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# Nematicidal Activity of Oxygen-Containing Aliphatic Compounds on *Bursaphelenchus xylophilus*, *B. mucronatus* and *B. fraudulentus* †

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**Abstract:** The pinewood nematode (PWN), *Bursaphelenchus xylophilus*, the causal agent of pine wilt disease (PWD), is a threat to *Pinus* forests in Asia and Europe. *Bursaphelenchus mucronatus* and *B. fraudulentus* are closely related non-pathogenic pine wood nematodes. In the present work, four medium chain aliphatic alcohols (C10 to C13) were evaluated in direct contact bioassays against *B. xylophilus*, *B. mucronatus* and *B. fraudulentus*. The compounds showed high nematicidal activity against the species tested. The lowest values for half maximal effective concentrations ($EC_{50}$) were determined for *B. xylophilus* and *B. fraudulentus*, suggesting a higher sensibility to these compounds. Further bioassays will include compounds with different chain lengths and functional groups to explore the diversity in the activity of oxygen-containing aliphatic compounds for a more targeted sustainable control strategy for the PWN.

**Keywords:** biopesticides; Bursaphelenchus fraudulentus; Bursaphelenchus mucronatus; Bursaphelenchus xylophilus; nematicides; pinewood nematode

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## 1. Introduction

The pinewood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner & Bührer) Nickle, is the causal agent of pine wilt disease (PWD). This migratory endoparasite is believed to be indigenous to North America, where it causes PWD to non-endemic pine species. In the beginning of the 20th century, the PWN spread to Japan and later to China, Korea, and more recently to Portugal, in 1999, and is temporarily contained at border Spanish pine forests [1–6]. Since its introduction, European authorities have established phytosanitary plans to control the progression of the PWN by heavily restricting and regulating wood transportation and export, eliminating symptomatic trees, creating buffer zones adjacent to affected areas, and controlling vector beetle populations, longhorn beetles *Monochamus* spp. (Coleoptera; Cerambycidae). However, these measures had only a limited impact on PWN spread, and new pockets of infection continue to be detected by

the official national monitoring programs. Thus, PWD continues to be considered a serious threat to the strongly susceptible pine forests of Europe and Asia [7–9].

*Bursaphelenchus* genus (Nematoda: Aphelenchidae) is mostly mycetophagous, yet some can also feed on plants (wood), e.g., *B. cocophilus*, pathogen of coconuts and palms; and the PWN, parasitizing pines. *Bursaphelenchus mucronatus* Mamiya & Enda and *B. fraudulentus* Rühm are taxonomically closely related to the PWN, sharing many morphological traits and tree hosts. Contrary to the PWN, these are considered non-pathogenic [10]. *Bursaphelenchus xylophilus* and *B. mucronatus* nematodes feed on epithelial cells and reproduce in the resin canals of pine trees or feed on fungi present in their hosts [11], however only the PWN commonly increases its population uncontrollably, damaging the tree. Conversely, *B. fraudulentus* is mycetophagous and does not cause harm to the host trees, being even considered a biological control agent of *Armillaria* spp., the causal agent of Armillaria root rot in a multitude of woody hosts [12].

Currently, pest management in agriculture and forestry is still heavily dependent on the application of synthetic pesticides. For the control of the PWN, highly damaging insecticides can be used to reduce the insect vector's populations, that transmits the PWN; or strong nematicides, of synthetic or hemisynthetic sources, are used by direct application through trunk injection of infected or endangered pines. The commonly used nematicides include morantel tartrate, levamisole hydrochloride, mesulfenfos, emamectin benzoate or nemadectin [13]. Despite their high effectiveness in eliminating the PWN, synthetic pesticides are non-specific and can effectively target numerous beneficial microorganisms, and negatively influence food chains, ultimately damaging several other organisms, e.g., bees, birds, plants, and even humans. In general terms, downstream effects result in the loss of endemic biodiversity in forest ecosystems, potentiate acquired resistance on the target pests, and bioaccumulation of residues above the safety limits in human food chains [14–17].

The establishment of more sustainable management strategies is thus essential to minimize the effects of pesticide misuse, by developing novel biopesticides and biocontrol agents [18–22]. In recent years, a growing demand for "greener" pesticides has fueled the search for novel biopesticides. These Plant Protection Products (PPPs) are regarded as a more sustainable control since they can allow specific activities against the target pest. Moreover, PPPs have shown fewer adverse effects on the environment and are reported to be less concerning for human health when compared to conventional pesticides [23,24]. Against the PWN, essential oils (EOs) have been screened with relative success, and several of these complex mixtures of volatiles have shown higher activities than common use nematicides [15]. Plants synthesize volatile organic compounds (VOCs) with several important environmental roles, particularly when subjected to biotic stress. VOCs function as chemical signals between neighboring plants and/or other organisms and are essential in plant defense mechanisms [25–27]. For example, under the attack of phytophagous parasites, small and medium chain aliphatic aldehydes and alcohols can have a protective role [28], and their nematicidal strength against the PWN has begun to be screened [29].

In the present study, the nematicidal activity of four medium chain aliphatic alcohols (C10 to C13) was investigated against *B. xylophilus*, but also against its closest relatives *B. mucronatus*, and *B. fraudulentus*. Their chemical structures were compared considering the nematicidal activity to uncover and explore potentially differential susceptibilities to these compounds, for the development of targeted nematotoxic compounds.

## 2. Materials and Methods

### *2.1. Nematicidal Chemicals*

The pure analytical-grade standards 1-decanol (purity ≥98%), 1-undecanol (purity 99%), 1-dodecanol (purity ≥98%), and 1-tridecanol (purity 97%) were acquired from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade methanol was acquired from Fisher Chemicals (Hampton, NH, USA).

*2.2. Bursaphelenchus spp. In Vitro Culture*

Nematodes were obtained in vitro by culturing on axenically grown fungal mats of a non-sporulating strain of *Botrytis cinerea* (de Bary) Whetzel. The fungus was grown 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), at 25 ± 1 °C, for 7 to 10 days, until the surface of the cereal was fully colonized. Following, ca. 1000–2000 surface sterilized [30] *B. xylophilus* [Portuguese isolate BX013.003 (N 39°43′338″, W 9°01′557″)], *B. mucronatus* (Chinese isolate BmCh3FJ12), or *B. fraudulentus* (German isolate BfG1DE10W) from the reference collection maintained at the Plant Nematology Laboratory of the National Institute for Agrarian and Veterinary Research (INIAV, I.P.) at Oeiras, Portugal, were added to these cultures and kept at 25 ± 1 °C, in darkness, for 7 to 10 days, until the fungal mat was consumed, and then extracted through the modified Baermann funnel technique [31]. Aqueous suspensions of each nematode species were used for the direct contact assays, for further inoculations or stored at 4 °C. The assessment of nematode numbers and/or survival rates was performed using an Olympus SZX12 (Tokyo, Japan) stereomicroscope. For nematode morphological traits, at least 10 specimens of each species were heat-killed and placed in a drop of water on a glass slide and observed using an Olympus BX-51 bright field light microscope (Hamburg, Germany) and photographed with an Olympus DP10 digital camera.

*2.3. Toxicological Characterization of Nematicidal Compounds*

Direct contact bioassays were employed to determine the activity of the nematicidal compounds, by assessing compound mortality at different concentrations. Stock solutions of 20 mg of compound per mL of methanol were prepared and screened for activity in flat-bottom 96-well microtiter plates (Carl Roth GmbH & Co. KG, Karlsruhe, Germany). In each well, 100 ± 10 mixed life-stage nematodes in 95 µL aqueous suspension were added to 5 µL of stock solution, for a final concentration of 1 µL/mL. Control assays were performed with 5 µL of pure methanol. 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 compounds, covered with aluminum foil to establish complete darkness and maintained at 25 ± 1 °C in an orbital shaker at 50 r.p.m., for 24 h. Following, dead and live nematodes were counted under a stereomicroscope. Nematodes 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 compound concentrations were screened using the same procedure. Stock solutions for 0.500, 0.250, 0.125, 0.063, 0.031, 0.016, 0.008 and 0.004 µL/mL were obtained by serial dilutions with a dilution factor of two.

*2.4. Data Treatment and Statistical Analysis*

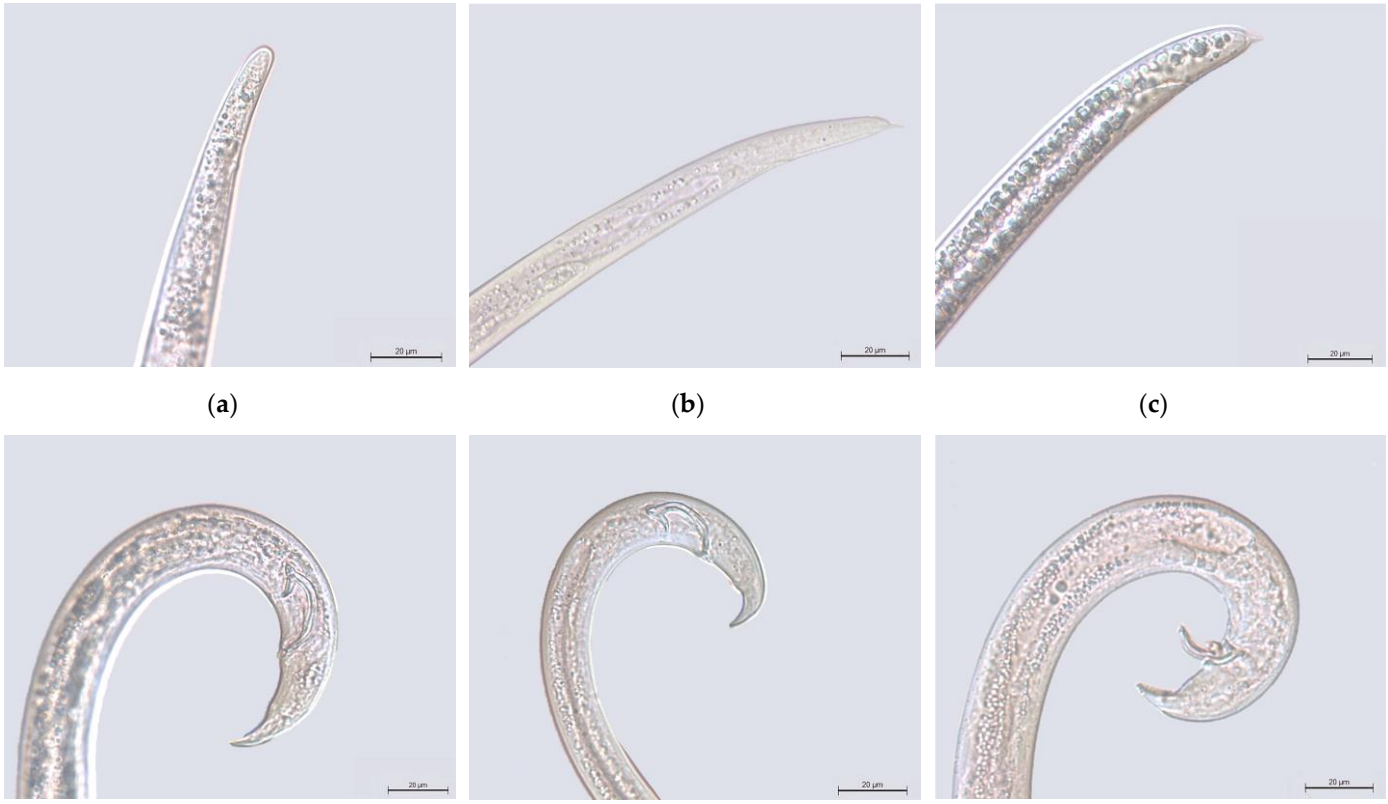
Nematode mortality percentages were determined according to the formula, mortality% = 100 × [(dead nematodes)/(live + dead nematodes)]. The corrected mortality percentages were determined for mortalities below 100%, using the formula, corrected mortality% = 100 × [(mortality% in treatment − mortality% in control)/(100 − mortality% in control)]. The toxicological strength of each compound was evaluated according to the classification established by Kong et al. [32] 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 $EC_{50}$ values 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 compound concentration values and fitting a dose-response log-logistic equation, $y = C + (D - C)/1 + \exp\{b [\log(x) - \log(EC_{50})]\}$ [33], where C and D are the lower- and the upper limit of the sigmoidal dose-response curve, respectively, b is the slope and $EC_{50}$ is

the compound concentration which induces a response halfway between the lower- and the upper limits.

## 3. Results

### *3.1. Bursaphelenchus spp. Morphology*

*Bursaphelenchus xylophilus*, *B. mucronatus* and *B. fraudulentus* are commonly grouped in a unit designated as the "pinewood nematode complex", within the xylophilus group (that groups nine morphologically similar *Bursaphelenchus* species). Within the "pinewood nematode complex" these three species can be identified by their different female tail morphology and more minutely by the shape of the spicules in the male tail (Figure 1). Under the microscope, amongst other features, *B. mucronatus* females showed a round mucronated tail, *B. fraudulentus* a pointed tail, and *B. xylophilus* a rounded tail, that may be mucronated (Figure 1).



(**a**) (**b**) (**c**)

(**d**) (**e**) (**f**)

**Figure 1.** Light microscopy micrographs of the tails of female (**a**–**c**) and male (**d**–**f**) *Bursaphelenchus xylophilus* (**a**,**d**), *B. mucronatus* (**b**,**e**) and *B. fraudulentus* (**c**,**f**). Bar = 20 µm.

### *3.2. Toxicity of Oxygen-Containing Aliphatic Compounds*

The four medium chain aliphatic alcohols screened showed high nematicidal activities (Table 1). Among the tested compounds, 1-dodecanol showed the lowest $EC_{50}$ values for *B. xylophilus* or *B. fraudulentus*, while for *B. mucronatus*, 1-tridecanol had the highest effect. For *B. xylophilus* or *B. fraudulentus*, 1-dodecanol was followed by 1-tridecanol, 1-undecanol and 1-decanol, respectively, in terms of activity. For *B. mucronatus*, 1-tridecanol was followed by 1-undecanol, 1-dodecanol and 1-decanol, respectively, in terms of activity. The results suggest that the nematoxicity of these compounds against *B. xylophilus* and *B. fraudulentus* may have some homology.

**Table 1.** Nematicidal activity of 1-decanol, 1-undecanol, 1-dodecanol and 1-tridecanol against *Bursaphelenchus* spp.

| Compounds | *Bursaphelenchus* spp. | $EC_{50\ 24h}$ (mg/mL) | $R^2$ of Fit |
|---|---|---|---|
| $C_{10}H_{22}O$ | *B. xylophilus* | 0.045 | 0.98 |
| | *B. mucronatus* | 0.082 | 0.96 |
| | *B. fraudulentus* | 0.042 | 0.99 |
| $C_{11}H_{24}O$ | *B. xylophilus* | 0.014 | 0.99 |
| | *B. mucronatus* | 0.022 | 0.98 |
| | *B. fraudulentus* | 0.020 | 0.99 |
| $C_{12}H_{26}O$ | *B. xylophilus* | 0.009 | 0.99 |
| | *B. mucronatus* | 0.023 | 0.99 |
| | *B. fraudulentus* | 0.011 | 0.99 |
| $C_{13}H_{28}O$ | *B. xylophilus* | 0.012 | 0.99 |
| | *B. mucronatus* | 0.019 | 0.98 |
| | *B. fraudulentus* | 0.012 | 0.99 |

## 4. Discussion

Oxygen-containing aliphatic compounds are known antagonists of the PWN. Seo et al. [34] uncovered several structure-activity relationships between medium carbon chain length alcohols, aldehydes and carboxylic acids and mortality on the PWN. Depending on compound chain length and functional group, activity towards the PWN could be almost completely lost after a certain number of C in the aliphatic compound. For medium carbon chain alcohols, this cut-off effect occurred at C12 chain length, *n*-dodecanol, with *n*-undecanol showing the strongest activity. This effect contradicts the Meyer-Overton rule, in which n-alkanols with increasing chain length are expected to induce a constant increase in potency, and can be explained by the increase in molecule volume exceeding that of its putative protein binding site [35,36]. So, this activity loss can be related to the oxygen-containing molecule's suitability to its target receptor. In the present work, this effect was not detected, however, 1-undecanol was identified as a highly nematicidal n-alkanol against the PWN, similarly, to some extent, to the study of Seo et al. [34].

To the best of our knowledge, this is the first time that C10 to C13 n-alkanols were screened against *B. mucronatus* and *B. fraudulentus*. Although some tendencies were identified that distinguished the response of these species to the tested compounds, no substantial difference could be ascertained.

In future research, transcriptomics and/or metabolomics approaches will be used to identify the molecular mechanisms of the activity of these compounds on the PWN. Also, different oxygen-containing compounds, with similar carbon chain lengths, will be tested to determine possible analogous nematotoxic effects, and screen for possible variations in susceptibility among *Bursaphelenchus* spp. Finally, evaluating the phytotoxicity of these compounds on the host tree [37] is crucial before a greener nematicide against PWN can be formulated.

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