J. of Supercritical Fluids 159 (2020) 104784

Contents lists available at ScienceDirect

The Journal of Supercritical Fluids

journal homepage: www.elsevier.com/locate/supflu

Impact of the pinewood nematode on naturally-emitted volatiles and $scCO_2$ extracts from *Pinus pinaster* branches: a comparison with *P. pinea*

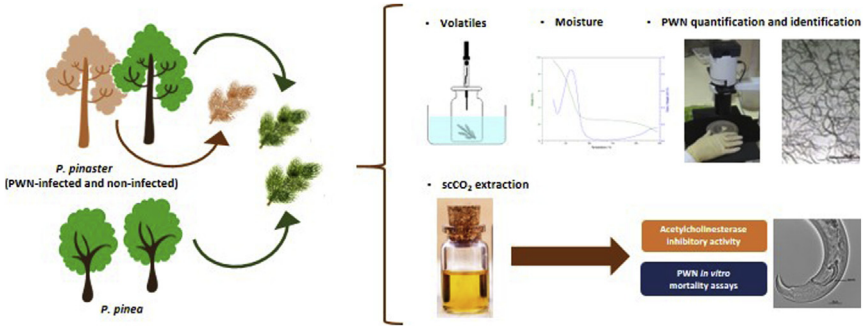
M.C. Gaspar[a], B. Agostinho[a], L. Fonseca[b], I. Abrantes[b], H.C. de Sousa[a], M.E.M. Braga[a,*]

[a] CIEPQPF, Chemical Process Engineering and Forest Products Research Centre, Department of Chemical Engineering, University of Coimbra, 3030-790, Coimbra, Portugal
[b] CFE, Centre for Functional Ecology, Department of Life Sciences, University of Coimbra, 3000-456, Coimbra, Portugal

HIGHLIGHTS

- Moisture content of PWN-infected *P. pinaster* is related to the PWN number.
- Limonene was naturally-emitted by all *P. pinea* and some infected *P. pinaster* branches.
- Abietadiene was identified in some $scCO_2$ extracts of PWN-infected branches.
- All extracts were able to inhibit the acetylcholinesterase enzyme.
- All extracts revealed no effects on PWN-mortality.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 18 October 2019
Received in revised form 6 February 2020
Accepted 6 February 2020
Available online 7 February 2020

Keywords:
P. pinaster
P. Pinea
Volatiles
$scCO_2$ extraction
Acetylcholinesterase inhibitory activity
Pinewood nematode

ABSTRACT

In Portugal, the pinewood nematode (PWN), *Bursaphelenchus xylophilus*, has been affecting *Pinus pinaster*, leading to economic and ecological losses, while *P. pinea* is a less susceptible species. The main goal of this study was to assess volatile composition of PWN-infected *P. pinaster*, and both non-infected *P. pinaster* and *P. pinea* branches, by using supercritical carbon dioxide extraction compared with solid-phase microextraction (SPME). Volatile profile was associated to nematodes number, which was related to moisture content. Extracts effects on acetylcholinesterase (AChE) inhibitory activity, and *in vitro* PWN-mortality were analyzed. Limonene was the main volatile naturally-emitted by *P. pinea* and by some PWN-infected *P. pinaster* samples. Abietadiene was identified in some PWN-infected *P. pinaster* extracts, which may be due to a tree defensive response. Supercritical CO_2 extracted ~5–20 heavier compounds than those identified by the SPME-GC/MS technique. Extracts revealed AChE inhibition, corresponding to a possible insecticidal/nematicidal effect. However, no PWN-mortality was found.

1. Introduction

The forest area corresponds to around 35 % of the continental Portuguese territory, with the pine area representing almost 1 million ha. In spite of the decrease in recent years in maritime pine, *Pinus pinaster*, area, mainly due to wildfires and to pine wilt disease (PWD), this species is the third most abundant, after euca-

* Corresponding author.
E-mail address: marabraga@eq.uc.pt (M.E.M. Braga).

https://doi.org/10.1016/j.supflu.2020.104784
0896-8446/

lyptus and cork oak. According to the latest published data from the National Forest Services (*Instituto de Conservação da Natureza e Florestas*, ICNF), *P. pinaster* corresponded to 714,000 ha, in 2015 (less 88,000 ha, when compared to 2005), while the stone pine, *P. pinea,* accounted for 193,000 ha (with an increase of 20,500 ha from 2005 to 2015) [1,2]. *Pinus pinaster* is a coniferous tree native to the western Mediterranean basin, and it is also found in other parts of central and southern Europe and in North Africa [3]. This species has crucial importance for the Portuguese economy, with almost 2000 million € of forest-based products exportations per year [1], being widely used for furniture and construction, as well as in pulp and paper industries [2,4]. Resin from its wood is tapped to make rosin and turpentine, and bark is used to prepare low-weight substrates for plant nursery production. A wide range of products can be obtained from rosin and turpentine, such as oils, varnishes, adhesives, waxes, soaps and bioactive compounds with medicinal applications [3,5–8]. *Pinus pinea* is also distributed near the Mediterranean basin, and particularly abundant in south Western Europe, including Portugal. This pine species is mostly known for its edible seeds, the pine nuts, which are economically important, being predominantly produced by Spain, Portugal and Italy. These trees are slow growth, and therefore, despite the good wood quality, their commercial timber plantations are scarce [5]. Moreover, in Europe, *P. pinaster* and *P. pinea* trees are also used for stabilization of dunes, slopes and near coastal areas [3,5].

Considering all the applications, a sustainable management of pine forests is needed in order to guarantee the future of this relevant economic segment. However, pests, diseases and wildfires are serious threats to pine forests, leading to huge economic and ecological impacts [9,10]. The pinewood nematode (PWN), *Bursaphelenchus xylophilus*, is the causal agent of the PWD, the most serious worldwide conifer disease, which leads to great losses in coniferous forests. This nematode is considered an harmful organism to plants or plant products within the European Union (EU) and it is included in the list of harmful pests and referenced as a quarantine organism (List A2) by the European and Mediterranean Plant Protection Organization (EPPO) [10,11]. This microscopic filiform nematode (less than 1 mm length) is an endoparasitic migratory nematode considered native to North America, where it has caused little damage due to the resistance or tolerance of native conifers. Then, it dispersed to Japan, in the early 20th century and, later, to China, Korea and Taiwan. In Europe, the PWN was first identified in Continental Portugal in 1999 [12], and then it spread to Spain [13] and Madeira Island [14]. The main hosts are trees belonging to the genus *Pinus* which vary among geographical locations, *P. densiflora, P. luchuensis, P. massoniana* and *P. thunbergii,* for Asian countries and *P. nigra, P. sylvestris* and *P. pinaster* for Europe [15,16]. *Bursaphelenchus xylophilus* is vectored by longhorn beetles belonging mainly to the genus *Monochamus* (order Coleoptera, family Cerambycidae). In Portugal, the only known vector is *M. galloprovincialis* [17]. During insect maturation feeding on young pine branches, nematodes are transmitted to the host and spread through the vascular system and resin canals. Under favorable conditions (~20 °C), nematodes reproduce exponentially causing water conductance blocking, tracheid cavitation, and rupture of the resin canals, leading to resin exudation reduction, yellowing and wilting of needles, partial or total dryness of the crown and plant death, in a more advanced stage. The declining or dead tree become attractive for insect oviposition [10,16,18]. In Portugal, this nematode has been severely affecting the *P. pinaster* forest area and thus emergency measures and control actions are being implemented, in order to prevent further PWN dispersal to non-affected areas [10,19,20]. However, the disease does not affect other local pine species, such as the *P. pinea*, which is considered a less susceptible species [21,22]. The low susceptibility may rely on (non-) suitability of *P. pinea* as a host for the insect vector feeding and oviposition [21], but some studies have reported similar feeding preferences, when compared to *P. pinaster* trees [23]. On the other hand, *M. galloprovincialis* females appear to reject this pine species to lay their eggs, and limonene, the main emitted volatile, is a feeding deterrent for many conifer insects [24].

Despite of control and management measures, that have been implemented in the field, PWD affected areas are still increasing, and eradication is no longer considered to be possible. Emergence of this disease and respective control is based on an interaction between three factors: host pine tree, PWN and insect vector. However, despite the efforts that have been made in this area, the basic ecology of this correlation is poorly known [19]. Control measures can be directed to the nematode or to its insect vector. Some methods include fumigation of dead trees [25], and trunk injection with synthetic nematicides [26], but practical issues and ecological concerns exist. Synthetic insecticides could also be applied, but their lack of selectivity, repeated use and residual contamination have been resulting in poisoning of non-targeted organisms, and increased resistance in insects [27,28]. Nowadays, one of the most environmentally friendly strategies to control this disease is the use of traps baited with attractants (e.g. pheromones) for the capture of the insect vector [29,30]. Some of those compounds correspond to volatiles naturally-emitted by pine trees, such as α-pinene, which has been reported as an attractant for this insect [31,32]. The use of repellents can be useful to avoid the oviposition behavior of the PWN insect vector, but only a few studies have assessed their use to control the referred insect [31]. Thus, a promising alternative may be low-toxicity natural compounds with attractive, repellent or insecticidal/nematicidal activity, extracted from a wide number of plants, including pine species. Those extracts can be obtained by supercritical fluid extraction, and are usually composed of hydrophobic compounds: alcohols, aldehydes, aromatic phenols, mono- and diterpenes, sesquiterpenes, among others [33]. In fact, several previous studies have showed nematicidal or insecticidal activity from plant constituents/ volatile oils [33–35]. Moreover, volatile composition is distinct among plant species [24], and when trees are stressed or attacked by pathogens or insects, the sort and content of volatile compounds are also modified [36,37]. Abietic acid, for example, is produced by conifer species as a defensive secretion, when attacked by insects and other pathogens [38].

Some plant extracts revealed a nematicidal effect against PWN, despite being the mechanism involved in this action unclear. Nevertheless, some enzymes appear to be affected, namely the acetylcholinesterase (AChE) [34,39,40]. This enzyme is responsible for the hydrolysis of the neurotransmitter acetylcholine, which is involved in the neuronal excitement at the postsynaptic membrane. When AChE is inhibited, an accumulation of acetylcholine occurs, producing a rapid twitching of voluntary muscles, and eventually paralysis and death of animals with a cholinergic nervous system [41,42]. It is by this mechanism that some pesticides, such as organophosphates and carbamates, act [34,43].

Therefore, the aims of this study were to assess the PWN impact on naturally-emitted pine volatiles composition and on supercritical carbon dioxide ($scCO_2$) extracts of PWN-infected *P. pinaster* branches, comparing with both non-infected *P. pinaster* and *P. pinea*, and to evaluate the effect of the extracts on AChE activity and on *in vitro* PWN mortality.

2. Materials and methods

2.1. Pinus *spp. wood samples and chemicals*

Pine trees were selected in collaboration with the National Forest Services (*Instituto de Conservação da Natureza e Florestas*, ICNF), and samples were collected in pine forests of each species, to avoid interferences from other tree volatiles. *Pinus pinaster* branches

were collected from PWD-symptomatic (4 samples) and non-symptomatic (3 samples) trees at the Central Region of Continental Portugal, in Oleiros (-39°55′38.20″N, 7°53′02.8″W; -39°55′34.62″N, 7°53′07.77″W; -39°55′30.50″N, 7°53′11.82″W; -39°55′23.75″N, 7°53′11.61″W), in August 2017, while *P. pinea* branches were obtained from non-symptomatic trees, in Leiria (39°51′14.321″N, 8°45′37.598″W; 39°51′13.392″N, 8°45′37.530″W) in July 2019. Wood samples were milled (cross beater mill, Retsch, Germany) in order to obtain a particle size smaller than 2 mm, and then stored at – 20 °C until further analysis.

Chemicals and reagents used for PWN extraction, isolates establishment and maintenance and morphological/molecular identification were: bacto malt extract and agar granulated from Difco (Sparks, MD, USA); glycerol (≥ 99.5 %) from Fischer Scientific (Geel, Belgium); ampicillin sodium salt from Sigma Aldrich (Sintra, Portugal), dream taq DNA polymerase (5U/μl) and dream taq DNA polymerase buffer from Thermo Scientific (Loures, Portugal); and resctrictions endonucleases (10 U/μl) (*Hinf*I, *Alu*I, *Hae*III, *Rsa*I, *Msp*I) from Bioron (Ludwigshafen, Germany).

Chemicals and solvents used for volatile compounds extraction from pine branches and for further analyses were: carbon dioxide (≥ 99.5 %) from Praxair (Maia, Portugal); ethyl acetate (≥ 99.9 %, HPLC grade), and hydrochloric acid (HCl, 37 %, p.a.) from Carlo Erba (Val de Reuil, France); acetylcholinesterase (AChE, Type VI-S, 500 U/mg protein), 5,5′-dithiobis[2-nitrobenzoic acid] (DTNB, ≥ 98 %), acetylthiocholine iodide (AChI, ≥ 98 %), ethanol (≥ 99.8 %, p.a.), tris(hydroxymethyl)aminomethane (Tris Buffer), and Triton X-100 from Sigma-Aldrich (Sintra, Portugal).

2.2. Nematode extraction, identification and quantification

Nematodes were extracted from PWD-symptomatic and non-symptomatic *P. pinaster* trees, and from non-symptomatic *P. pinea* trees, using the Whitehead and Hemming tray method [16,44], that is based on the motility of the nematodes, which migrate, at room temperature, from the wood samples into the water. Briefly, wood samples were wrapped in a tissue paper and placed on a plastic mesh overlaying a plastic tray. The trays were then filled with water until wood samples were immersed. After 48 h, samples were removed, and entire water suspensions poured into glass beakers and passed through a 20 μm sieve. After sieving, nematodes retained on the sieves were collected in 20 mL of water for observation under a stereoscopic microscope. All nematodes present in the suspensions were quantified and *B. xylophilus* was quantified and identified, based on the species specific morphological diagnostic characters [16,45].

2.3. Pinewood nematode isolates and molecular identification

Four PWN isolates, corresponding to the four PWN-infected *P. pinaster* trees, PWN-tree 1; PWN-tree 2; PWN-tree 3; PWN-tree 4, were established and maintained, at 25 °C, in cultures of *Botrytis cinerea* grown on malt extract agar (MEA) medium with ampicillin. Subcultures of each isolate were carried out every 3 weeks by transferring small plugs with nematodes to new MEA medium colonised with *B. cinerea* [46]. Isolate characterization and molecular identification were based on Polymerase Chain Reaction-Internal Transcribed Spacers technique (PCR-ITS) followed by Restriction Fragment Length Polymorphisms (RFLP) analyses with five restrictions endonucleases following the EPPO standard [14,45]. DNA from 10 nematodes of each isolate was extracted with the DNeasy Blood and Tissue Mini kit (Qiagen, Hilden, Germany) following manufacturer's instructions. The ITS rDNA regions containing partial 18S and 28S and complete ITS1, 5.8S and ITS2 sequences were amplified using 50 ng extracted DNA and 1 U Dream Taq DNA polymerase (Fermentas, Hanover, NH, USA) in 1X Dream Taq buffer, 0.2 mM each dNTP and 1 lM primers 18SF 5′-CGTAACAAGGTAGCTGTAG-3′ [47] and 28SR 5′-TTTCACTCGCCGTTACTAAGG-3′ [48]. All reactions were carried out in a Thermal Cycler (Bio-Rad, Madrid, Spain) with an initial denaturation step of 95 °C for 2.5 min followed by 40 reaction cycles of 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min. The resulting PCR-ITS amplification product was used for RFLP analyses, using *Afa*I, *Alu*I, *Hae*III, *Hinf*I and *Msp*I endonucleases, according to the manufacturer's instructions. PCR and restriction products were separated by electrophoresis on 1.5 % agarose gel.

2.4. Moisture content

The moisture content of pine branches was evaluated by simultaneous thermal analyzer (differential scanning calorimetry/ thermogravimetric analysis, DSC/TGA, TA Instruments, model Q600), in duplicate. Samples (6–12 mg) were heated at 10 °C/min, from 25 °C up to 600 °C, under a nitrogen atmosphere (100 mL/min). Water content was calculated based on weight loss that occurred close to 100 °C. Moisture values were associated with the PWN number, since the dryness of the tree increases with the degree of infection.

2.5. Volatile composition of pine branches

2.5.1. Naturally-emitted compounds

The solid-phase microextraction (SPME) technique was performed to evaluate the volatiles naturally emitted by *P. pinaster* (PWN-infected and non-infected) and (non-infected) *P. pinea* branches. In order to avoid modifications in the volatile composition, samples were carefully handled and only a single intact portion was used for these assays. In fact, the disruption of plant tissues activates some enzymatic processes leading to a different volatiles production [49]. Concisely, the previously-weighed branch portion was transferred to an headspace empty flask to ensure a 1/100 (w/v) ratio, i.e., 1.0 g of sample/ 100 cm^3 of air. Then, volatiles were adsorbed for 5 min using a 65 μm polydimethylsiloxane/divinylbenzene (PDMS/DVB) coated fiber (Sigma-Aldrich) in the headspace mode (35 °C), without using solvents.

2.5.2. Volatile compounds extracted with supercritical carbon dioxide

Pine branches volatile compounds were extracted with $scCO_2$ by using a laboratory-scale equipment provided by Separex (Champigneulles, France) [50]. Milled pine branches (4.0×10^{-3} kg, dry basis, d.b.) were placed in a 20×10^{-6} m^3 thermostatic stainless-steel cell, and the outlet CO_2 flow rate was measured by a gas flow meter (Alexander Wright, UK), and set to 2.5 L/min ($\sim 7.5 \times 10^{-5}$ kg/s), at ambient conditions. A static period of 15 min was defined, followed by 360 min of dynamic one, totalizing 375 min of extraction time, and resulting in a solid-to-solvent ratio of 1:401 (w/w, d.b.). Extractions were carried out, in duplicate, at a $scCO_2$ density of 750 kg/m^3, and temperature of 45 °C, corresponding to a pressure of 154 bar. These conditions were chosen according to previous pine samples extractions, performed by the authors.

Pine extracts were collected in cooled flasks at pre-determined sampling times to infer about the extraction curves profile. Note that these fractions were collected with ethanol due to practical aspects in recovering the extract from the flask. A glass wool packed column was placed after the collection flask for preventing the loss of volatiles with the exiting CO_2 stream. After depressurization, the tubing lines were also rinsed with ethanol after each extraction experiment. All the ethanol-containing extract fractions (recovered during the extraction, and from line rising) were later evaporated at 40 °C (Rotovap R-210, Büchi), while glass wool packed columns

Table 1
Moisture content and nematodes numbers (pinewood nematode, PWN, and other nematodes) of PWN-infected and non-infected *Pinus pinaster* and *P. pinea* trees.

Pinus spp.			Moisture content*(%)	PWN/100 g wood	Other nematodes/100 g wood
P. pinaster	**PWN non-infected**	1	41.04 ± 1.85	0	0
		2	35.69 ± 1.14	0	0
		3	40.41 ± 0.79	0	0
	PWN-infected	1	33.22 ± 2.09	94	168
		2	11.27 ± 0.40	2267	1436
		3	30.74 ± 2.42	28	62
		4	9.69 ± 0.18	12992	3240
	P. pinea	1	59.81 ± 0.51	0	0
		2	51.33 ± 3.89	0	0
		3	59.70 ± 0.24	0	0
		4	57.61 ± 3.78	0	0

*Results are expressed as mean ± standard deviation.

were rinsed with ethyl acetate in the fuming hood, under ventilation and at room temperature. For the calculation of total yields, the ratio between the total extracted mass and the initial raw material mass, on a dry basis, was considered. The total mass of extract includes the volatile fractions recovered in the collection flask, from the final depressurization and line rising, and from the glass wool packed column. Before performing the analyses, the fractions recovered in the collection flask were mixed with the ones from the depressurization and rinsing, and stored, protected from light, at – 20 °C.

2.5.3. Volatile compounds analysis by gas chromatography

Identification of naturally-emitted and $scCO_2$ extracted volatiles from pine branches was carried out by coupled gas chromatography mass spectrometry (GC/MS 7890A, 5975 C inert MSD with triple axis-detector, Agilent Technologies). In the case of SPME experiments, the fiber was introduced in the injection port of the GC equipment, immediately after the adsorption period, and the trapped compounds were then desorbed at 250 °C, for 1 min. Separation was achieved on a DB5-MS fused silica capillary column (30 m ×0.25 mm i.d. × 0.25 μm, Agilent J & W Scientific), using helium as the carrier gas, at a flow rate of 1 mL/min. The temperature program included an isothermal period of 5 min at 50 °C, followed by a temperature increase of 10 °C/min and up to 270 °C, where it was held for 5 min [51].

For the extracted volatiles by $scCO_2$, they were dissolved in ethyl acetate (5 mg/mL) and analyzed by GC/MS with an injection volume of 0.2 μL. The volatiles trapped in the glass wool column were also identified by GC/MS, using the same solvent, concentration and injection volume. The temperature program was the same as the one described for the SPME experiments.

The prior identification of volatile compounds was based on a comparison of their mass spectra with those of libraries (NIST and Flavors and Fragrances of Natural and Synthetic Compounds, FFNSC2.L). A semi-quantitative analysis was performed, based on peak relative areas of the identified compounds. All samples were analyzed, in duplicate.

2.6. Acetylcholinesterase inhibitory activity assay

AChE inhibitory activity assay was conducted to test the nematicidal/insecticidal potential of *P. pinaster* and *P. pinea* $scCO_2$ extracts. The experiments were performed according to the procedure described by Ellman et al. [52] and modified by Ferreira et al. [53]. Briefly, DTNB (3 mM, 500 μL), AChI (15 mM, 100 μL), Tris–HCl buffer at pH 8 (50 mM, 275 μL), and the extract (previously dissolved in ethanol:Tris-HCl buffer (50:50, v/v), 100 μL) were added to a 1 mL cuvette. The enzyme AChE (0.28 U/mL, 25 μL) was then added to start the reaction, which was monitored for 5 min (405 nm) for determination of the reaction rate. The AChE activity was calculated as a percentage of this velocity compared to that of the assay using buffer instead of extract (the inhibitor), and the inhibitory activity was then calculated by subtraction. For each extract, the procedure was done in triplicate, with three extract concentrations and results were expressed as IC_{50} values.

2.7. Pinewood nematode mortality in vitro *assay*

$scCO_2$ pine extracts nematicidal activity against PWN was assessed by an *in vitro* mortality assay, as already tested for other plant extracts [35]. Extract solutions from the four PWN-infected *P. pinaster*, one non-infected *P. pinaster* and one *P. pinea* samples were prepared in Triton X-100 (5 mg/mL). Different concentrations of selected pine extracts, ranging from 0.125 mg/mL up to 10 mg/mL, were firstly tested. Based on preliminary results and on literature data [54], three final concentrations of each extract were defined to be used in these assays: 0.5, 1 and 2 mg/mL. Five replicates were used for each extract concentration and Triton-X100 (5 mg/mL) was used as control solution. Twenty nematodes (mixed developmental stages) were placed on a glass-staining block containing 1 mL of each pine extract at different concentrations, or 1 mL of control solution. Glass-staining blocks were maintained in a moist chamber, in the dark, at room temperature (20–22 °C), and nematode mortality was monitored at 24, 48, and 72 h after exposure. Pine extracts solutions were not replaced as it was assumed that their activity was preserved during the tested period. Nematodes not showing movements when touched with a bristle were transferred to water and considered dead if they still failed to react. Corrected mortality was calculated by comparing the percentage mortality promoted by the extract to the percentage mortality obtained in the control, using the Schneider-Orelli formula [55]:

$$\text{Corrected mortality}(\%) = [(\text{mortality with extract} - \text{mortality with control})/(100 - \text{mortality with control})] \times 100.$$

3. Results and discussion

3.1. Pinewood nematode identification/quantification and Pinus spp. branches moisture content

Pine branches were analyzed regarding their PWN and other nematodes number, and moisture content. PWN was detected in the four PWD-symptomatic *P. pinaster* trees, while no nematodes were detected in both non-symptomatic *P. pinaster* and *P. pinea*. (Table 1). The identification of PWN, based on the species-specific morphological diagnostic characters revealed that both females and males presented the species-specific diagnostic characters of the species [45]: males with narrow spicules, evenly arcuate, with a

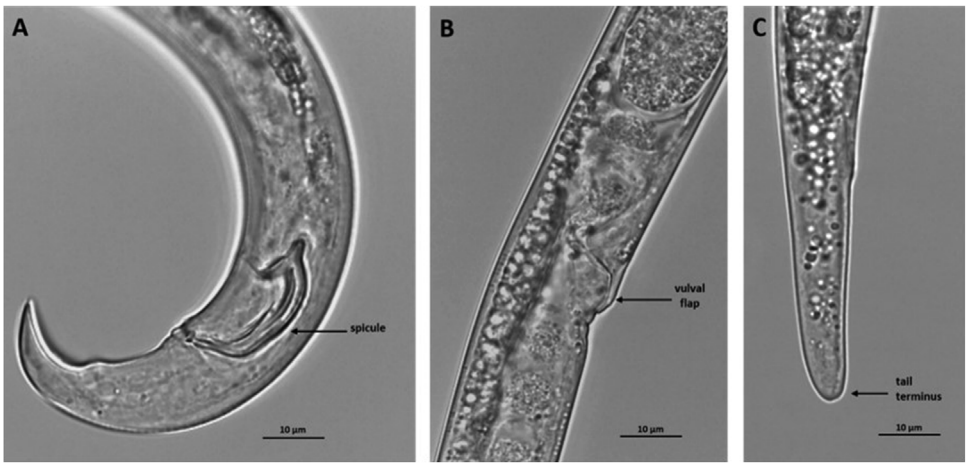


Fig. 1. Light microscope photographs of pinewood nematode (PWN), *Bursaphelenchus xylophilus,* from PWN-infected *Pinus pinaster* trees. A: male tail; B: female vulval region; C: female rounded tail terminus.

Table 2
Total extraction yield and AChE inhibitory activity of $scCO_2$ extracts from PWN-infected and non-infected and *Pinus pinaster* and *P. pinea* samples.

Sample			Total extraction yield*(% wt., d.b.)	AChE inhibitory activity(IC_{50}, mg/mL)
P. pinaster	**PWN non-infected**	**1**	5.25 ± 0.04	9.20 ± 0.65
		2	4.43 ± 0.40	10.59 ± 0.45
		3	5.96 ± 0.49	7.23 ± 0.53
	PWN-infected	**1**	5.72 ± 0.43	9.10 ± 0.43
		2	5.58 ± 2.06	9.50 ± 0.41
		3	4.52 ± 0.57	8.17 ± 0.50
		4	4.25 ± 0.20	9.04 ± 0.08
	P. pinea	**1**	1.55 ± 0.08	1.55 ± 0.08
		2	2.43 ± 0.54	44.37 ± 3.48
		3	1.50 ± 0.55	11.74 ± 0.51
		4	1.93 ± 0.47	> 40 mg/mL**

*Results are expressed as mean ± standard deviation; **30 % inhibition at 40 mg/mL.

disc-like projection (cucullus) at the distal end (Fig. 1A) and females with a distinct overlapping anterior lip (vulval flap) (Fig. 1B) and with the tail terminus with sub-cylindrical form with a broadly rounded tip (Fig. 1C).

Results from DSC/TGA analyses and number of nematodes/100 g of wood are included in Table 1. *P. pinea* samples had the highest water content, with values between 51 and 60 %, and absence of nematodes. On the other side, *P. pinaster* wood samples showed lower moisture contents. For the PWN non-infected trees, no nematodes were detected, and moisture content was in the 36–41 % range. In spite of the absence of nematodes, these values are lower than the ones observed for *P. pinea* samples. These differences may be attributed to their different morphology and to the soil composition and climate conditions at collection time. Other authors have found 59 % of water content for both *P. pinaster* and *P. pinea* samples, from many regions in Portugal [56]. Nevertheless, that value was observed for needles and not for branches (which also include wood in their composition and therefore may reduce the global water content that was observed). The lowest values were achieved for the PWN-infected *P. pinaster* samples (10–33 %), and a relationship between the PWN number and the moisture content appears to exist because the driest samples presented the highest PWN number (~10 % of water content corresponded to thousands of PWN/ 100 g of wood), while the infected samples with ~ 30 % humidity had lower PWN number (less than 100/ 100 g of wood). In these samples, other nematodes were quantified, and their number increased with the PWN number increment. This proliferation of other nematodes (mainly fungivorous) is related to the modifications that occur in the tree after PWN infection [18,57].

The reduction of water content in pine trees have already been noticed by other authors, in the case of PWD [58], and when PWN artificial inoculation is carried out [37,59]. According to the literature, this effect was more pronounced in *P. pinaster* and *P. sylvestris* trees in relation to controls (stem water reduction of 40 % and 24 %, respectively), but did not significantly modified water content in *P. pinea* stems [59]. Based on the results and considering other studies, the moisture content tends to decrease with the PWN number increase. When PWN enters into the host tree, they start reproducing exponentially causing water conductance blocking in the xylem, and tracheid cavitation, leading to yellowing and wilting of needles, and partial or total dryness of the crown [18].

3.2. Pinewood nematode isolates and molecular identification

Molecular characterization of the four PWN isolates (PWN-tree 1, PWN-tree 2, PWN-tree 3 and PWN-tree 4), established and maintained on the fungus *B. cinerea,* was performed based on PCR ITS-RFLP. Amplification of ITS regions yielded a single DNA fragment of approximately 950 bp for the four PWN isolates (data not shown). After restriction digestion with the restriction endonucleases *Alu*I, *Hae*III, *Hinf*I, *Msp*I and *Rsa*I, the isolates presented a *B. xylophilus* specific ITS restriction pattern (Fig. 2) [14,45,60].

3.3. Extracted and naturally-emitted volatiles from pine branches

Pine branches volatiles were extracted by SPME and $scCO_2$, followed by GC/MS analysis. Supercritical extraction curves profiles are depicted in Fig. 3 and total extraction yields included in

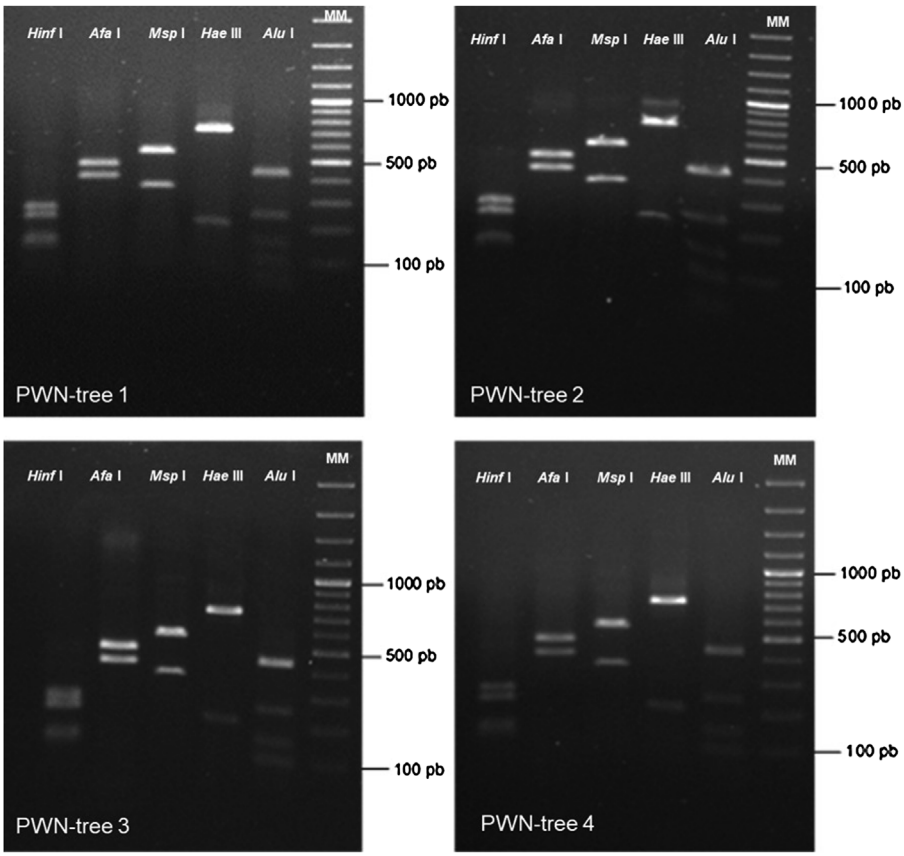


Fig. 2. Restriction Fragment Length Polymorphisms patterns, with five restriction endonucleases, of pinewood nematode (PWN), *Bursaphelenchus xylophilus,* from the four isolates (PWN-tree 1, PWN-tree 2, PWN-tree 3, PWN-tree 4). MM: DNA size marker (100 bp ladder, Fermentas).

Table 2. Main identified compounds are included in chromatograms (Figs. 4 and 5), and complete composition is included in Table S1.

It should be noted that before performing the supercritical extractions, raw material was milled, but not dried because air and oven drying and even freeze-drying, usually show a limited ability to preserve volatile bioactive compounds [61]. Total extraction yields (which include the amount of extract collected in the collection flask, trapped in the glass wool column, and from depressurization and line rinsing) were in the 1.5–6% range, after 375 min (Table 2, section 3.4). The lowest values were achieved for *P. pinea* samples, with the maximum close to only 2.5 %. *Pinus pinaster* extracts were obtained in higher yields, with values around ~ 4–6%, independently of being infected or non-infected branches. Since the temperature, pressure, and CO_2 flow rate were the same for all the extractions, these differences between species are related to differences among species, and eventually to the distinct geographical origin. Moreover, the higher *P. pinea* samples water content may have reduced the extract yield by hindering the diffusion of CO_2 into the vegetable matrix, as well as the diffusion of soluble compounds into the solvent bulk [62]. Obtained yield values were in general higher than those reported in the literature: 0.97–1.37% for *P. pinaster* bark [63] and 1.60 % for *P. nigra* needles [64]. This yield from *P.nigra* needles is, however, close to the values achieved in this study for *P. pinea* branches. In the literature, and as far as we know, there is no study using $scCO_2$ extraction for branches from these two pine species (*P. pinaster* and *P. pinea*), and, therefore, a direct comparison between these yield results and other published studies cannot be performed.

The extraction kinetic curves showed higher extraction yields for PWN-infected *P. pinaster* samples, when compared to the non-infected samples (Fig. 3). However, a major variability of extraction yield values was observed in the infected samples and this finding can be attributed to their distinct degrees of moisture content. The *P. pinea* samples showed the lowest extraction yields (Fig. 3A), similarly to the results obtained for the total yields (Table 2). Different extraction yields were obtained probably because the solubility of compounds that are extracted with the $scCO_2$, and the water content, are different. The extraction profiles indicate that the concentration of extract in the solvent bulk decreases continuously due to increasing mass transfer resistances, and consequently extraction rate decreases [65]. In general, the three extractions periods were observed in the kinetic curves with an initial extraction rate period, followed by a falling extraction rate period and finally by a diffusion controlled rate period. Extraction curves from PWN-infected *P. pinaster* samples reached a plateau (diffusional period), Fig. 3B, corresponding to the maximum amount of extractable compounds. For non-infected trees (both *P. pinaster* and *P. pinea* samples), the diffusional period appears to exist, but it is less pronounced (Fig. 3C and Fig. 3A, respectively). The retention of extract within the exit line and the clusters formation may have altered some extraction profiles, and therefore extraction curves were probably affected by those events.

The evaluation of naturally-emitted volatiles by PWN-infected *P. pinaster* trees and both non-infected *P. pinaster* and *P. pinea* trees revealed the presence of about 20–40 volatile compounds (from C4 up to C15), including many terpenes. Most of them were identified between 7 and 18 min (Fig. 4), and the total identified peak areas corresponded to 77–95 %, depending on the sample (Table S1). No relevant differences in naturally-emitted volatiles were detected among non-infected trees from the same species (*P. pinaster* or *P. pinea*) and, therefore, only one chromatogram for each non-infected pine species is present (Fig. 4), for simplification of presented data.

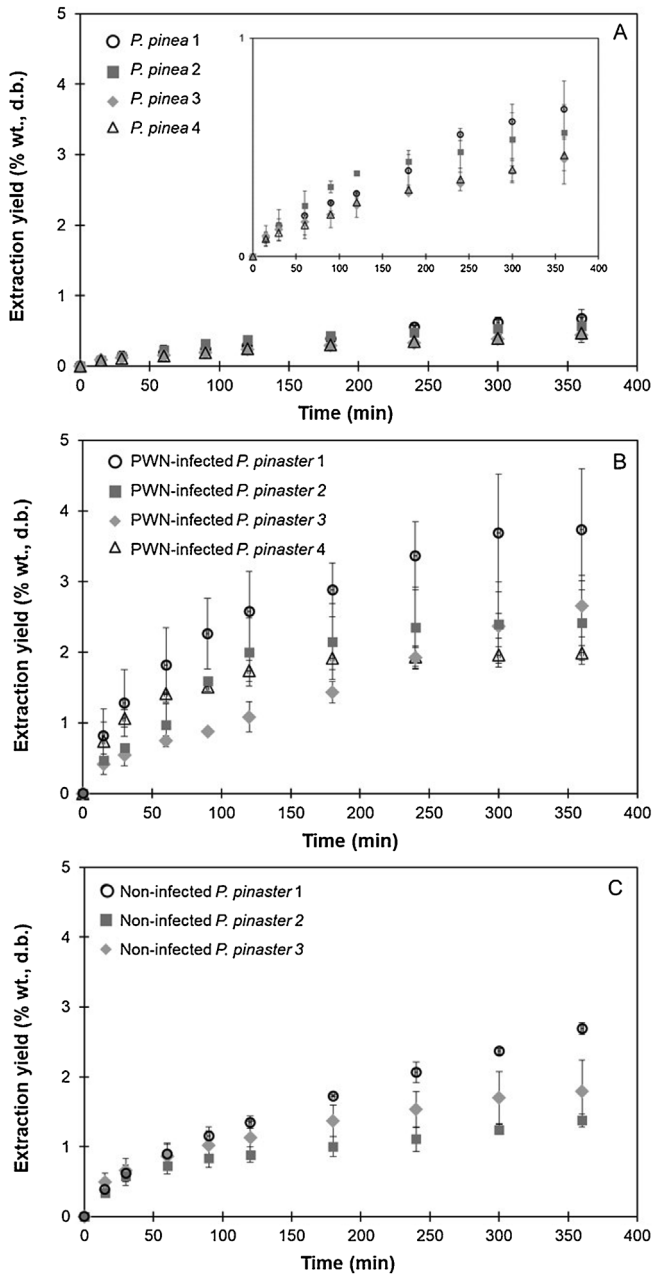


Fig. 3. Overall $scCO_2$ extraction kinetic curves of *Pinus pinea* branches (A); and pinewood nematode (PWN)-infected (B) and non-infected *P. pinaster* branches (C).

Further identified compounds are included in the Supplementary Material (Table S1). However, for PWN-infected samples, some differences in the emitted volatiles were observed, probably due to the different moisture content and PWN number.

Three monoterpenes, α-pinene, β-pinene, and α-terpinolene were identified in all samples, as well as the sesquiterpene β-caryophyllene, independently of the pine species. The emission of these volatiles by the two analyzed pine species has been reported [66,67] and for β-caryophyllene repellent activity against the pine shoot beetle, *Tomicus destruens* was suggested [68]. Interestingly, two PWN-infected samples, with the lowest number of nematodes (PWN-tree 1 and PWN-tree 3) showed the presence of limonene, a known repellent for some insects, including the PWN insect vector (Fig. 4) [69,70]. This result may be related to a defensive secretion produced by the tree. The other two PWN-infected trees may be too weakened to be able to fight against the infection, and therefore, volatiles such as limonene, are no longer emitted. This finding is in accordance with the literature, since other authors that evaluated the expressed genes associated with the PWN in susceptible (and inoculated) and resistant pine species, found that the limonene synthase might play a positive role in the resistance against PWN infection [71]. In the case of fungal pathogens in *Pinus* species, high concentration of limonene has been found in resistant species, when compared to the susceptible ones, and the initial infection appears to induce the limonene production, to inhibit the disease progression [72].

Additionally, the diversity of emitted volatiles by PWN-infected branches is, in general, lower, when compared to the non-infected *P. pinaster*. Some volatiles, such as β-phellandrene and thymol methyl ether, were identified in all non-infected *P. pinaster* samples but they were not detected in PWN-infected trees (Fig. 4 and Table S1). Actually, other authors have already found lower and distinct volatile production by pine stems inoculated with PWN, when compared to non-inoculated trees [73].

Limonene was also emitted by all *P. pinea* samples (Fig. 4), as expected, and according to other published results [70,74,75]. It accounts for ~ 60 % of the total peaks area in *P. pinea* samples (Table S1) and may be a reason for the less susceptibility of this pine species. However, longifolene was also identified in all *P. pinea* samples, and in one PWN-infected *P. pinaster* sample. This compound is a plausible attractor for the *M. galloprovincialis* [51]. Nevertheless, the relative concentrations of this volatile in *P. pinea* samples are very low (~ 0.2–1.2%), when compared to limonene, for example.

Other volatiles have been also detected in most of the pine samples, namely β-myrcene and α-terpinolene [74,75].

Around 30 compounds were identified in each $scCO_2$ extract, accounting for ~55–90 % of the total peaks area, depending on the extracted sample. Similar compounds were extracted from non-infected trees, from the same pine species. Therefore, only one chromatogram for extracts from each non-infected pine species is present (Fig. 5), and additional identified compounds are included in Table S1.

Low-molecular weight compounds were found in lower amounts when compared to the naturally emitted volatiles, being this difference more pronounced in the *P. pinea* extracts. In general, most of the extracted compounds eluted between 15 and 30 min, and for *P. pinea*, most compounds were identified between 20 and 30 min (Fig. 5 and Table S1). This result may be related to the high-water content, which in contact with the CO_2 makes the medium acidic and may modify the nature of extracted compounds or degrade acid-labile compounds. Also, the dissolution of CO_2 into the aqueous phase may turn the raw material into a mucilaginous state due to the interaction with the carbohydrate fraction at higher temperatures [62]. Another possibility may rely on some equipment limitations, which may result in loss of the most volatile compounds, despite the glass wool column, that was used to trap those volatiles. Moreover, the extraction conditions affect the $scCO_2$ solubility of monoterpenes, such as α-pinene and limonene, which increases with pressure and decreases with temperature [76]. In this way, the selected conditions (154 bar and 45 °C) could not be the most favorable to extract these compounds by $scCO_2$.

Some extracted compounds, namely from *P. pinaster* samples, were similar to those naturally-emitted by the pine branches, including α-cubebene, α-copaene, longifolene, β-caryophyllene, β-copaene, α-humulene, γ-cadinene, Δ-cadinene, among others (Fig. 5 and Table S1). These compounds are usually present in *Pinus* foliage essential oils, in distinct amounts, depending on the pine species [74,75,77]. For non-infected *P. pinaster* extracts, main compounds eluted between 15 and 18 min, while for infected *P. pinaster* and for non-infected *P. pinea* extracts, a second group of compounds was identified between 22 and 28 min. For *P. pinea* extracts, the

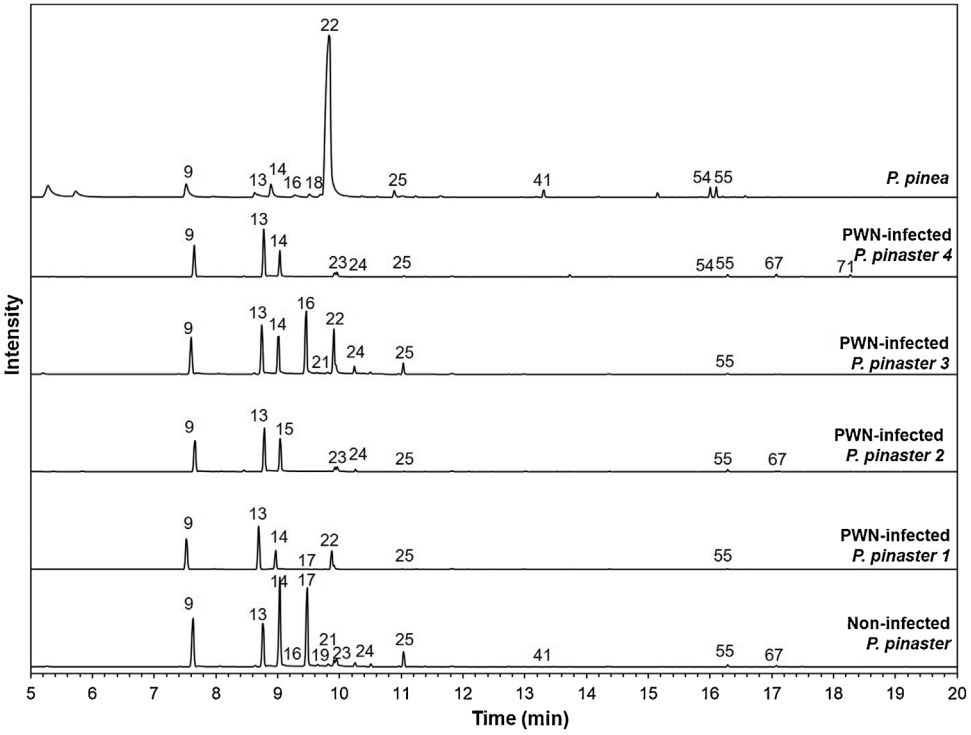


Fig. 4. Main naturally-emitted volatiles (SPME technique) by a sample of *Pinus pinea* tree and by pinewood nematode (PWN)-infected *P. pinaster* trees and a non-infected *P. pinaster* tree. Main compounds identified between 5 and 20 min (numbered according to Table S1): 9. α-pinene; 13. β-pinene; 14. β-myrcene; 15. tricyclene; 16. γ-terpinene; 17. Δ-3-carene; 18. α-terpinene; 19. β-phellandrene; 21. cymene; 22. limonene; 23. α-phellandrene; 24. β-ocimene; 25. α- terpinolene; 41. thymol methyl ether; 54. longifolene; 55. β-caryophyllene; 67. cis-muurola-4(14),5-diene; 71. Δ-cadinene.

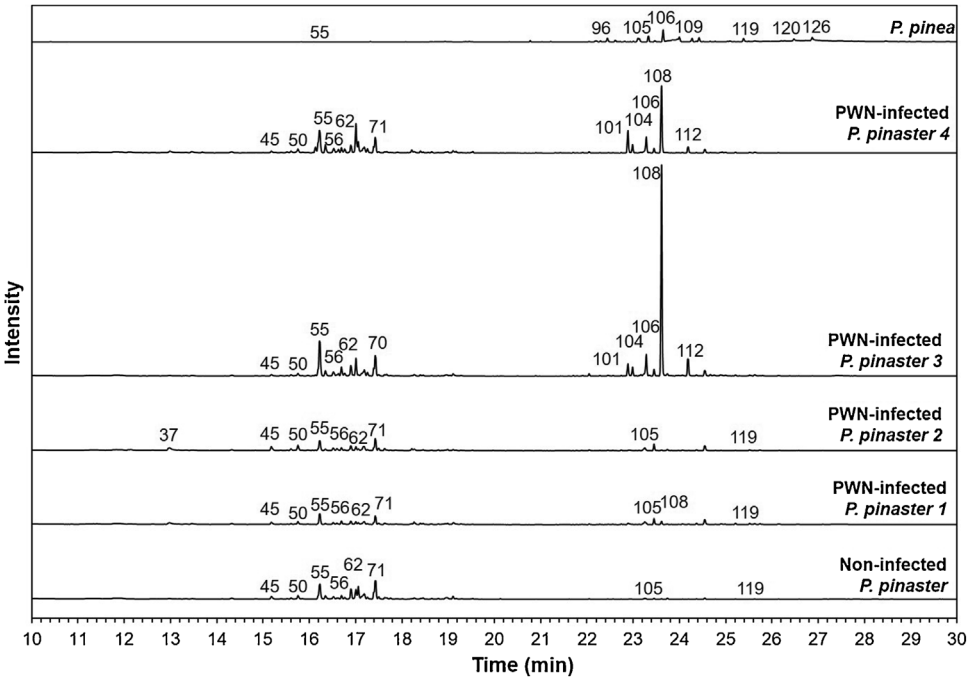


Fig. 5. Main volatile compounds extracted by $scCO_2$ from *Pinus pinea* trees, and pinewood nematode (PWN)-infected and non-infected *P. pinaster* trees. Main extracted compounds between 10 and 30 min (numbered according to Table S1): 37. α- or β-fenchene; 45. α-cubebene; 50. γ-amorphene; 55. β-caryophyllene; 56. β-copaene; 62. α-humulene; 70. cadina-1(6),4-diene; 71. Δ-cadinene; 96. di(methylphenyl)ester phtalic acid; 101. rimuene; 104. lovopimaradiene; 105. simonellite; 106. abietatriene; 108. abietadiene; 109. (11E,13Z)-labdadien-8-ol; 112. abieta-8(14),13(15)-diene; 119. methyl dehydroabietate; 120. dehydroabietic acid; 126. abietic acid.

presence of manool oxide, abietatriene, and other compounds was already reported in needles [75], as well as the identification of (11E,13Z)-labdadien-8-ol in foliage essential oils [74].

Interestingly, abietadiene was identified in some $scCO_2$ extracts from the PWN-infected *P. pinaster* samples (Fig. 5 and Table S1). This compound is the precursor of abietic acid, which is produced by conifer species as a defensive secretion against insect and pathogen

attacks [78]. In the PWN-trees 3 and 4, this compound was the predominant extracted volatile, accounting for 34 and 15 % of the total peak areas, respectively. The presence of this compound may also be associated to a response produced by the tree against pathogenic attack, and other authors have already identified this compound in volatile oils of *P. pinaster* wood, cones and needles [7]. Moreover, other abietic acid-related compounds such as methyl dehydroabietate and abietatriene were present in some infected *P. pinaster* samples and in *P. pinea*, and this finding is in accordance with the literature [75,79]. This may, once again, be related to a defensive response from the trees. In the case of *P. pinea* trees, it is related with their natural morphological and physiological properties, while for infected *P. pinaster* trees, a modification in their composition appear to occur, when the infection is established.

Drying the samples carefully before the extraction procedure is another possibility to evaluate whether the water has some influence on the volatile compounds that are extracted.

Similarly, to the extracts, the lowest molecular weight compounds were not identified in the correspondent glass wool packed columns, which indicates the most volatile compounds are not extracted with $scCO_2$, in the applied conditions and with the equipment that was used.

In addition, $scCO_2$ extraction removed from branches around 5–20 compounds with higher molecular weight, which were not extracted by SPME technique. In spite of SPME being a fast procedure to separate volatile compounds, the $scCO_2$ extraction could also be used to compare samples according to their composition, since this technique can separate a wide range of compounds (from C8 to C21, or more).

3.4. Acetylcholinesterase inhibitory activity

Based on volatile composition of pine samples, the AChE inhibitory activity assay was carried out to infer about the possible nematicidal/insecticidal activity of $scCO_2$ extracts. Results are expressed as IC_{50} values and included in Table 2.

The inhibition of this enzyme leads to accumulation of acetylcholine and extreme neuro excitation, resulting in tremors, convulsions and paralysis, and utmost in death. It is by this mechanism that some insecticides/nematicides act, including on the PWN, which also present AChE enzymes [43,80,81].

Pinus pinaster extracts had IC_{50} values in the 7−11 mg/mL range (Table 2), independently of PWN infection. On the other side, *P. pinea* volatile oils revealed a wide range of IC_{50} values, from 2 to > 40 mg/mL. Note that for *P. pinea*, sample 4, an extract concentration of 40 mg/mL resulted in only 30 % AChE inhibition and therefore, the IC_{50} value was not calculated.

The observed variability may be related to higher molecular weight compounds, which may be present in the extracts, and were not detected by GC/MS analysis. Such compounds may be waxes that may be responsible for the effect and/or for some degree of turbidity conferred by the extract to the assay. In fact, another explanation may be the interference from the yellow color (and turbidity) of some extracts, in spite of the control that was included in the AChE assay, with all reagents and the extract (without the enzyme).

The obtained IC_{50} values of the pine branches $scCO_2$ extracts revealed their possible action as insecticides/nematicides. Furthermore, insecticidal activity of *P. pinea* resin essential oils was already found using *in vitro* methodologies [82]. The effect of several organophosphates and carbamates on three recombinant AChE enzymes of PWN have already been studied by other authors, which concluded that the inhibition results are dependent on the enzyme [43]. The inhibition of AChE extracted from the PWN, has been also tested *in vitro*, with plant-extracts [83]. The effect of many compounds, including monoterpenes, phenylpropenes, sesquiterpenes and sulfides, on three different PWN AChE enzymes has also been determined and the monoterpene α-pinene revealed high inhibitory effects [34]. This volatile compound is, however, not present in high percentages in the obtained pine extracts. Other authors have found inhibition of AChE by phenolic compounds extracted from the plant *Urginea maritima,* with IC_{50} = 66 μg/mL [84], which indicates that extract appears to be more potent than those obtained in this study. Others evaluated the larvicidal activity of the hydrodistillate from *P. densiflora* needles and obtained IC_{50} < 2 mM, for thymol and δ-3-carene [81].

Further studies with AChE enzymes extracted from *B. xylophilus* should be conducted to confirm if this effect is observed.

3.5. In vitro *pinewood nematode mortality*

The effect of pine extracts on PWN mortality was also assessed, and mortality data was converted to percentage cumulative mortality and corrected by Schneider Orelli's formula considering the Triton X-100. Corrected PWN mortality in all extracts at different concentrations (0.5, 1 and 2 mg/mL) from the two *Pinus* species was evaluated. In preliminary studies, higher concentrations (5 and 10 mg of extract per mL of Triton X-100 solution at 5000 ppm) were tested for one non-infected and one PWN-infected tree. However, the extract-Triton X-100 solutions were too cloudy/opaque making unfeasible the nematode visualization and quantification. Thus, three concentrations were defined for all the extracts (0.5, 1 and 2 mg/mL). Results from these three concentrations revealed no effects on nematode mortality (Table S2), in spite of the potential activity of *P. pinaster* and *P. pinea* volatile oils against the AChE. Nevertheless, the concentrations that were found to be active against the AChE enzyme, in the *in vitro* assay, were in general higher (IC_{50} ∼ 10 mg/mL) than those tested with the PWN (0.5–2 mg/mL). Other authors have already tested the effect of many plant-extracts on PWN mortality [85], and some of them compared those results with their action on the AChE enzyme, by using extracted enzymes [35,83]. Some plant-extracts revealed no PWN mortality with a concentration of 2.5 mg/mL, similarly to our results [54]. Moreover, essential oils from two gymnosperms genera *Cryptomeria* and *Juniperus,* also showed no nematode mortality [86]. In order to better understand the possible effects of pine extracts on the PWN development, more studies focused on biological effects on nematode morphological/physiological characteristics (reproduction, population growth, fecundity, egg hatchability and dispersal ability) in addition to gene expression and function analyses, should be performed.

4. Conclusions

Distinct moisture contents, volatile composition and supercritical kinetic profiles were achieved for PWN-infected and non-infected *P. pinaster* trees, as well as for *P. pinea* trees. High moisture contents were found for *P. pinea* samples (> 50 %), and slightly lower values were accomplished for non-infected *P. pinaster* samples (∼ 35–40 %). The lowest moisture contents were obtained for PWN-infected *P. pinaster* samples (∼ 10–30 %), being this related to the PWN number. *P. pinea* and some PWN-infected *P. pinaster* branches emitted limonene in high percentages, being this terpene known as repellent. Lower extraction yields were observed for *P. pinea* samples, and the higher moisture content may explain this finding. Abietadiene was present in some extracts from the PWN-infected *P. pinaster* samples, which may correspond to a tree defensive response against the insect/nematode attack. Also, other compounds structurally-related to abietic acid, were identified in *P. pinea* and in some PWN-infected *P. pinaster* volatile oils. More-

over, $scCO_2$ was able to extract about 5–20 compounds with higher molecular weight than those extracted by SPME technique.

Extracts revealed inhibitory activity on AChE, which could be associated to a potential insecticidal/nematicidal effect. However, no nematicidal activity was found. Further studies are, therefore, needed to conclude about the influence of extracts on PWN physiological/morphological characteristics.

Acknowledgements

This work was financially supported by COMPETE 2020, Fundação para a Ciência e Tecnologia (FCT, Portugal), through the Programa de Atividades Conjuntas (PAC), Project "MultiBiorefinery - POCI-01-0145-FEDER-016403"; Project ECOVECTOR - POCI-01-0145-FEDER-016820; Project ReNATURE-CENTRO-01-0145-FEDER-000007; and FCT-MEC (PEst-C/EQB/UI0102/2013, Est-C/EQB/UI0102/2018, PEst-C/EQB/UI0102/2019 and UID/BIA/04004/2019), and IATV-Instituto do Ambiente, Tecnologia e Vida. M. C. Gaspar acknowledges FCT for the financial support through the Scientific Employment Stimulus 2017 (CEECIND/00527/2017), and post-doctoral fellowship from Multi-Biorefinery project. The authors would like to thank to João Leocádio for the help provided in the pinewood nematode mortality assays. The authors thank to Engineer José Bernardino Dias (ICNF) for his contribution in the trees selection and branches collection.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.supflu.2020.104784.

References

[1] Centro PINUS, A Fileira Do Pinho Em, Indicadores Da Fileira Do Pinho, 2018 www.centropinus.org.
[2] ICNF, 6º Inventário Florestal Nacional: Áreas Dos Usos Do Solo E Das Espécies Florestais De Portugal Continental, Resultados preliminares, Instituto da Conservação da Natureza e das Florestas, I.P., Lisboa, 2013, pp. 34.
[3] R. Abad Viñas, G. Caudullo, S. Oliveira, D. de Rigo, Pinus pinaster in Europe: distribution, habitat, usage and threats, in: J. San-Miguel-Ayanz, D. de Rigo, G. Caudullo, T.H. Durrant, A. Mauri (Eds.), European Atlas of Forest Tree Species, Publication Office of the European Union, Luxembourg, 2016.
[4] G. Louro, M. Monteiro, L. Constantino, F. Rego, The Portuguese forest based chains: sector analyses, in: F. Reboredo (Ed.), Forest Context and Policies in Portugal, Springer, Cham, Switzerland, 2014, pp. 39–65, http://dx.doi.org/10.1007/978-3-319-08455-8.
[5] R. Abad Viñas, G. Caudullo, S. Oliveira, D. de Rigo, Pinus pinea in Europe: distribution, habitat, usage and threats, in: J. San-Miguel-Ayanz, D. de Rigo, G. Caudullo, T.H. Durrant, A. Mauri (Eds.), European Atlas of Forest Tree Species, Publication Office of the European Union, Luxembourg, 2016.
[6] F. Aljos, A Handbook of the World's Conifers (2 vols.), Brill, Leiden, The Netherlands, 2010, http://dx.doi.org/10.1163/9789047430629.
[7] İ. Tümen, E.K. Akkol, H. Taştan, I. Süntar, M. Kurtca, Research on the antioxidant, wound healing, and anti-inflammatory activities and the phytochemical composition of maritime pine (*Pinus pinaster* Ait), J. Ethnopharmacol. 211 (2018) 235–246, http://dx.doi.org/10.1016/j.jep.2017.09.009.
[8] S. Xiao, R. Gao, Y. Lu, J. Li, Q. Sun, Fabrication and characterization of nanofibrillated cellulose and its aerogels from natural pine needles, Carbohydr. Polym. 119 (2015) 202–209, http://dx.doi.org/10.1016/j.carbpol.2014.11.041.
[9] D. Pimentel, S. McNair, J. Janecka, J. Wightman, C. Simmonds, C. O'Connell, E. Wong, L. Russel, J. Zern, T. Aquino, T. Tsomondo, Economic and environmental threats of alien plant, animal, and microbe invasions, Agriculture, Ecosystems & Environ. 84 (2001) 1–20, http://dx.doi.org/10.1016/S0167-8809(00)00178-X.
[10] C. Vicente, M. Espada, P. Vieira, M. Mota, Pine wilt disease: a threat to european forestry, Eur. J. Plant Pathol. 133 (2012) 89–99, http://dx.doi.org/10.1007/s10658-011-9924-x.
[11] European and Mediterranean Plant Protection Organization (E.P.P.O.), EPPO A2 List of pests recommended for regulation as quarantine pests. Version 2019/09. https://www.eppo.int/ACTIVITIES/plant_quarantine/A2_list.
[12] M.M. Mota, H. Braasch, M.A. Bravo, A.C. Penas, W. Burgermeister, K. Metge, E. Sousa, First report of *Bursaphelenchus xylophilus* in Portugal and in Europe, Nematology 1 (1999) 727–734, http://dx.doi.org/10.1163/156854199508757.
[13] A. Abelleira, A. Picoaga, J.P. Mansilla, O. Aguin, Detection of *Bursaphelenchus Xylophilus*, Causal agent of pine wilt disease on *Pinus pinaster* in Northwestern Spain, Plant Dis. 95 (2011) 776, http://dx.doi.org/10.1094/PDIS-12-10-0902.
[14] L. Fonseca, J. Cardoso, A. Lopes, M. Pestana, F. Abreu, N. Nunes, M. Mota, I. Abrantes, The pinewood nematode, *Bursaphelenchus xylophilus*, in Madeira Island, Helminthologia 49 (2012) 96–103, http://dx.doi.org/10.2478/s11687-012-0020-3.
[15] H.F. Evans, D.G. McNamara, H. Braasch, J. Chadoeuf, C. Magnusson, Pest Risk Analysis (PRA) for the territories of the European Union (as PRA area) on *Bursaphelenchus xylophilus* and its vectors in the genus *Monochamus*, Eppo Bull. 26 (1996) 199–249, http://dx.doi.org/10.1111/j.1365-2338.1996.tb00594.x.
[16] L. Fonseca, J. Cardoso, I. Abrantes, Plant-nematode, in: E. Sousa, F. Vale, I. Abrantes (Eds.), Pine Wilt Disease in Europe – Biological Interactions and Integrated Management, Federação Nacional das Associações de Proprietários Florestais (FNAPF), Portugal, 2015, pp. 35–78.
[17] E. Sousa, M. Bravo, J. Pires, P. Naves, A. Penas, L. Bonifacio, M. Mota, *Bursaphelenchus xylophilus* (Nematoda; Aphelenchoididae) associated with *Monochamus galloprovincialis* (Coleoptera; Cerambycidae) in Portugal, Nematology 3 (2001) 89–91, http://dx.doi.org/10.1163/156854101300106937.
[18] K. Kuroda, Physiological incidences related to symptom development and wilting mechanism, in: B.G. Zhao, K. Futai, J.R. Sutherland, Y. Takeuchi (Eds.), Pine Wilt Disease, Springer, Tokyo, 2008, pp. 204–222, http://dx.doi.org/10.1007/978-4-431-75655-2.
[19] P.N. Firmino, T. Calvão, M.P. Ayres, C.S. Pimentel, *Monochamus galloprovincialis* and *Bursaphelenchus xylophilus* life history in an area severely affected by pine wilt disease: implications for forest management, For. Ecol. Manage. 389 (2017) 105–115, http://dx.doi.org/10.1016/j.foreco.2016.12.027.
[20] J.M. Rodrigues, E. Sousa, I. Abrantes, Pine wilt disease historical overview, in: E. Sousa, F. Vale, I. Abrantes (Eds.), Pine Wilt Disease in Europe: Biological Interactions and Integrated Management, FNAPF - Federação Nacional das Associações de Proprietários Florestais, Portugal, 2015, pp. 13–32.
[21] E. Sanchez-Husillos, G. Álvarez-Baz, I. Etxebeste, J.A. Pajares, Shoot feeding, oviposition, and development of *Monochamus galloprovincialis* on *Pinus pinea* relative to other pine species, Entomol. Exp. Appl. 149 (2013) 1–10, http://dx.doi.org/10.1111/eea.12105.
[22] C.S. Santos, M. Pinheiro, A.I. Silva, C. Egas, M.W. Vasconcelos, Searching for resistance genes to *Bursaphelenchus xylophilus* using high throughput screening, BMC Genomics 13 (2012) 599.
[23] P. Naves, E. Sousa, J. Quartau, Feeding and oviposition preferences of *Monochamus galloprovincialis* for certain conifers under laboratory conditions, Entomol. Exp. Appl. 120 (2006) 99–104, http://dx.doi.org/10.1111/j.1570-7458.2006.00430.x.
[24] A.M. Santos, M. Vasconcelos, E. Mateus, H. Farrall, M. Silva, M. Paiva, M. Branco, Characterization of the volatile fraction emitted by phloems of four *pinus* species by solid-phase microextraction and gas chromatography-mass spectrometry, J. Chromatogr. A 1105 (2006) 191–198, http://dx.doi.org/10.1016/j.chroma.2005.10.049.
[25] L.F. Bonifacio, E. Sousa, P. Naves, M.L. Inacio, J. Henriques, M. Mota, P. Barbosa, M.J. Drinkall, S. Buckley, Efficacy of sulfuryl fluoride against the pinewood nematode, *Bursaphelenchus xylophilus* (Nematoda: aphelenchidae), in *Pinus pinaster* boards, Pest Manag. Sci. 70 (2014) 6–13, http://dx.doi.org/10.1002/ps.3507.
[26] R. James, N. Tisserat, T. Todd, Prevention of pine wilt of scots pine (*Pinus sylvestris*) with systemic abamectin injections, Arboric. Urban For. 32 (2006) 195–201.
[27] G. Hussain Mallah, Review of the current status of insecticide resistance in insect pests of cotton and their management, Pak. J. Bot. 39 (2007) 2699–2703.
[28] J.R. Roberts, J.R. Reigart, Recognition and Management of Pesticide Poisonings, Environmental Protection Agency, 6th edition, USA, 2013.
[29] L.M. Torres-Vila, C. Zugasti, J.M. De-Juan, M.J. Oliva, C. Montero, F.J. Mendiola, Y. Conejo, Á. Sánchez, F. Fernández, F. Ponce, G. Espárrago, Mark-recapture of *Monochamus galloprovincialis* with semiochemical-baited traps: population density, attraction distance, flight behaviour and mass trapping efficiency, Forestry: An Int. J. Forest Res. 88 (2015) 224–236, http://dx.doi.org/10.1093/forestry/cpu049.
[30] D. Rassati, E.P. Toffolo, A. Battisti, M. Faccoli, Monitoring of the pine sawyer beetle *Monochamus galloprovincialis* by pheromone traps in Italy, Phytoparasitica 40 (2012) 329–336, http://dx.doi.org/10.1007/s12600-012-0233-5.
[31] G. Álvarez, D. Gallego, D.R. Hall, H. Jactel, J.A. Pajares, Combining pheromone and kairomones for effective trapping of the pine sawyer beetle *Monochamus galloprovincialis*, J. Appl. Entomol. 140 (2016) 58–71, http://dx.doi.org/10.1111/jen.12297.
[32] E. Sousa, F. Vale, I. Abrantes, Pine Wilt Disease in Europe: Biological Interactions and Integrated Management, 1 ed., FNAPF - Federação Nacional das Associações de Proprietários Florestais, Portugal, 2015.
[33] C. Regnault-Roger, C. Vincent, J.T. Arnason, Essential oils in insect control: low-risk products in a high-stakes world, Annu. Rev. Entomol. 57 (2012) 405–424.
[34] J.S. Kang, E. Kim, S.H. Lee, I.-K. Park, Inhibition of acetylcholinesterases of the pinewood nematode, *Bursaphelenchus xylophilus*, by phytochemicals from

plant essential oils, Pestic. Biochem. Physiol. 105 (2013) 50–56, http://dx.doi.org/10.1016/j.pestbp.2012.11.007.
[35] J.M.S. Faria, P. Barbosa, R.N. Bennett, M. Mota, A.C. Figueiredo, Bioactivity against *Bursaphelenchus xylophilus*: nematotoxics from essential oils, essential oils fractions and decoction waters, Phytochemistry 94 (2013) 220–228, http://dx.doi.org/10.1016/j.phytochem.2013.06.005.
[36] X.-J. Li, G.-P. Dong, J.-M. Fang, H.-J. Liu, W.-L. Guo, Comparison of volatile organic compounds from uninfested and *Monochamus alternatus Hope* infested *Pinus massoniana Lamb*, Entomol. Res. 47 (2017) 203–207, http://dx.doi.org/10.1111/1748-5967.12209.
[37] C. Santos, M. Vasconcelos, Resposta fisiológica de *Pinus* spp. Nas primeiras horas após infecção com *Bursaphelenchus xylophilus* (Nematoda: aphelenchoididae), Silva Lusit. 19 (2011) 99–110, http://dx.doi.org/10.1016/j.chroma.2005.10.049.
[38] J.U. Rehman, A. Ali, I.A. Khan, Plant based products: use and development as repellents against mosquitoes: a review, Fitoterapia 95 (2014) 65–74, http://dx.doi.org/10.1016/j.fitote.2014.03.002.
[39] J.S. Kang, D.W. Lee, Y.H. Koh, S.H. Lee, A soluble acetylcholinesterase provides chemical defense against xenobiotics in the pinewood nematode, PLoS One 6 (2011), e19063, http://dx.doi.org/10.1371/journal.pone.0019063.
[40] C. Mills, B.J. Cleary, J.F. Gilmer, J.J. Walsh, Inhibition of acetylcholinesterase by Tea Tree oil, J. Pharm. Pharmacol. 56 (2004) 375–379, http://dx.doi.org/10.1211/0022357022773.
[41] T. Nabeshima, A. Mori, T. Kozaki, Y. Iwata, O. Hidoh, S. Harada, S. Kasai, D.W. Severson, Y. Kono, T. Tomita, An amino acid substitution attributable to insecticide-insensitivity of acetylcholinesterase in a Japanese encephalitis vector mosquito, *Culex tritaeniorhynchus*, Biochem. Biophys. Res. Commun. 313 (2004) 794–801, http://dx.doi.org/10.1016/j.bbrc.2003.11.141.
[42] J. Massoulié, L. Pezzementi, S. Bon, E. Krejci, F.-M. Vallette, Molecular and cellular biology of cholinesterases, Prog. Neurobiol. 41 (1993) 31–91, http://dx.doi.org/10.1016/0301-0082(93)90040-Y.
[43] J.S. Kang, Y.S. Moon, S.H. Lee, Inhibition properties of three acetylcholinesterases of the pinewood nematode *Bursaphelenchus xylophilus* by organophosphates and carbamates, Pestic. Biochem. Physiol. 104 (2012) 157–162, http://dx.doi.org/10.1016/j.pestbp.2012.06.002.
[44] A.G. Whitehead, J.R. Hemming, A comparison of some quantitative methods of extracting small vermiform nematodes from soil, Ann. Appl. Biol. 55 (1965) 25–38, http://dx.doi.org/10.1111/j.1744-7348.1965.tb07864.x.
[45] European and Mediterranean Plant Protection Organization (E.P.P.O.), PM 7/4 (3) bursaphelenchus xylophilus, Eppo Bull. 43 (2013) 105–118, http://dx.doi.org/10.1111/epp.12024.
[46] L. Fonseca, C. Vieira dos Santos, M.N. Santos, R. Curtis, I. Abrantes, Morpho-biometrical characterisation of Portuguese *Bursaphelenchus xylophilus* isolates with mucronate, digitate or round tailed females, Phytopathol. Mediterr. 47 (2009) 223–233, http://dx.doi.org/10.14601/Phytopathol_Mediterr-2726.
[47] V.R. Ferris, J.M. Ferris, J. Faghihi, Variation in spacer ribosomal DNA in some cyst-forming species of plant parasitic nematodes, Fundam. Appl. Nematol. 16 (1993) 177–184.
[48] T.C. Vrain, Restriction fragment length polymorphism separates species of the Xiphinema americanum group, J. Nematol. 25 (1993) 361–364.
[49] Q. Ren, Y. Jin, Y. Hu, H. Chen, Z. Li, Rapid changes of induced volatile organic compounds in *Pinus massoniana*, Front. For. China 2 (2007) 459–465, http://dx.doi.org/10.1007/s11461-007-0073-5.
[50] N.A. Gañán, A.M.A. Dias, F. Bombaldi, J.A. Zygadlo, E.A. Brignole, H.C. de Sousa, M.E.M. Braga, Alkaloids from *Chelidonium majus* L.: fractionated supercritical CO_2 extraction with co-solvents, Sep. Purif. Technol. 165 (2016) 199–207, http://dx.doi.org/10.1016/j.seppur.2016.04.006.
[51] R. Szmigielski, M. Cieslak, K.J. Rudziński, B. Maciejewska, Identification of volatiles from *Pinus silvestris* attractive for *Monochamus galloprovincialis* using a SPME-GC/MS platform, Environ. Sci. Pollut. Res. - Int. 19 (2012) 2860–2869, http://dx.doi.org/10.1007/s11356-012-0792-5.
[52] G.L. Ellman, K.D. Courtney, V. Andres, R.M. Featherstone, A new and rapid colorimetric determination of acetylcholinesterase activity, Biochem. Pharmacol. 7 (1961) 88–95, http://dx.doi.org/10.1016/0006-2952(61)90145-9.
[53] A. Ferreira, C. Proença, M.L.M. Serralheiro, M.E.M. Araújo, The *in vitro* screening for acetylcholinesterase inhibition and antioxidant activity of medicinal plants from Portugal, J. Ethnopharmacol. 108 (2006) 31–37, http://dx.doi.org/10.1016/j.jep.2006.04.010.
[54] E. Kim, Il-K. Park, J. Kim, Nematicidal activity of Korean domestic plant extracts against the pine wood nematode, *Bursaphelenchus xylophilus*, Nematology 13 (2011) 377–380, http://dx.doi.org/10.1163/138855410X543517.
[55] W. Puntener, Manual for Field Trials in Plant Protection, 2 ed., Ciba-Geiji Limited, Basle, Switzerland, 1981, pp. 205.
[56] N. Ratola, J.M. Amigo, M.S.N. Oliveira, R. Araújo, J.A. Silva, A. Alves, Differences between *Pinus pinea* and *Pinus pinaster* as bioindicators of polycyclic aromatic hydrocarbons, Environ. Exp. Bot. 72 (2011) 339–347, http://dx.doi.org/10.1016/j.chroma.2006.03.110.
[57] C. Shi, C. Wang, X. Xu, B. Huang, L. Wu, D. Yang, Comparison of bacterial communities in soil between nematode-infected and nematode-uninfected *Pinus massoniana* pinewood forest, Appl. Soil Ecol. 85 (2015) 11–20, http://dx.doi.org/10.1016/j.apsoil.2014.08.008.
[58] L.L. Zhao, W. Wei, L. Kang, J.H. Sun, Chemotaxis of the Pinewood nematode, *Bursaphelenchus xylophilus*, to volatiles associated with host pine, *Pinus massoniana*, and its vector *Monochamus alternatus*, J. Chem. Ecol. 33 (2007) 1207–1216, http://dx.doi.org/10.1007/s10886-007-9289-y.
[59] M. Nunes da Silva, A. Solla, L. Sampedro, R. Zas, M.W. Vasconcelos, Susceptibility to the pinewood nematode (PWN) of four pine species involved in potential range expansion across Europe, Tree Physiology 35 (2015) 987–999, http://dx.doi.org/10.1093/treephys/tpv046.
[60] W. Burgermeister, H. Braasch, K. Metge, J. Gu, T. Schröder, E. Woldt, ITS-RFLP analysis, an efficient tool for differentiation of *Bursaphelenchus* species, Nematology 11 (2009) 649–668, http://dx.doi.org/10.1163/156854108/399182.
[61] S.M. Pourmortazavi, S.S. Hajimirsadeghi, Supercritical fluid extraction in plant essential and volatile oil analysis, J. Chromatogr. A 1163 (2007) 2–24, http://dx.doi.org/10.1016/j.chroma.2007.06.021.
[62] O. Catchpole, S. Tallon, P. Dyer, F. Montanes, T. Moreno, E. Vági, W. Eltringham, J. Billakanti, Integrated supercritical fluid extraction and bioprocessing, Am. J. Biochem. Biotechnol. 8 (2013) 263–287, http://dx.doi.org/10.3844/ajbbsp.2012.263.287.
[63] I.J. Seabra, A.M.A. Dias, M.E.M. Braga, H.C. de Sousa, High pressure solvent extraction of maritime pine bark: study of fractionation, solvent flow rate and solvent composition, J. Supercrit. Fluids 62 (2012) 135–148, http://dx.doi.org/10.1016/j.supflu.2011.10.016.
[64] E. Duquesnoy, B. Marongiu, V. Castola, A. Piras, S. Porcedda, J. Casanova, Supercritical CO_2 extract from needles of *Pinus nigra* ssp. Laricio : combined analysis by GC, GC–MS and ^{13}C NMR, Nat. Prod. Res. 21 (2007) 834–837, http://dx.doi.org/10.1080/14786410701427280.
[65] G. Brunner, Gas Extraction: an Introduction to Fundamentals of Supercritical Fluids and the Application to Separation Processes, Steinkopff ; Springer, Darmstadt; New York, 1994, pp. 387, http://dx.doi.org/10.1007/978-3-662-07380-3.
[66] Ö. Özgenç, S. Durmaz, G. Çelik, B. Korkmaz, N. Yaylı, Comparative phytochemical analysis of volatile organic compounds by SPME-GC-FID/MS from six coniferous and nine deciduous tree bark species grown in Turkey, South Afr. J. Bot. 113 (2017) 23–28, http://dx.doi.org/10.1016/j.sajb.2017.07.004.
[67] F. Macchioni, P.L. Cioni, G. Flamini, I. Morelli, S. Maccioni, M. Ansaldi, Chemical composition of essential oils from needles, branches and cones of *Pinus pinea*, *P. halepensis, P. Pinaster* and *P. Nigra* from central Italy, Flavour Fragr. J. 18 (2003) 139–143, http://dx.doi.org/10.1002/ffj.1178.
[68] M. Faccoli, G. Anfora, M. Tasin, Stone pine volatiles and host selection by *Tomicus destruens* (Wollaston) (Coleoptera: curculionidae, Scolytidae), Silva Lusit. 19 (2011) 61–73.
[69] M.A. Ibrahim, P. Kainulainen, A. Aflatuni, Insecticidal, repellent, antimicrobial activity and phytotoxicity of essential oils: with special reference to limonene and its suitability for control of insect pests, Agric. Food Sci. 10 (2001) 243–259.
[70] A.M. Rodrigues, M.D. Mendes, A.S. Lima, P.M. Barbosa, L. Ascensão, J.G. Barroso, L.G. Pedro, M.M. Mota, A.C. Figueiredo, *Pinus halepensis*, *Pinus pinaster*, *Pinus pinea* and *Pinus sylvestris* essential oils chemotypes and monoterpene hydrocarbon enantiomers, before and after inoculation with the Pinewood nematode *Bursaphelenchus xylophilus*, Chem. Biodivers. 14 (2017), http://dx.doi.org/10.1002/cbdv.201600153, e1600153-n/a.
[71] Q. Liu, Y. Wei, L. Xu, Y. Hao, X. Chen, Z. Zhou, Transcriptomic profiling reveals differentially expressed genes associated with pine wood nematode resistance in masson pine (*Pinus massoniana Lamb.*), Sci. Rep. 7 (2017) 4693.
[72] L.S. Bullington, Y. Lekberg, R. Sniezko, B. Larkin, The influence of genetics, defensive chemistry and the fungal microbiome on disease outcome in whitebark pine trees, Mol. Plant Pathol. 19 (2018) 1847–1858, http://dx.doi.org/10.1111/mpp.12663.
[73] M.R.M. Lima, A.C.S. Ferreira, M.W. Vasconcelos, Produção diferencial de compostos voláteis em caules de *Pinus pinaster* inoculados com o nemátode da madeira do pinheiro (*Bursaphelenchus xylophilus*), Cuadernos de la Sociedad Española de Ciencias Forestales 36 (2012) 27–32.
[74] V. Roussis, P.V. Petrakis, A. Ortiz, B.E. Mazomenos, Volatile constituents of needles of five *Pinus* species grown in Greece, Phytochemistry 39 (1995) 357–361, http://dx.doi.org/10.1016/0031-9422(94)00885-W.
[75] N. Nasri, N. Tlili, S. Triki, W. Elfalleh, I. Chéraif, A. Khaldi, Volatile constituents of *Pinus pinea* L. Needles, J. Essent. Oil Res. 23 (2011) 15–19, http://dx.doi.org/10.1080/10412905.2011.9700441.
[76] J.C. Francisco, B. Sivik, Solubility of three monoterpenes, their mixtures and eucalyptus leaf oils in dense carbon dioxide, J. Supercrit. Fluids 23 (2002) 11–19, http://dx.doi.org/10.1016/S0896-8446(01)00131-0.
[77] I. Amri, S. Gargouri, L. Hamrouni, M. Hanana, T. Fezzani, B. Jamoussi, Chemical composition, phytotoxic and antifungal activities of *Pinus pinea* essential oil, J. Pest Sci. 85 (2012) 199–207, http://dx.doi.org/10.1007/s10340-012-0419-0.
[78] M.C. Gaspar, H.C. de Sousa, I.J. Seabra, M.E.M. Braga, Conference proceeding ON17. Extracting the natural insecticide abietadiene from *P. pinaster* branches using $scCO_2$, in: 12th International Symposium on Supercritical Fluids, Antibes-Juan-Les-Pins, France, 2018, p. 6.
[79] H. Erdtman, L. Westfelt, The neutral diterpenes from pine wood resin, Acta Chem. Scand. 17 (1963).
[80] T.R. Fukuto, Mechanism of action of organophosphorus and carbamate insecticides, Environ. Health Perspect. 87 (1990) 245–254, https://doi.org/10.1289ehp.9087245.
[81] C.D. Lee, Y.-J. Ahn, Laboratory and simulated field bioassays to evaluate larvicidal activity of *Pinus densiflora* hydrodistillate, its constituents and structurally related compounds against *Aedes albopictus*, *Aedes aegypti* and

Culex pipiens pallens in relation to their inhibitory effects on acetylcholinesterase activity, Insects 4 (2013) 217–229, http://dx.doi.org/10.3390/insects4020217.

[82] Z. Ulukanli, S. Karabörklü, F. Bozok, B. Ates, S. Erdogan, M. Cenet, M.G. Karaaslan, Chemical composition, antimicrobial, insecticidal, phytotoxic and antioxidant activities of Mediterranean *Pinus brutia* and *Pinus pinea* resin essential oils, Chin. J. Nat. Med. 12 (2014) 901–910, http://dx.doi.org/10.1016/S1875-5364(14)60133-3.

[83] Q. Guo, G. Du, H. Qi, Y. Zhang, T. Yue, J. Wang, R. Li, A nematicidal tannin from *Punica granatum* L. Rind and its physiological effect on pine wood nematode (*Bursaphelenchus xylophilus*), Pestic. Biochem. Physiol. 135 (2017) 64–68, http://dx.doi.org/10.1016/j.pestbp.2016.06.003.

[84] A.M. Maazoun, T.B. Hlel, S.H. Hamdi, F. Belhadj, J.M.B. Jemâa, M.N. Marzouki, Screening for insecticidal potential and acetylcholinesterase activity inhibition of *Urginea maritima* bulbs extract for the control of *Sitophilus oryzae* (L.), J. Asia Pac. Entomol. 20 (2017) 752–760.

[85] P. Barbosa, J.M.S. Faria, M.D. Mendes, L.S. Dias, M.T. Tinoco, J.G. Barroso, L.G. Pedro, A.C. Figueiredo, M. Mota, Bioassays against pinewood nematode: assessment of a suitable dilution agent and screening for bioactive essential oils, Molecules 17 (2012) 12312–12329, http://dx.doi.org/10.3390/molecules171012312.

[86] P. Barbosa, A.S. Lima, P. Vieira, L.S. Dias, M.T. Tinoco, J.G. Barroso, L.G. Pedro, A.C. Figueiredo, M. Mota, Nematicidal activity of essential oils and volatiles derived from Portuguese aromatic flora against the pinewood nematode, *Bursaphelenchus xylophilus*, J. Nematol. 42 (2010) 8–16.