 m length, 0.25 mm I.D., 0.25 µm film thickness) (Phenomenex, Torrance, CA, USA). The sample was injected using a split sampling technique (ratio 1:100) with the injector temperature set to 250 °C and a helium flow of 1.5 mL/min. The GC oven temperature program was set to increase from 45 to 175 °C, at 3 °C/min, and then up to 300 °C, at 15 °C/min, with a final isothermal step for 10 min [11]. The mass spectrometer was operated in EI mode (70 eV) and scanned from 40 to 850 *m*/*z*. The ion source temperature was set at 240 °C and the interface temperature was maintained at 280 °C. Peak assignment was performed using the National Institute of Standards and Technology (NIST) and Wiley mass spectra libraries, through AMDIS software (National Institute of Standards and Technology of the US Department of Commerce, Gaithersburg, MD, USA).

2.2. Pinewood Nematode

Bursaphelenchus xylophilus nematodes were obtained from the reference collection BX013.003 (N 39°43′338″, W 9°01′557″) maintained at the Plant Nematology Laboratory of the National Institute for Agrarian and Veterinary Research (INIAV, I.P.) at Oeiras, Portugal. PWN rearing and in vitro culture were performed according to Faria et al. [9]. Briefly, axenic cultures of the non-sporulating strain of *Botrytis cinerea* (de Bary) Whetzel were inoculated on steam-sterilized hydrated certified organic commercial barley grains (*Hordeum vulgare* L.) (ca. 15 g cereal/15 mL ultrapure water, in 250 mL Erlenmeyer flasks), and kept at 25 ± 1 °C for 7 to 10 days, until the surface of the cereal was fully colonized. For PWN inoculation, ca. 1000–2000 nematodes were surface sterilized with an ethanol solution in ultrapure water (50% *v*/*v*) for 5 min [12] to avoid undesirable microbial contamination and added to *B.*

cinerea axenic cultures. In vitro PWN cultures were kept at 25 ± 1 °C in darkness for 7 to 10 days until the fungal mat was consumed, and extracted using the modified Baermann funnel technique [13]. Aqueous solutions of the PWNs were used for the direct contact assays, for further inoculations, or stored at 4 °C. The assessment of PWN numbers and/or survival rates was performed using an Olympus SZX12 (Tokyo, Japan) stereomicroscope.

2.3. Direct Contact Bioassays

Stock solutions of the EOs, or combinations of EOs, were prepared in HPLC-grade methanol (Fisher Chemicals, Hampton, NH, USA) at 20 µL/mL. The nematotoxic activity was determined using flat-bottom 96-well microtiter plates (Carl Roth GmbH & Co. KG, Karlsruhe, Germany). In each well, 100 ± 10 mixed life-stage PWNs in 95 µL aqueous suspensions were added to 5 µL of EO stock solution to obtain a final concentration of 1 µL/mL. Control assays were performed in 5% methanol (*v*/*v*, methanol/nematode suspension). The plates were then mixed in an orbital shaker (IKA labortechnik, Staufen, Germany) at 800 cycles/min for 1 min, covered with plastic film to reduce volatilization of EOs, covered with aluminum foil to establish complete darkness, and maintained at 25 ± 1 °C in an orbital shaker at 50 rpm for 24 h. Subsequently, dead and live nematodes were counted under a stereomicroscope. PWNs were considered dead if no movement was detected even after physical prodding. A minimum of 10 assays were performed for each sample, in at least two separate trials. To determine toxicity thresholds, lower EO concentrations were screened using the same procedure. Stock solutions for 0.5, 0.25, 0.12, 0.06 and 0.03 µL/mL were obtained by serial dilutions with a dilution factor of two.

2.4. Data Treatment and Statistical Analysis

For each EO solution, PWN mortality percentages were determined according to the formula: mortality % = 100 × [(dead PWNs)/(live + dead PWNs)]. Subsequently, corrected mortality percentages were determined for mortalities below 100%, using the formula: corrected mortality % = 100 × [(mortality % in treatment − mortality % in control)/(100 − mortality % in control)]. The toxicological strength of the EOs tested against the PWNs was evaluated according to the classification established by Kong et al. [14], by considering mortality as complete when 100%, strong when above 80%, moderate between 80 and 61%, weak between 60 and 40%, and low or inactive below 40%. Determination of the half maximal effective concentration (EC_{50}) was performed with Version 2019 of Origin Graphing & Analysis software (OriginLab, Northampton, MA, USA). A nonlinear regression analysis was performed by plotting mean corrected mortality values against EO concentration values, and fitting a dose-response log-logistic equation: $y = C + (D - C)/1 + \exp\{b [\log(x) - \log(EC_{50})]\}$ [15], where C and D are the lower and upper limits of the sigmoidal dose-response curve, respectively; b is the slope, and EC_{50} is the EO concentration which induces a response halfway between the lower and upper limits.

3. Results

3.1. Composition of Essential Oils

The EOs analyzed showed different volatile profiles (Table 1). Lemongrass (*C. citratus*, Poaceae) EO was mainly composed of the monoterpene isomeric aldehydes geranial and neral, the alcohol geraniol, and the hydrocarbon *β*-myrcene. The EO of eucalyptus (*E. globulus*), which belongs to the Myrtaceae family, was rich in the monoterpenes 1.8-cineole, *α*-phellandrene, and *α*-pinene. The Lamiaceae *M. piperita* (peppermint) EO was mainly composed of the oxygen-containing monoterpenes menthone and pulegone. Lastly, the *S. montana* (winter savory) EO showed high amounts of carvacrol and *γ*-terpinene.

Table 1. Relative amounts (%) of volatile compounds, obtained by GC-MS, in the essential oils of *Cymbopogon citratus*, *Eucalyptus globulus*, *Mentha piperita* and *Satureja montana*.

Compounds [1]	*C. citratus*	*E. globulus*	*M. piperita*	*S. montana*
Aromadendrene		4.2		
Carvacrol				61.3
β-Caryophyllene				4.2
1.8-Cineole		60.7	3.1	
p-Cymene				7.9
Geranial	26.3			
Geraniol	10.9			
Limonene		3.7	1.5	
Linalool	1.6			
Menthofuran			9.0	
Menthol			7.3	
Menthone			58.0	
iso-Menthone			3.5	
β-Myrcene	35.4			1.5
Neomenthol			2.1	
Neral	24.1			
α-Phellandrene		10.9		
α-Pinene		14.8		
Pulegone			15.0	
α-Terpinene				1.7
γ-Terpinene				20.0
Identification (%)	98.3	94.3	99.5	96.6

[1] Only compounds with relative amounts ≥1% are presented.

3.2. Activity of Essential Oils and Combinations against the Pinewood Nematode

Toxicity against the PWN varied between the EOs and their combinations (Table 2). When screened solely, only *C. citratus* and *S. montana* EOs showed strong nematicidal activities (EC_{50} values of 0.3068 ± 0.0073 and 0.1602 ± 0.0034 µL/mL, respectively), while those of *E. globulus* and *M. piperita* can be considered low or inactive (<40%).

Table 2. Nematicidal activity of essential oils of *Cymbopogon citratus*, *Eucalyptus globulus*, *Mentha piperita*, *Satureja montana,* and respective combinations against the pinewood nematode.

EOs/EO Combinations	Mortality % at 1 µL/mL	$EC_{50\ 24h}$ (µL/mL)	Type of Interaction (Expected $EC_{50\ 24h}$) [1]
C. citratus	100.0 ± 0.0	0.3068 ± 0.0073	-
E. globulus	6.0 ± 0.2	-	-
M. piperita	1.1 ± 0.2	-	-
S. montana	100.0 ± 0.0	0.1602 ± 0.0034	-
C. citratus/*E. globulus*	100.0 ± 0.0	0.7246 ± 0.0341	Antagonistic (≈0.3)
C. citratus/*M. piperita*	100.0 ± 0.0	0.0956 ± 0.0011	Synergistic (≈0.3)
C. citratus/*S. montana*	100.0 ± 0.0	0.2242 ± 0.0037	Combined (<0.16)
E. globulus/*M. piperita*	17.2 ± 0.9	-	Combined
E. globulus/*S. montana*	100.0 ± 0.0	0.1921 ± 0.0027	Combined (<0.16)
M. piperita/*S. montana*	100.0 ± 0.0	0.1958 ± 0.0021	Combined (<0.16)

[1] based on the EC_{50} values of each single EO.

When the EOs were applied in combination—at the same concentration as when applied solely—the mixtures of *C. citratus*/*S. montana*, *E. globulus*/*M. piperita*, *E. globulus*/*S. montana* and *M. piperita*/*S. montana* showed activities that can be considered as the result of the respective combined activities. However, the EO mixtures of *C. citratus*/*E. globulus* and *C. citratus*/*M. piperita* revealed antagonistic and synergistic interactions, respectively. The EO of *E. globulus* reduced the nematicidal efficiency of the *C. citratus* EO, from ca. 0.31

to 0.72 µL/mL. The EO of *M. piperita* was seen to increase the biocidal potency of the *C. citratus* EO against the PWN, from ca. 0.31 to 0.10 µL/mL.

4. Discussion

The EOs of lemongrass and winter savory have previously shown high biocidal activities, including against plant parasitic nematodes, e.g., the PWN [7,9,16–21]. However, to the best of our knowledge, this is the first time that EO combinations were tested against the PWN. An important synergistic interaction was identified in the combination of lemongrass with peppermint EOs. This promising nematicidal activity can be attributed to the main compounds identified in these EOs, namely geranial, geraniol, menthone, β-myrcene, neral and pulegone. The activity of monoterpenes has previously been analyzed on the PWN [22–25], with high activities being obtained for geraniol and citral (a mixture of the isomers geranial and neral), but lower activities for menthone, β-myrcene or pulegone. However, the synergistic activities of these compounds have not yet been analyzed against the PWN. Against the soil dwelling nematode *Meloidogyne incognita*, geraniol has shown synergistic interactions with *trans*-anethole, carvacrol and estragol, but only additive interactions with pulegone, while pulegone showed synergistic activity with *trans*-anethole and antagonistic activity with carvacrol [10]. Due to the complexity of an EO in terms of its composition and quantity in volatile compounds, it is difficult to reach a definitive conclusion on the origin of the nematicidal strength in the combination of the EOs of lemongrass with peppermint.

In future research, different ratios of these EOs will be tested to assess their effects on anti-PWN activity, and pure chemical standards of the main compounds will be assayed alone or in combination to pinpoint which compounds can provide an optimized activity against PWNs. Additionally, their effect on the plant host must be screened before a successful bionematicide can be proposed [26,27].

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