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Profiling the Nematicidal Activity of Linear and Cyclic Compounds on the Pinewood Nematode †

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Abstract: The pinewood nematode (PWN), *Bursaphelenchus xylophilus*, has become one of the most damaging pests to forest health in Asia and Europe. In the screening for sustainable biopesticides, research has focused on highly active secondary metabolites. In the present work, 25 linear and cyclic pure compounds, commonly found in essential oils (EOs) and extracts, were preliminarily tested in direct contact bioassays against the PWN to understand how distinct chemical structures can be related to a stronger nematicidal activity. Their activity appeared to be strongly related to specific functional groups, isomerism, or the length of the linear carbon chain. Uncovering the variation in the structure–activity relationships of anti-PWN compounds contributes to the identification of the nematicidal mechanisms of action as a basis for improving sustainable pest management in forest ecosystems.

Keywords: biopesticides; enantiomers; forest health; metabolites; nematicide; pine wilt disease; pinewood nematode; sustainable pest management

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1. Introduction

Current management strategies for the most damaging pests in agriculture and forestry are being increasingly improved by integrating sustainable cultural practices, biopesticides, and biocontrol agents in an effort to reduce losses in ecosystem biodiversity and to contain environmental degradation. The ban imposed on the most widely used and highly effective broad-spectrum synthetic pesticides coupled with the recent boost in the development of integrated protection biopesticides has fueled the search for "greener" chemicals capable of being used in precision agroecology. To date, several natural compounds and formulations have been screened against the pinewood nematode (PWN) *Bursaphelenchus xylophilus* (Steiner an d Bührer) Nickle, responsible for pine wilt disease (PWD) [1,2]. This microscopic phytoparasite was introduced in 1905 to Asia and has since ravaged the susceptible pine forests of Japan, China, Taiwan, and Korea. In 1999, it was detected for the first time in Europe, in Portugal [3], which led European and national authorities to promptly formulate a phytosanitary plan for the control of the PWN and eradicate it in its established sites [4]. This strategic plan aimed at containing the dispersion of PWD by establishing a tighter control of national wood transportation, regulating the exportation of wood products, creating quarantine and buffer zones in affected forest areas, felling and burning of symptomatic trees, and managing populations of the PWN insect vector, *Monochamus galloprovincialis*. This approach showed a considerable impact on PWD dispersal, yet the nematode was able to reach Madeira island and Spain in the following years [5–7].

Currently, the use of nematicidal compounds for the control of PWN populations is considered one of the most reliable strategies in integrated PWD pest management. Direct control of the PWN can be performed in restricted areas by trunk injection of powerful compounds, e.g., levamisole hydrochloride, mesulfenfos, morantel tartrate, or nemadectin [8,9]. Nevertheless, many pesticides have shown negative ecological and human health effects, displaying toxicity to other organisms and accumulating in food chains. The screening of ecologically less damaging compounds is being performed and has already shown promising results. Research has identified highly active plant and microbial EOs and extracts that show higher activities than some commercial nematicides [10,11]. Essential oils and extracts are composed of natural compounds whose chemical structures are often difficult or impossible to synthetize commercially. Nevertheless, the activity of pure compounds from a commercial origin can provide much information on the mechanism of action of highly active natural compounds, and can serve as the basis for the discovery of novel nematicidal compounds with more advantageous properties. In the present study, 25 linear and cyclic pure compounds, commonly found in EOs and extracts, were preliminarily tested in direct contact bioassays against the PWN. Their chemical structures were compared to understand the chemical guidelines that induce a stronger nematicidal activity against the PWN.

2. Materials and Methods

2.1. Chemicals

Pure analytical-grade standard compounds were acquired from commercial sources: *trans*-anethole (purity $\geq$ 99.5%), (−)-*trans*-caryophyllene (purity $\geq$ 98%), citral (purity $\geq$ 96%), (*S*)-(−)-citronellal (purity 96%), (*R*)-(+)-citronellal (purity 90%), *p*-cymene (purity $\geq$ 99.5%), 1-decanol (purity $\geq$ 98%), *n*-decanoic acid (purity $\geq$ 99.5%), dodecanal (purity $\geq$ 99.8%), 1-dodecanol (purity $\geq$ 98%), dodecanoic acid (purity $\geq$ 99%), eugenol (purity $\geq$ 99.6%), geraniol (purity $\geq$ 98.5%), linalool (purity $\geq$ 99%), menthol (purity $\geq$ 98%), α-pinene (purity 98%), (+)-pulegone (purity $\geq$ 98.5%), α-terpineol (purity $\geq$ 95%), γ-terpinene (purity $\geq$ 98.5%), (−)-terpinen-4-ol (purity $\geq$ 95%), (+)-terpinen-4-ol (purity $\geq$ 98.5%), 1-tridecanol (purity 97%), undecanoic acid (purity $\geq$ 99%), 1-undecanol (purity 99%), and 2-undecanone (purity $\geq$ 98%) were acquired from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade methanol was acquired from Fisher Chemicals (Hampton, NH, USA).

2.2. Pinewood Nematodes

Mixed life-stage populations of *Bursaphelenchus xylophilus* nematodes were obtained according to [12], using reference collections maintained at the Nematology Laboratory of INIAV. Briefly, certified organic hydrated barley grains (*Hordeum vulgare* L.; 15 g/15 mL of ultrapure water) were steam-sterilized in 250 mL Erlenmeyer flasks. Then, *Botrytis cinerea* fungal mats were grown on the sterilized barley. After 7 days, 100 to 200 mixed life-stage PWNs were added to these cultures and allowed to reproduce and consume the fungus (c.a., 7 to 10 days, in darkness). Finally, the nematodes were extracted through the *Baermann* funnel technique and used in direct contact bioassays.

2.3. Direct Contact Bioassays

Bioassays were performed as previously described by [12], using flat-bottom 96-well microtiter plates (Carl Roth GmbH & Co. KG, Karlsruhe, Germany). In each well, 100 $\pm$ 10 mixed life-stage PWNs in 95 μL aqueous solution were added to 5 μL of a 20 mg compound/mL of methanol solution to obtain a final concentration of 1 mg/mL. The contents were thoroughly mixed, and the plates were covered with plastic film and aluminum foil and maintained at 25 ± 1 °C in an orbital shaker at 90 r.p.m. The control bioassays were performed with 5 µL of methanol. After 24 h, the nematodes were observed using an inverted microscope (Diaphot, Nikon, Japan (40×)). Complete mortality was considered only if every PWN showed no movement, even after mechanical stimulation. Each compound was bioassayed 10 times. Positive results were considered for compounds showing complete mortality in every bioassay.

3. Results and Discussion

The tested compounds, 11 cyclic and 14 linear molecules, consisted of alcohols (four cyclic and six linear), aldehydes (four linear), carboxylic acids (three linear), ethers (two cyclic), hydrocarbons (four cyclic), and ketones (one cyclic and one linear). After 24 h, complet mortality was obtained for 3 cyclic and 11 linear compounds (Table 1). The linear alcohols 1-decanol, 1-dodecanol, geraniol, linalool, 1-tridecanol, 1-undecanol, and the cyclic menthol showed complete mortality while the cyclic (−)-terpinen-4-ol, (+)-terpinen-4-ol, and α-terpineol were unable to kill all of the PWNs at 1 mg/mL after 24 h.

Table 1. PWN mortality after 24 h of direct contact with 25 cyclic and linear compounds (1 mg/mL).

Complete PWN Mortality		Partial or No PWN Mortality
	Alcohols	
menthol geraniol linalool 1-decanol 1-dodecanol 1-undecanol 1-tridecanol		(−)-terpinen-4-ol (+)-terpinen-4-ol α-terpineol
	Aldehydes	
S(−)-citronellal citral		*R*(+)-citronellal dodecanal
	Carboxylic acids	
undecanoic acid *n*-decanoic acid		dodecanoic acid
	Ethers	
trans-anethole		eugenol
	Hydrocarbons	
p-cymene		(−)-*trans*-caryophyllene α-pinene γ-terpinene
	Ketones	
2-undecanone		*R*(+)-pulegone

Menthol, geraniol, and linalool are monoterpenes known to have considerable biological activities. As components of EOs, they have previously shown activity against the PWN [12,13]. In a study that screened the activity of 26 monoterpenoids commonly found in EOs against the PWN, menthol and geraniol showed very high activities (half maximal lethal concentrations (LC_{50}) of 0.985 and 0.540 mg/mL, respectively) while linalool showed a weak activity [14]. The authors were able to establish that primary alcohols had a stronger anti-PWN activity than secondary and tertiary alcohols. In the present study, linalool showed a high activity, which may suggest the existence of variability in PWN response to nematicidal monoterpenes.

The aldehydes citral (a mixture of the geometric isomers geranial, the *trans*-isomer, and neral, the *cis*-isomer) and *S*(−)-citronellal showed complete PWN mortality, while its isomer *R*(+)-citronellal and the linear dodecanal were unable to achieve complete mortality. Compound isomerism is an extremely important parameter in biological activity and often only one isomer is capable of inducing activity. As a mixture, the isomers that compose citral may have different degrees of influence on the PWN and should be investigated further.

Undecanoic and *n*-decanoic acids, C11 and C10 carboxylic acids, respectively, showed complete PWN mortality, while the C12 dodecanoic acid did not. In a study that screened the activity of C6 to C14 alkanoic acids on the PWN, this same break in activity was also reported [15]. The authors detected very high mortalities for C7 to C11 alkanoic acids, but the C12 compound showed only minimal mortality at 0.5 mg/mL. The specificity of this cut-off effect should be explored for effective pest control of the PWN.

Complete mortality was also seen for the ether *trans*-anethole, the hydrocarbon *p*-cymene, and the ketone 2-undecanone. In previous works, EOs rich in these compounds were also seen to possess important anti-PWN activities [11–13].

The activity of the tested linear and cyclic compounds allowed the determination of preliminary guidelines for anti-PWN strength, namely, that specific functional groups appear to be more successful, that compound isomerism can dictate PWN mortality, and that the length of the linear carbon chain significantly influences the activity of linear aliphatic alkanoic acids.

The chemically guided screening of compounds and EOs may contribute to the discovery of novel active molecules as well as important mechanisms of action to be used for an outline of successful sustainable pest management strategies.

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