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Nematotoxic and phytotoxic activity of *Satureja montana* and *Ruta graveolens* essential oils on *Pinus pinaster* shoot cultures and *P. pinaster* with *Bursaphelenchus xylophilus in vitro* co-cultures

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ABSTRACT

Satureja montana (winter savory) and *Ruta graveolens* (rue) nematotoxic essential oils (EOs) (0.5 μl EO/ml culture medium) were assessed for the first time in *Pinus pinaster in vitro* shoot cultures (Ppi) and *P. pinaster* shoots with *Bursaphelenchus xylophilus* co-cultures (PpiBx). The EOs nematotoxic effect was evaluated on *B. xylophilus* population density in PpiBx co-cultures and the phytotoxic activity to the host was assessed by evaluating relative water content and volatile profiles both on Ppi cultures and on PpiBx co-cultures. Carvacrol-rich *S. montana* EO showed phytotoxicity, by inducing shoot chlorosis and drooping, whereas no major morphological changes were detected on *R. graveolens* EO-added Ppi and PpiBx *in vitro* cultures. Both EOs maintained the nematotoxicity during all experimental phases. *R. graveolens* EO proved to be an effective PWN antagonist to be further evaluated for pine wilt disease control, given its less phytotoxicity while maintaining nematoxicity.

1. Introduction

The pine wilt disease (PWD) pathogenic agent, pinewood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner and Buhrer) Nickle was classified as an A2 type quarantine pest by the European and Mediterranean Plant Protection Organization (EPPO, 2012). It is commonly controlled by controlling the insect vector or the nematode through the use of insecticides or nematicides, yet these are associated with environmental pollution and undesirable influences on human health or non-target organisms (Mota and Vieira, 2008). As potential phytochemical alternatives, essential oils (EOs) show low toxicity to mammals, are biodegradable, and do not accumulate in the environment (Figueiredo et al., 2008). EOs PWN nematoxicity has been extensively researched, mainly by means of direct contact bioassays (Barbosa et al., 2010, 2012; Andrés et al., 2012). From screening 59 plant species EOs, Faria et al. (2013) identified highly PWN nematotoxic *Satureja montana* L. (winter savory) and *Ruta graveolens* L. (rue) EOs, with $LC_{100/24h}$ <0.4 μl/ml. EO fractions were also evaluated revealing, in general, oxygen-containing molecules fractions with higher activities than hydrocarbon molecules fractions, the later fractions contributing, on a plant specific manner, to the overall EO nematoxicity. Nevertheless, direct contact bioassays do not take into account toxicity for the host or the plant's capability to biotransform the nematotoxic active substances. On the other hand, greenhouse and field assays are very laborious and, many times, environment-dependent. *In vitro* co-cultures constitute a laboratory model, allowing analysis of metabolomic interplay between plant and nematode at various levels, namely to follow directly the host and nematode response to phytonematotoxics application, at various stages of infection (Faria et al., 2014, 2015).

The present work is the first report on the use of plant with nematode co-cultures as models for screening effective nematotoxic EOs. Using previously established (Faria et al., 2015), *Pinus pinaster in vitro* shoot cultures and *P. pinaster* shoots with PWN co-cultures, the present study aims at evaluating the nematotoxic and phytotoxic activities of *S. montana* and *R. graveolens* EOs by assess-

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ing the nematode density in the co-cultures, the relative water content and volatile profiles of both *in vitro* cultures types.

2. Material and methods

2.1. Pine shoot cultures and pine shoots with nematode co-cultures

P. pinaster in vitro shoot cultures (Ppi) and *P. pinaster* shoots with PWN co-cultures (PpiBx) were established as detailed in Faria et al. (2015), and maintained in Combiness® (Belgium) microboxes [8 cm base diameter *per* 6 cm height, with the green filter (XXL+) on the lid, to facilitate air exchange], containing 20 ml SH solid medium (Schenk and Hildebrandt, 1972) with 30 g/l sucrose, at 24 ± 1 °C under a 16 h light photoperiod [cool fluorescent lamps (32 μE/m^2/s)]. Routine subculture was performed every four weeks.

2.2. R. graveolens *and* S. montana *essential oils nematotoxic and phytotoxic activity*

The effect of *R. graveolens* (Rg) and *S. montana* (Sm) EOs, at 0.5 μl/ml, was assessed both on (a) Ppi cultures and (b) PpiBx co-cultures, Fig. 1. The EOs nematotoxic effect was evaluated on *B. xylophilus* population density in PpiBx co-cultures, and the phytotoxic activity to the host was assessed both on Ppi cultures and on PpiBx co-cultures. For Ppi cultures, after 7 days of growth in regular SH solid medium (Phase 1), the cultures were transferred, for 7 days, to SH solid medium without, or with EO (Phase 2), and then again transferred to regular SH solid medium, for 7 days (Phase 3, recovery time), Fig. 1. PpiBx co-cultures were established by adding a 100 μl suspension (250 ± 50 PWNs) into a small hole made in the culture medium into which the cut end of *P. pinaster* shoot (Ppi) was inserted, and maintained for 7 days for nematode infection (Phase 1). PpiBx co-cultures Phase 2 and 3 were run as detailed for Ppi cultures. Sampling was performed at the beginning (time 0), at days 1, 2 and 7 of Phase 2 and at the end of recovery time (day 7 of Phase 3). In both cases, control cultures (without addition of EO) were maintained simultaneously, under the same growth conditions. To prepare SH solid medium with EO, a solution of EO in methanol (1:1, v/v) was added, in asepsis with agitation, to previously autoclaved medium (121 °C for 15 min) after reaching room temperature, in such a way as to give 0.5 μl EO/ml culture medium. EO evaporation and decomposition control experiments were performed by adding the same amount of EO to microboxes containing only regular culture medium, and keeping them in the same conditions as the experimental ones. Two independent experiments were separately run and 4 replicates *per* experimental time-point were used in each experiment. The data shown were calculated as mean values of all experiments. All statistical analyses were performed using Microsoft Excel 2013. The effect of adding *S. montana* and *R. graveolens* EOs to Ppi cultures and PpiBx co-cultures was followed by measuring relative water content (RWC), nematode density in the culture medium and *in vitro* volatiles production as in Faria et al. (2015).

2.3. R. graveolens *and* S. montana *essential oils*

Satureja montana and *R. graveolens* EOs were isolated from the dried aerial parts sold in local herbal shops and the chemical profiling was performed as detailed in Faria et al. (2013). Volatiles were analyzed by gas chromatography (GC), for component quantification, and gas chromatography coupled to mass spectrometry (GC–MS) for component identification.

Gas chromatographic analyses were performed using a Perkin Elmer Autosystem XL gas chromatograph (PerkinElmer, Shelton, CT, USA) equipped with two flame ionization detectors (FIDs), a data handling system, and a vaporizing injector port into which two columns of different polarities were installed: a DB-1 fused-silica column (30 m × 0.25 mm i.d., film thickness 0.25 μm; J & W Scientific Inc., Rancho Cordova, CA, USA) and a DB-17HT fused-silica column (30 m × 0.25 mm i.d., film thickness 0.15 μm; J & W Scientific Inc., Rancho Cordova, CA, USA). Oven temperature was programmed to increase from 45 to 175 °C, at 3 °C/min increments, then up to 300 °C at 15 °C/min increments, and finally held isothermal for 10 min. Gas chromatographic settings were as follows: injector and detectors temperatures, 280 °C and 300 °C, respectively; carrier gas, hydrogen, adjusted to a linear velocity of 30 cm/s. The samples were injected using a split sampling technique, ratio 1:50. The volume of injection was 0.1 μl of a pentane-oil solution (1:1). The percentage composition of the oils was computed by the normalization method from the GC peak areas, calculated as a mean value of two injections from each volatile oil, without response factors.

The GC–MS unit consisted of a PerkinElmer Autosystem XL gas chromatograph, equipped with DB-1 fused-silica column (30 m × 0.25 mm i.d., film thickness 0.25 μm; J & W Scientific, Inc., Rancho Cordova, CA, USA) interfaced with PerkinElmer Turbomass mass spectrometer (software version 4.1, Perkin Elmer). GC–MS settings were as follows: injector and oven temperatures were as above; transfer line temperature, 280 °C; ion source temperature, 220 °C; carrier gas, helium, adjusted to a linear velocity of 30 cm/s; split ratio, 1:40; ionization energy, 70 eV; scan range, 40–300 u; scan time, 1 s. The identity of the components was assigned by comparison of their retention indices relative to C_8–C_{25} *n* alkane indices, and GC–MS spectra from a laboratory made library based upon the analyses of reference oils, laboratory-synthesized components, and commercial available standards.

2.3.1. Isolation and identification of 8-phenyl-2-octanone

Given the presence of an unidentified compound >7%, in *R. graveolens* EO (compound UI E, in Faria et al., 2013 supplementary table), this was further fractionated for compound isolation and identification. Hydrocarbon molecules (HM) and oxygen-containing molecules (OCM) were fractionated according to Faria et al. (2013). EOs were fractionated on a silica gel column by successive elution with distilled *n*-pentane and diethyl ether. The fractions were analyzed by gas chromatography coupled to mass spectrometry (GC–MS) and purified according to Figueiredo et al. (1992). OCM fractions (approximately 0.2 ml each in a total of 1.4 ml) were separated twice on silica gel plates (60 F_{254}, Merck, 20 × 20 cm, 1 mm layer thickness) using *n*-hexane : ethyl acetate (95:5) as eluent. The fraction obtained (7.4 mg) was characterized and identified by NMR spectroscopy and GC–MS spectrometry. 1D NMR (^{1}H, ^{13}C and APT) and 2D NMR (HSQC, HMBC and COSY) spectra were recorded on Bruker spectrometer CXP400 operating at 400.13 MHz (^{1}H) and 100.61 MHz (^{13}C). All chemical shifts are given at ppm and using CD_2Cl_2 signals as reference (δ = 5.30 ppm). Identification was as follows: ^{1}H RMN (400 MHz, CD_2Cl_2) δ 7.29–7.21 (m, 2H, H-11, H-11′), 7.20–7.12 (m, 3H, H-10, H-10′, H-12), 2.59 (t, 2H, *J* = 7.6 Hz, CH_2-8), 2.39 (t, 2H, *J* = 7.6 Hz, CH_2-3), 2.08 (s, 3H, CH_3-1), 1.65–1.48 (m, 4H, CH_2-7 and CH_2-4), 1.36–1.27 (m, 4H, CH_2-5 and CH_2-6); ^{13}C RMN (101 MHz, CD_2Cl_2) δ 209.5 (C-2), 143.5 (C-9), 128.9 (C-10, C-10′), 126.1 (C-12), 128.7 (C-11, C-11′), 44.1 (C-3), 36.4 (C-8), 30.1 (C-1), 32.0, 24.3 (C-7 and C-4), 29.6, 29.5 (C-5 and C-6); MS (EI, 70 eV) *m*/*z* ($C_{14}H_{20}O$): 204 $(M)^+$ (0), 186 $(M\text{-}H_2O)^+$ (30), 130 (10), 105 $(C_6H_5CO)^+$ (20), 104 $(C_7H_7)^+$ (98), 91 $(C_7H_7)^+$ (100), 82 (14), 71 (30), 65 (15), 58 (10), 43 (60).

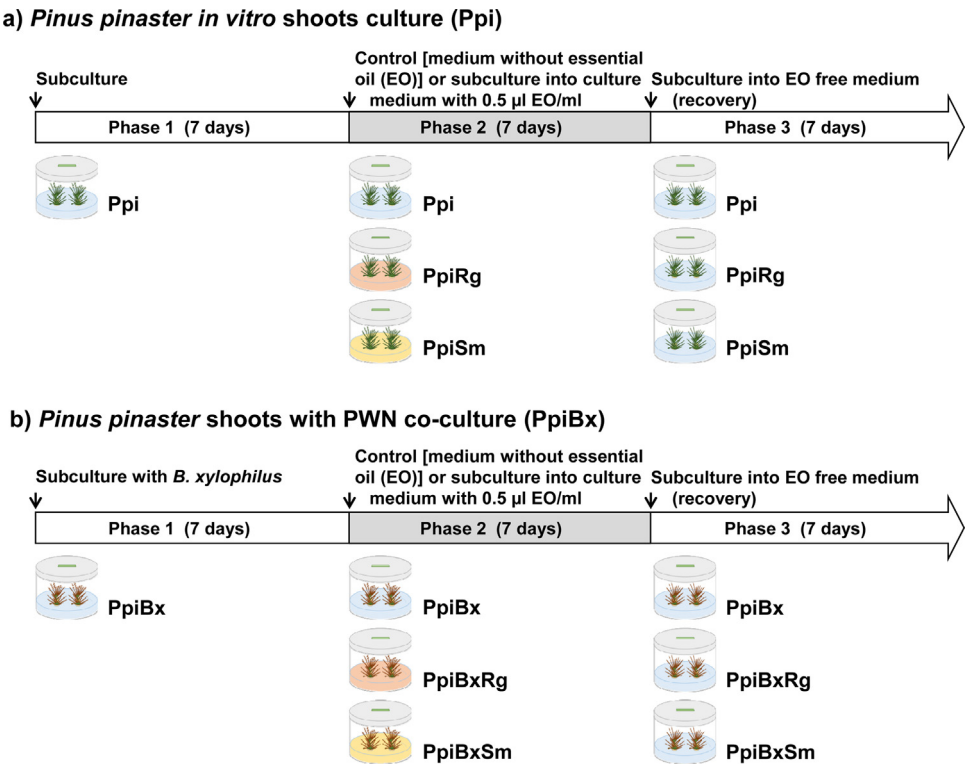

Fig. 1. Schematic representation of experimental design (for details see experimental section). The effect of *Ruta graveolens* (Rg) and *Satureja montana* (Sm) EOs was assessed both on (a) *Pinus pinaster in vitro* shoot cultures (Ppi) and (b) *P. pinaster* shoots with PWN co-cultures (PpiBx).

3. Results and discussion

3.1. *Phytotoxicity to* P. pinaster *shoot cultures*

P. pinaster shoots (Ppi) showed both the typical *in vitro* pine macroscopic aspect, green upright shoots with straight pine needles (Fig. 2a), and similar volatiles composition (Table 1), to that previously reported (Faria et al., 2015). The isolated constitutive volatiles showed no substantial differences in composition throughout experimental time. Table 1 reports the full chemical characterization of the volatiles isolated, in a total of 46 compounds. Volatiles were dominated by β-pinene (35–47%), α-pinene (18–29%), an unidentified compound (UI B Ppi) (3–10%), germacrene D (3-9%) and β-caryophyllene (1–3%).

The phytotoxic effect of adding *S. montana* EO to the culture medium was noticeable by day 2 and the chlorotic and drooping shoots observable at day 7 of Phase 2 (Fig. 2b) were not able to recover after 7 days in EO-free culture medium (Phase 3). Despite this aspect, Ppi shoots RWC range was 80-85% (Fig. 3), probably due to the *in vitro* culture generally fully saturated water content environment.

In addition to *S. montana* EO compounds, and to Ppi shoots constitutive volatiles, also new volatiles were detected in Ppi shoots *S. montana* EO added cultures. Of the main *S. montana* EO components (carvacrol 64% and γ-terpinene 18%) (Faria et al., 2013), only carvacrol was detected in high relative amounts, increasing to the end of the experimental time (67–80%) (Fig. 4a). γ-Terpinene maximum detected was 0.1%, at 0, 1 and 2 days of Phase 2, and remained at trace amounts thereafter. Part of this decrease can be due to volatilization, as this was also detected in controls of EO evaporation and decomposition (carvacrol 95–97% and γ-terpinene 0.4–0.5%). Nevertheless, since *in vitro* cultures are known to have biotransformation capacity (Giri et al., 2001; Faria et al., 2009; Nunes et al., 2009), the conversion of γ-terpinene into a non-volatile glycosylated form, can also partly explain the difference between γ-terpinene percentage in *S. montana* EO and in *S. montana* EO added cultures (PpiSm). Despite the transference of the shoots to EO-free medium, at the end of phase 3 (recovery time), carvacrol was still detected in high relative amount (19%). As a probable phytotoxic effect, and opposite of constitutive volatiles from control Ppi shoots, the PpiSm monoterpenes percentage was lower than that of sesquiterpenes, palmitic acid, or the unidentified compound B. Moreover, two new volatiles were detected in PpiSm shoots, 2-undecane, detected in trace amounts, and pentadecanal, <2% throughout the experimental time.

S. montana EOs are commonly dominated by carvacrol, γ-terpinene and *p*-cymene which have been associated to its phytotoxic activity. In assays analyzing the activity of EOs in weeds and crops seeds germination, *S. montana* EO showed to be damaging to both, making it a poor choice for an herbicidal pesticide. Angelini et al. (2003) tested the EO at 0.5 mg/ml, and also its main compound carvacrol (57%), and found it to be highly inhibitory against *in vitro* seed germination. Grosso et al. (2010) also obtained high phytotoxic activity applying carvacrol-rich *S. montana* EO (52%) to 4 crops and 3 weed seeds and seedlings *in vitro*. Both germination and seedling root/shoot growth were affected negatively making this herbicidal EO only appropriate for uncultivated fields. Albuquerque et al. (2012) tested both carvacrol and its isomer thymol, at 0.4 µl EO *per* ml culture medium, on *in vitro* shoot cultures of *Heliconia psittacorum* × *Heliconia spathocircinata* var. Golden Torch. Their highly damaging effect was due to general destruction of the cell membranes and coagulation of the cytoplasm, detected through transmission electron microscopy. *S. montana* EOs appear not to be a sound choice for application as nematode biopesticides given their very negative effect on plant growth and development.

No macroscopic negative effect was detected after transferring Ppi shoots into *R. graveolens* EO-added culture medium (PpiRg) at phase 2 (Fig. 2c). As for *S. montana* EO, also in this case, in addition

Fig. 2. Aspect of (a) *Pinus pinaster* control shoot (Ppi), *P. pinaster* shoots grown in (b) *S. montana* and (c) *R. graveolens* EOs-added culture media (PpiSm and PpiRg, respectively), at 0.5 μl/ml, at day 7 of Phase 2, and (d) *P. pinaster* with PWN control co-culture shoot (PpiBx). PpiBx co-cultures transferred to EO-added culture medium showed morphology similar to Ppi shoots. Scale bar 1 cm.

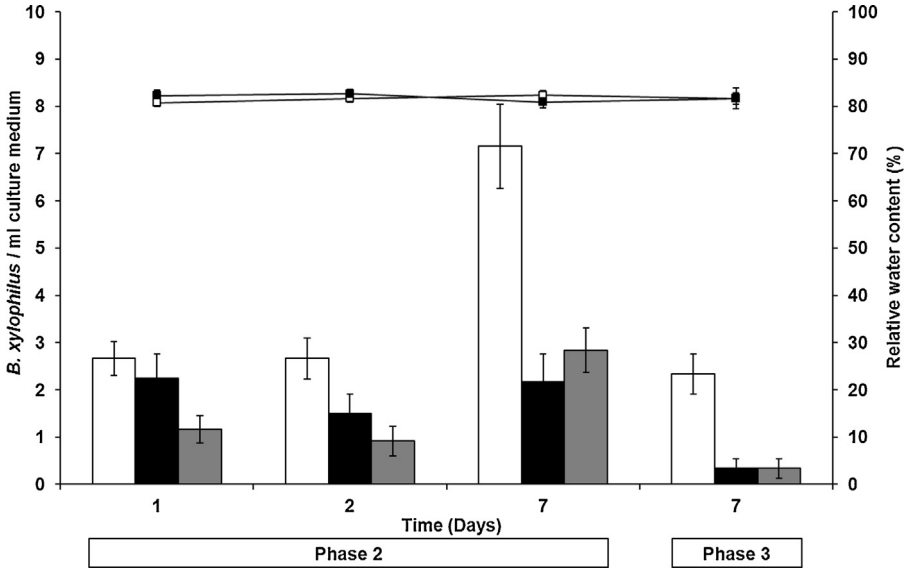

Fig. 3. PWN population density in the culture medium of *P. pinaster* shoots with PWN co-cultures (PpiBx), without- (Control, white column), and with *S. montana* (black column) and *R. graveolens* (gray column) added EOs, at 0.5 μl EO/ml culture medium, and relative water content average of all *P. pinaster in vitro* cultures (empty square) and PpiBx co-cultures (filled square), at the different days of Phase 2 and at the end of Phase 3 (recovery time) (for phases details see Fig. 1).

Table 1
Percentage composition of volatiles isolated from *Pinus pinaster in vitro* shoot cultures (Ppi Shoots) and *P. pinaster* shoots with PWN co-cultures (PpiBx Co-cultures) sampled at time 0, and days 1, 2 and 7 of Phase 2 and at the end of recovery time (R, day 7 of Phase 3). For experimental design see Fig. 1.

Components	RI	Ppi Shoots					PpiBx Co-cultures				
		0	1	2	7	R	0	1	2	7	R
trans-2-Hexenal	866	0.1	0.1	t	0.1	t	0.1	0.1	t	t	t
α-Thujene	924	0.1	0.2	0.2	0.3	0.2	0.1	0.2	0.1	0.4	t
α-Pinene	930	24.4	26.6	25.9	28.7	24.6	25.4	28.2	17.7	29.4	20.5
Camphene	938	0.5	0.6	0.6	0.7	0.5	0.6	0.8	0.4	0.7	0.2
Sabinene	958	t	t	t	t	t	t	t	t	t	t
β-Pinene	963	42.4	44.1	41.3	45.7	40.2	39.7	45.5	35.3	47.2	34.7
β-Myrcene	975	1.5	1.3	1.0	1.1	1.1	1.1	1.0	1.1	1.1	1.0
β-Phellandrene	1005	1.2	1.3	1.4	1.3	1.6	1.1	1.2	1.6	1.2	1.9
Limonene	1009	1.1	1.2	1.3	1.3	1.3	1.2	1.5	1.3	1.2	1.3
Butyl isovalerate	1022	t	t	t	t	t	t	t	t	t	t
trans-β-Ocimene	1027	t	t	t	t	t	t	t	t	t	t
γ-Terpinene	1035	0.1	0.1	0.1	t	t	0.1	t	t	t	t
Terpinolene	1064	0.4	0.3	0.5	0.3	0.1	0.3	0.5	0.6	0.4	t
Linalool	1074	0.2	0.2	0.1	0.1	0.1	t	t	0.4	0.1	t
Isopentyl isovalerate	1084	0.1	0.1	t	t	t	t	t	t	t	t
trans-Pinocamphone	1121	t	t	t	t	t	t	t	t	t	t
δ-Terpineol	1134	t	t	t	t	t	t	t	t	t	t
Borneol	1134	t	t	t	t	t	t	t	t	t	t
α-Terpineol	1159	1.5	1.1	1.7	0.7	1.5	1.3	1.8	2.6	0.9	2.2
Methyl thymol	1210	t	t	0.1	t	t	t	t	0.1	0.1	t
Phenyl ethyl acetate	1228	0.1	0.1	t	t	t	0.1	t	t	t	t
Bornyl acetate	1265	0.3	0.3	0.2	0.3	0.2	0.2	t	0.2	0.1	t
Carvacrol	1286	t	0.1	0.1	0.1	0.2	t	0.4	0.5	0.6	0.3
trans-Pinocarvyl acetate	1278	0.1	0.1	t	t	t	0.1	t	t	0.4	t
α-Longipinene	1338	t	t	t	t	t	t	t	t	t	t
α-Cubebene	1345	0.1	0.1	t	t	t	t	t	0.1	0.1	t
α-Copaene	1375	0.1	0.1	0.1	t	t	0.1	0.1	t	0.1	t
Longifolene	1399	t	t	t	0.1	t	t	0.1	0.1	t	t
β-Caryophyllene	1414	2.9	2.4	2.0	1.9	2.3	3.2	2.4	3.1	1.4	2.6
α-Humulene	1447	0.2	0.1	t	t	t	0.9	0.2	0.5	0.4	t
trans-β-Farnesene	1455	0.1	0.1	t	t	t	0.3	t	t	t	t
Phenyl ethyl 2-methyl butanoate	1467	0.3	0.3	0.1	0.3	0.2	0.5	0.6	1.0	0.3	t
Phenyl ethyl isovalerate	1468	1.1	0.9	0.6	0.7	0.6	1.1	1.6	2.0	1.0	0.4
γ-Muurolene	1469	t	t	t	t	t	t	t	t	t	t
Germacrene-D	1474	4.4	4.2	6.6	4.1	6.7	4.5	3.5	8.7	3.1	9.3
α-Muurolene	1494	0.4	0.3	0.2	0.2	0.1	0.5	0.2	0.6	0.1	t
γ-Cadinene	1500	1.2	1.0	0.7	0.7	0.4	1.5	0.7	1.1	0.7	t
δ-Cadinene	1505	0.9	0.9	1.9	0.8	1.4	1.0	1.7	1.8	0.5	1.9
β-Caryophyllene oxide	1561	0.3	1.1	1.8	1.9	2.5	1.0	0.4	2.5	2.8	3.0
epi-Cubenol	1600	0.3	0.2	t	0.1	0.1	0.1	0.3	0.3	t	t
epi-α-Cadinol	1616	0.2	0.2	t	0.2	0.1	0.2	0.4	0.6	0.3	t
α-Muurolol	1618	0.3	0.2	0.2	0.1	0.8	0.3	0.2	0.1	t	1.4
α-Cadinol	1626	0.2	0.2	0.5	0.3	2.0	0.4	0.3	0.8	0.4	3.8
Palmitic acid	1908	0.9	0.8	0.5	0.6	1.0	0.7	0.6	0.9	0.6	1.4
UI A Ppi*	2006	t	0.1	0.6	0.2	0.4	t	t	0.3	0.1	0.6
UI B Ppi*	2309	7.2	5.6	4.2	4.0	6.8	7.8	4.1	8.2	2.6	9.5
% Identification		88.0	90.4	89.7	92.9	89.4	87.7	93.9	85.9	95.6	85.9
Grouped components											
Monoterpene hydrocarbons		71.7	75.6	72.3	79.4	69.5	69.6	78.7	57.9	81.6	59.6
Oxygen-containing monoterpenes		2.1	1.7	2.2	1.3	1.9	1.6	2.2	3.9	2.1	2.5
Sesquiterpene hydrocarbons		10.3	9.1	11.5	7.8	10.8	12.0	8.7	15.9	6.4	13.8
Oxygen-containing sesquiterpenes		1.3	2.0	2.5	2.6	5.4	2.0	1.6	4.3	3.5	8.2
Others		2.6	2.1	1.2	1.7	1.7	2.5	2.8	4.0	1.9	1.8

RI: In-lab calculated retention indices relative to C9-C24 n-alkanes on the DB-1 column, t: trace (<0.05%), UI: Unidentified compound.

to *R. graveolens* EO volatiles, and to Ppi shoots constitutive volatiles, also new volatiles were detected in PpiRg shoots. 2-Undecanone, the major component in *R. graveolens* EO (91%) (Faria et al., 2013), increased throughout Phase 2 (18-38%) (Fig. 4b), decreasing after transference to Phase 3 (29%). During this study, the identity of a previously unidentified component from *R. graveolens* EO (Faria et al., 2013), was ascertained as 8-phenyl-2-octanone by NMR (Figs. 4 d and Fig. 5). There was no major variation in the relative amount of this compound during Phase 2 and 3 (3–5%). Four new, as yet unidentified, volatiles (0–3%) were detected in PpiRg shoots, whose relative amount either declined, increased or remained relatively stable throughout time.

de Feo et al. (2002) tested *in vitro* phytotoxic activity of *R. graveolens* essential oil and some of its constituents on *Raphanus sativus* germination and radicle growth and found activity only in the EO and some minor constituents but not with the EO major components, 2-undecanone (47%) or 2-nonanone (19%). While showing no detectable phytotoxic activity, *R. graveolens* EO used in the present work differed from that used by de Feo et al. (2002) by displaying a higher amount of 2-undecanone and the presence of the ketone 8-phenyl-2-octanone.

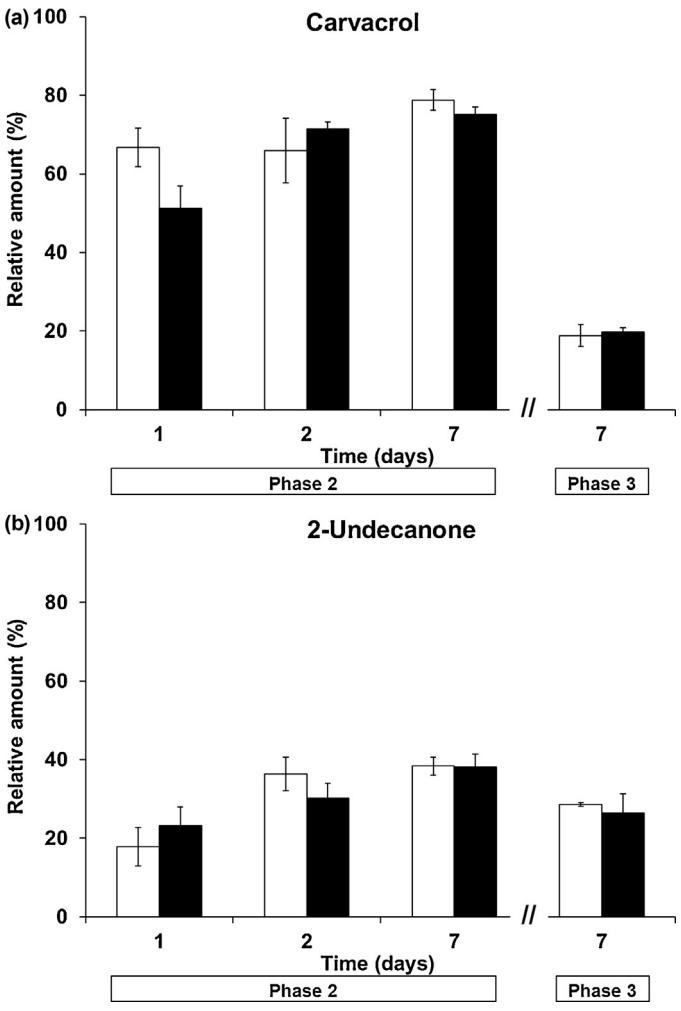

Fig. 4. Variation in the percentage composition of the main components of the EOs added to the culture media. (a) Carvacrol from *Satureja montana* and (b) 2-undecanone from *Ruta graveolens* EOs added, at 0.5 μl EO/ml culture medium, to *P. pinaster* shoots cultures (Ppi) (white columns) and to *P. pinaster* shoots with PWN co-cultures (PpiBx) (black columns).

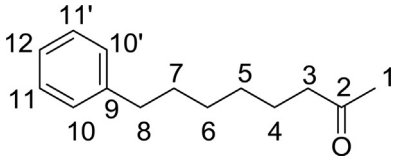

Fig. 5. Chemical structure of 8-phenyl-2-octanone as determined by NMR.

3.2. *Phytotoxicity and nematotoxicity to* P. pinaster *with* B. xylophilus *co-cultures*

PpiBx co-cultures and Ppi shoots showed similar morphology (Fig. 2a and d) and volatile profile (Table 1). Likewise, PpiBx co-cultures response to EOs addition to culture media was similar at morphological and volatile levels. Again, PpiBx co-cultures shoots transferred to *S. montana* EO-added culture medium developed toxicity symptoms, while *R. graveolens* EO induced no apparent tissue damage.

S. montana and *R. graveolens* EOs showed high nematotoxicity in direct contact bioassays (Faria et al., 2013) and were now assessed on *B. xylophilus* population density in PpiBx co-cultures, during Phase 2 and at the end of Phase 3 (Fig. 1). In PpiBx co-cultures grown in EO-free SH medium (control cultures), PWNs showed a 62% increase from day 1 to day 7 of Phase 2 (Fig. 3). Independently of the added EO, PWN population was always lower in EOs-added culture media, comparatively to control cultures (Fig. 3).

In *S. montana* EO-added culture medium PWN population showed no increase while in *R. graveolens* EO-added culture medium increased from 1.2 ± 0.3 to 2.8 ± 0.5 PWNs/ml culture medium, from day 1 to day 7 in Phase 2. Comparing with PpiBx shoots (control) at day 7 in Phase 2, 7.2 ± 0.9 PWNs/ml culture medium, *R. graveolens* EO effectively inhibited 61% PWN population increase. After a 7 days recovery (Phase 3), PWN population from EO-added cultures was detected below 15% that of control, indicating that treatment with EOs was effective in controlling PWN population (Fig. 3). EO activity was maintained which may be due to nematotoxic EO components being retained in the shoot tissue. Of the compounds retained in shoots, putative nematotoxic 2-undecanone is known for its biocidal activity. It was first registered in the United States in 1966 for use as a dog and cat repellant and is currently used in households and on ornamental plants as an insecticide. Its use was approved for indoor repellents, in 2014, in the European Union, although with high concerns due to its activity as a mammalian toxicant (European Commission, 2012).

Overall, activity against *B. xylophilus* is generally attributed to the EOs oxygen-containing terpenic fractions (Faria et al., 2013). According to Ntalli and Caboni (2012), these types of compounds (for ex. compounds with alcohol or phenol functional groups) are known to induce cytotoxicity, damage to the cellular and organelle membranes, act as pro-oxidants on proteins and DNA, and produce reactive oxygen species (ROS).

In the present work, two EOs nematotoxic and their phytotoxic activity were evaluated in a host with parasite *in vitro* co-culture environment. While *S. montana* EO revealed to be both nematotoxic and phytotoxic, the nematotoxic *R. graveolens* EO controlled PWN population showing negligible phytotoxic effects to *P. pinaster in vitro* shoot cultures and to *P. pinaster* shoots with PWN co-cultures. Given these characteristics, *R. graveolens* EO should be further evaluated as an effective nematotoxic pesticide against the PWN. Moreover, *P. pinaster* shoots with PWN co-cultures is a feasible system that allows a preview of how the plant host will react to nematotoxic pesticide application and so contributes in better designing *in vivo* bioassays on PWD affected plants.

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