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Bioactivity against *Bursaphelenchus xylophilus*: Nematotoxics from essential oils, essential oils fractions and decoction waters

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ABSTRACT

The Portuguese pine forest has become dangerously threatened by pine wilt disease (PWD), caused by the pinewood nematode (PWN), *Bursaphelenchus xylophilus*. Synthetic chemicals are the most common pesticides used against phytoparasitic nematodes but its use has negative ecological impacts. Phytochemicals may prove to be environmentally friendly alternatives. Essential oils (EOs) and decoction waters, isolated from 84 plant samples, were tested against *B. xylophilus*, in direct contact assays. Some successful EOs were fractionated and the fractions containing hydrocarbons or oxygen-containing molecules tested separately. Twenty EOs showed corrected mortalities ⩾96% at 2 μL/mL. These were further tested at lower concentrations. *Ruta graveolens*, *Satureja montana* and *Thymbra capitata* EOs showed lethal concentrations (LC_{100}) < 0.4 μL/mL. Oxygen-containing molecules fractions showing corrected mortality ⩾96% did not always show LC_{100} values similar to the corresponding EOs, suggesting additive and/or synergistic relationships among fractions. Nine decoction waters (remaining hydrodistillation waters) revealed 100% mortality at a minimum concentration of 12.5 μL/mL. *R. graveolens*, *S. montana* and *T. capitata* EOs are potential environmentally friendly alternatives for *B. xylophilus* control given their high nematotoxic properties. Nematotoxic activity of an EO should be taken in its entirety, as its different components may contribute, in distinct ways, to the overall EO activity.

1. Introduction

The pinewood nematode (PWN), *Bursaphelenchus xylophilus* L. is a highly pathogenic plant parasite that infects, mainly, *Pinus* species, and causing pine wilt disease (PWD) (Mota and Vieira, 2008). In 1999, Portugal became its entry point to the European pine forests (Mota et al., 1999). Since then, this phytoparasite has been progressing through the country, having been found in Madeira Island, in 2010 (Fonseca et al., 2012), and in Spain, in 2011 (Abelleira et al., 2011). It has been classified as an A2 type quarantine pest by the European Plant Protection Organization (EPPO, 2012). In Portugal, *Pinus pinaster* Aiton, maritime pine, is the susceptible species.

Phytoparasitic nematode control is very complex, generally relying upon synthetic chemicals, as broad-spectrum nematicides which are extremely damaging (Chitwood, 2003). The PWN is commonly controlled by controlling the insect vector through aerial application of synthetic insecticides, by fumigation of infected trees, pine tree-free strips, use of vector natural enemies, or by controlling the nematode through trunk injection of anti-nematicidal compounds such as abamectins (Lee et al., 2003; Takai et al., 2003; Mota and Vieira, 2008). Nevertheless, the use of synthetic pesticides is associated with environment pollution and undesirable influences on human health or against non-target organisms (Zhao, 2008). The use of plant derived natural products for pest control is not recent, but has gained ground in modern pest management due to growing ecological concerns about the use of synthetic pesticides, and their consequent phasing out in the market and banning. Essential oils (EOs) are complex mixtures of volatiles, mainly products of plant secondary metabolism. Commonly, they are comprised of terpenes, mono- and sesquiterpenes, and phenolic compounds, such as phenylpropanoids, although other groups of compounds can also occur in relevant amounts. They are generally biodegradable, have low toxicity to mammals and do not accumulate in the environment (Figueiredo et al., 2008). The biological activities of EOs can frequently exceed the sum of their single constituent's activities, due to synergy. As complex mixtures, EOs may display several biological activities which makes them desirable biopesticides, being able to control not just the targeted pest but also opportunistic species and resistant strains. This is of particular interest in phytoparasitic nematode

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control since complex disease symptoms are also commonly associated with accompanying pathogenic microbiota (Back et al., 2002; Vicente et al., 2011). Several EOs, such as those of *Cinnamomum zeylanicum* (Kong et al., 2006); *Boswellia carterii, Paeonia moutan*, *Perilla frutescens*, *Schizonepeta tenuifolia* (Choi et al., 2007a); *Thymus vulgaris* (Kong et al., 2007); *Litsea cubeba, Pimenta dioica*, *Trachyspermum ammi* (Park et al., 2007); *Coriandrum sativum* and *Liquidambar orientalis* (Kim et al., 2008) have revealed strong activities against *B. xylophilus*.

Containing this pest is of the utmost importance for the European pine forest safeguard. With the purpose of finding alternative means of controlling this phytoparasite, without further destabilizing the forest ecosystem, the present study was aimed at screening several plant taxa, some of which from the Portuguese flora, for natural phytochemicals with PWN nematotoxic properties. In view of this, (a) essential oils and decoction waters (remaining hydrodistillation water), isolated from 84 samples, were evaluated through direct contact assays, and (b) fractions containing hydrocarbons or oxygen-containing molecules from the most successful essential oils were further assessed, separately, against the PWN.

2. Results and discussion

2.1. Composition of essential oils and fractions containing hydrocarbons or oxygen-containing molecules

The essential oils isolated from 59 plant species (13 families), revealed yields that ranged from <0.05% to 9% (v/w). The highest yields were obtained from *Syzygium aromaticum* (9%), *Eucalyptus radiata* (6%), *E. dives* (3%) and *Cymbopogon citratus* (3%) (Table 1).

All 84 EOs isolated were fully chemically characterized (detailed relative amounts of all the identified components are listed in the Supplementary Table, ST), although Table 1 reports only their main components (⩾10%). For some species, duly identified in Table 1, the EO composition was previously reported (Barbosa et al., 2010, 2012; Faria et al., 2011). Some species can show several EO chemotypes, such as *Thymus caespititius* carvacrol, thymol or α-terpineol-rich chemotypes (Table 1), which may provide different biological properties. These chemotypes were separately assessed for their nematotoxic activity.

Some EOs were further chosen for fractionation, aiming to assess the independent input of the EO fractions containing hydrocarbons or oxygen-containing molecules against PWN. The main components (⩾10%) in each separate EO fraction are featured in Table 2 (detailed relative amounts are listed in the Supplementary Table, ST).

The high chemical diversity of the analyzed EOs, and of the corresponding fractions containing hydrocarbons or oxygen-containing molecules, was supported by the agglomerative cluster analysis based on their full chemical composition (Fig. 1). Despite this chemical diversity, the analyzed EOs were predominantly terpene-rich, although other chemical groups also achieved important percentages, such as in *Ruta graveolens* EO, dominated by the methyl nonyl ketone, 2-undecanone, or in *S. aromaticum* and in *Foeniculum vulgare* EOs, rich in the phenylpropanoids eugenol and *trans*-anethole, respectively (Table 1).

Cluster analysis showed two main uncorrelated clusters (S_{corr} < 0.2) (Fig. 1). Cluster I with only five out of the 94 samples analyzed, included EOs characterized by high percentages of specific compounds, usually not present in such high amounts in the other EOs. This was the case of 2-undecanone (91–94%) in *R. graveolens*, eugenol (93%) in *S. aromaticum* and 4aα, 7α, 7aα-nepetalactone (89%) in *Nepeta cataria* EOs.

Cluster II grouped the remaining EOs, and related fractions containing hydrocarbons or oxygen-containing molecules, representing 95% of the samples analyzed. Having been sub-divided into several sub-clusters, some of which also highly uncorrelated (S_{corr} < 0.2), the essential oils from this cluster were predominately terpene-rich.

2.2. PWN mortality and LC_{100} assessment

2.2.1. Essential oils

Essential oils were tested for activity against *B. xylophilus* through direct contact bioassays. Assays, performed with ultrapure water, showed an average mortality of 8 ± 4%, considered to be natural mortality. The mortality due to methanol, used as the EO solvent, was 10 ± 6%, which can be considered negligible, when compared to natural mortality.

At the highest concentration, 2 μL/mL, some of the ineffective EOs or EO fractions assessed, with corrected mortalities <40% showed dominant proportions of e.g. the monoterpenes limonene, α-pinene, 1,8-cineole, camphor, terpinolene, or sabinene (Table 1, Table 2 and Fig. 1).

The most active EOs, or EO fractions, showing corrected mortalities ⩾96% at 2 μL/mL, occurred both in cluster I and in sub-clusters IIa, IIc, IIe, and IIq from cluster II (Table 1, Table 2 and Fig. 1). Within cluster I, *R. graveolens* (91–94% 2-undecanone) and *S. aromaticum* (93% eugenol) EOs were highly effective (100% corrected mortality).

Sub-cluster IIa included EOs, or fractions with oxygen-containing molecules, with ⩾93% corrected mortality, which were chemically characterized by dominant contents of carvacrol (35–96%), *p*-cymene (traces-20%), γ-terpinene (traces-18%) and thymol (not detected-15%). Only three out of the seven samples from sub-cluster IIc showed corrected mortalities ⩾96%. These differed from the remaining members of the same cluster by showing high amounts of thymol (12–23%) and carvacrol (6–15%). Sub-cluster IIe integrated EOs rich in thymol (42–50%), *p*-cymene (14–20%), thymol acetate (traces-15%) and γ-terpinene (6–12%), which showed corrected mortalities ⩾99%. *Th. caespititius* chemotypes rich in carvacrol and/or thymol showed high nematotoxic activities while α-terpineol-rich chemotypes showed corrected mortalities ⩽60%. The occurence of chemotypes must be taken into account when choosing a nematotoxic EO bearing-species, since EO particular chemotype proved to be determinant in nematotoxic activity.

High PWN mortality (100%) was also observed with *C. citratus* EOs, and the related oxygen-containing molecules fraction. These EOs grouped in sub-cluster IIq and were dominated by geranial (23–45%), β-myrcene (traces-38%), neral (20–36%), and geraniol (1–18%).

Three other EOs showed corrected mortalities ⩾96%, although this toxicity was not shown by the EOs of other members of the same sub-clusters. *Mentha cervina* (96% corrected mortality) characterized by high contents of pulegone (80%), *E. citriodora* (97% corrected mortality) citronellal (36%), isopulegol (13%), citronellol (12%) and 1,8-cineole (11%) rich, and *M. arvensis* EOs (100% corrected mortality), dominated by piperitenone oxide (56%).

Despite the chemical diversity of the assessed EOs, it is noteworthy that sub-clusters IIa, IIc and IIe gathered EOs that had in common the presence of carvacrol, thymol, *p*-cymene and/or γ-terpinene. Separately or combined, these compounds can be partially responsible for each EOs nematotoxic properties.

EOs that attained nematotoxic activity ⩾96%, namely those of *C. citratus* 1, 2, and 3, *E. citriodora*, *M. arvensis*, *M. cervina* 1, *Origanum vulgare* subsp. *virens*, *Origanum vulgare* 2, *R. graveolens* 1, 2 and 3, *Satureja montana* 1 and 2, *S. aromaticum*, *T. capitata*, *Th. caespititius* 2, 5 and 7 (carvacrol and/or thymol-rich), *Th. vulgaris* and *Th. zygis* were further tested at lower concentrations (Table 1).