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ORIGINAL ARTICLE

In vitro co-cultures of *Pinus pinaster* with *Bursaphelenchus xylophilus*: a biotechnological approach to study pine wilt disease

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Abstract
Main conclusion **Co-cultures of *Pinus pinaster* with *Bursaphelenchus xylophilus* were established as a biotechnological tool to evaluate the effect of nematotoxics addition in a host/parasite culture system.**

The pinewood nematode (PWN), *Bursaphelenchus xylophilus*, the causal agent of pine wilt disease (PWD), was detected for the first time in Europe in 1999 spreading throughout the pine forests in Portugal and recently in Spain. Plant in vitro cultures may be a useful experimental system to investigate the plant/nematode relationships in loco, thus avoiding the difficulties of field assays. In this study, *Pinus pinaster* in vitro cultures were established and compared to in vivo 1 year-old plantlets by analyzing shoot structure and volatiles production. In vitro co-cultures were established with the PWN and the effect of the phytoparasite on in vitro shoot structure, water content and volatiles production was evaluated. In vitro shoots showed similar structure and volatiles production to in vivo maritime pine plantlets. The first macroscopic symptoms of PWD were observed about 4 weeks after in vitro co-culture establishment. Nematode population in the culture medium increased and PWNs were detected in gaps of the callus tissue and in cavities developed from the degradation of cambial cells. In terms of volatiles main components, plantlets, *P. pinaster* cultures, and *P. pinaster* with *B. xylophilus* co-cultures were all β- and α-pinene rich. Co-cultures may be an easy-to-handle biotechnological approach to study this pathology, envisioning the understanding of and finding ways to restrain this highly devastating nematode.

Keywords Maritime pine · Monoxenic culture · Pinewood nematode · Relative water content · Shoots structure · Volatiles

Abbreviations

BAP	6-Benzylaminopurine
DAI	Days after inoculation
EPPO	European and Mediterranean Plant Protection Organization

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GC	Gas chromatography
GC–MS	Gas chromatography coupled to mass spectrometry
IBA	Indole-3-butyric acid
LM	Light microscopy
PAS	Periodic acid–Schiff's reagent
PWD	Pine wilt disease
PWN	Pinewood nematode (*Bursaphelenchus xylophilus*)
RI	Retention index
RWC	Relative water content
SEM	Scanning electron microscopy
SH	Schenk and Hildebrandt medium
SHe	Schenk and Hildebrandt elongation medium
SHm	Schenk and Hildebrandt multiplication medium
t	Trace

Introduction

The pine wilt disease (PWD) is caused by the pinewood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle, which is a highly pathogenic, migratory, facultative endoparasite which generally infects some *Pinus* species. In Portugal, maritime pine, *Pinus pinaster* Aiton, is highly susceptible to infection. In 1999, the nematode was detected in Portugal (Mota et al. 1999) endangering European pine forests and has progressed throughout large areas of the country (Mota and Vieira 2008). In 2010, it was also found in Madeira island (Fonseca et al. 2012), and in 2011 for the first time in Spain (Abelleira et al. 2011). It was classified as an A2 type quarantine pest by the European and Mediterranean Plant Protection Organization (EPPO 2012).

The PWN dispersal and life cycle are dependent on vectors, cerambycid *Monochamus* spp., that include *M. alternatus* in East Asia, *M. saltuarius* in Japan, *M. carolinensis* in North America and *M. galloprovincialis*, abundant in the Portuguese pine forest (Mota and Vieira 2008, Petersen-Silva et al. 2014). After feeding on the fungus growing on dead or decaying wood (mycophagous phase), the nematodes molt into dispersal "third-stage dauer juvenile", J_{III}, able to outstand adverse conditions. Gathering around the developing insect, "fourth-stage dauer juvenile" (J_{IV}) enters the tracheal system of the emerging young callow adult through its spiracles. Infection of susceptible *Pinus* spp. occurs in the dispersal phase when adult beetles transmit the J_{IV} to other trees while feeding on young tree branches (Futai 2013). At this stage, PWNs are attracted to pine volatile cues that seem to determine changes in their development, particularly major terpenes ratio (Zhao et al. 2007) and/or β-myrcene content, as well as internal PWN neutral lipid energy reserves (Stamps and Linit 2001).

Once inside the host plant, the nematodes reproduce and multiply at a very high rate in the resin canals, consuming the epithelial cells (phytophagous phase), thus damaging internal pine structure. As infection progresses, embolized tracheids rapidly enlarge and water potential decreases ultimately leading to abrupt cavitation in the whole xylem area (Umebayashi et al. 2011). At this stage, cavitation effects appear to be promoted by increase in production of terpenes by ethylene cues (Wang et al. 2010).

As the tree very quickly begins displaying the characteristic wilting symptoms, "drying out" and yellowing of the pine needles, the oleoresin exudation decreases and as a consequence nematodes are able to move freely through the dying tree (Ikeda and Oda 1980; Kuroda 2008). Although stem anatomy is thought to be linked to variations in pine susceptibility, for e.g. the arrangement of the resin canals (Kuroda 2004) or lignification of infected pine cell walls (Kusumoto et al. 2014), it is not yet established which anatomy characteristics influence PWN progression. The trees showing intensified wilting and yellowing of the needles may collapse within 1–4 months (EPPO 2012). The decaying trees are hosts to the oviposition of female beetles and the remaining life cycle progresses as described above (Mota and Vieira 2008).

The effect of nematotoxic compounds on this phytoparasite has been well-documented, mainly using direct contact bioassays (Choi et al. 2007; Barbosa et al. 2010, 2012; Andrés et al. 2012; Faria et al. 2013). However, research is commonly performed on the nematode species alone and very seldom on the host–parasite system, not taking into account the cytotoxicity to the plant host or the plant's capability to metabolize or biotransform the nematotoxic active substances.

By co-culturing host and parasite at the same time, simulating the host-pathogen conditions, in vitro culture can be a useful system to study plant/nematode interactions, since it allows (a) eliminating variables due to environmental conditions, (b) having a contaminant-free system, which, by being in a monoxenic culture, excludes the diverse-associated microbiota (Amerson and Mott 1982; Vicente et al. 2012), (c) manipulating single variables, making possible the direct observation of plant/nematode responses in a controlled environment, which is very difficult to achieve in greenhouse or in field conditions, and also (d) attaining more biomass using fewer resources.

The present study aimed at developing a reliable host/pathogen system for PWD phytopathological research. To accomplish this, in vitro *P. pinaster* and in vitro *P. pinaster*/*B. xylophilus* co-cultures were established. PWN density in the co-culture medium was followed as well as in vitro pine relative water content. Healthy 1-year-old plantlets, pine in vitro cultures and pine/PWN co-culture

structure and volatile production were also determined. The present work proposes maritime pine/PWN co-cultures as an adequate biotechnological tool to study the PWD, capable of simulating many conditions of the ex vitro nematode infection.

Materials and methods

In vitro cultures establishment

Pinus pinaster cultures (shoots)

Seeds from maritime pine trees grown at Mata Nacional do Escaroupim, Portugal, were washed with running tap water for 5 min, then immersed in a commercial detergent (surfactants: anionic ≥15 and <30 %, non-ionic ≥5 and <15 %, disinfectant: triclosan 0.1 %) solution (10 drops per 100 ml of distilled water) for 10 min and dipped in an ultrasonic bath, 5 times for about 1 min at a time. After rinsing with running tap water, the seeds were surface sterilized by immersion in ethanol 96 %, in an ultrasound bath for 10 min, as before. In asepsis, the seeds were rinsed, three times, with ultrapure sterile water, approx. 100 ml each, and the outer seed coat was broken with a mechanical lathe. Pine nuts were hydrated in sterile ultrapure water, stratified at 4 °C for 2 days and sown in sterile wetted filter paper in covered glass jars. Seedlings were maintained in darkness, at 24 ± 1 °C, for 1 week and then transferred to a 16 h light photoperiod [cool fluorescent lamps (32 μE m^{-2} s^{-1})].

The seedling from one genotype was sectioned and the upper portion (hypocotyl and cotyledon) was maintained on multiplication medium (SHm), that is, on solid SH culture medium (Schenk and Hildebrandt 1972) with 30 g l^{-1} sucrose, supplemented with 0.5 mg l^{-1} 6-benzylaminopurine (BAP) and 0.1 mg l^{-1} indole-3-butyric acid (IBA). The pH was adjusted to 5.8 prior to the addition of 0.8 % (w/v) agar and autoclaved at 121 °C for 15 min. *P. pinaster* shoots were maintained in Combiness® (Belgium) microboxes [9.7 cm base diameter per 8 cm height and green filter (XXL+) on the lid, to facilitate air exchange], in a growth chamber with temperature and photoperiod as above. Under routine culture conditions, every 4 weeks, each shoot cluster was subdivided into 3–4 smaller clusters and transferred to microboxes with 100 ml fresh culture medium.

For shoot elongation, *P. pinaster* shoot masses (7–10 shoots) were transferred to an elongation medium (SHe), that is, to solid SH medium, without growth hormones and with activated charcoal (3 g l^{-1}), adapted from Tereso et al. (2006). Elongation allowed shoots to be detached from the main mass and individualized. In vitro cultures were maintained as described above and subculture was performed monthly. Elongation rate was followed monthly by measuring individual shoot length, for 32 months. A minimum of 30 in vitro shoots were measured per month. The data were statistically analyzed using Microsoft Excel 2013.

Pinus pinaster with Bursaphelenchus xylophilus co-cultures (co-cultures)

Bursaphelenchus xylophilus (isolate BxPt51T, retained at NemaLab and available on request) was obtained as described by Faria et al. (2013). Surface sterilization was performed in aliquots of 500 μl, with 3250 ± 250 mixed-stage PWNs in ultrapure water. In asepsis, nematodes were suspended in a 50 % ethanol/ultrapure sterile water solution (v/v) (20 ml), for 5 min in a 20 μm mesh sieve, and then washed 5 times in ultrapure sterile water, 20 ml each, resuspended in 1 ml sterile water. PWNs sterilization was tested on potato dextrose agar plates for 4 days at 25 °C. Sterilized PWNs were used for inoculating in vitro *P. pinaster* cultures.

Establishment of co-cultures was initiated by transferring *P. pinaster* shoots, maintained for 5–7 months in SHe, with monthly subculture, to activated charcoal-free solid SHe medium. A 100 μl suspension (250 ± 50 PWNs) was added into a small hole made in the culture medium into which the cut end of each shoot was inserted (Fig. 1). Cultures were maintained as described above. For further subculture, 4-weeks co-culture-grown PWNs were resuspended in 1 ml sterile water and used as described above.

Characterization of in vitro cultures and plantlets

Pinus pinaster cultures and *P. pinaster* with *B. xylophilus* co-cultures were evaluated in terms of structure, relative water (RWC), PWN population growth and volatiles content. For in vitro culture characterization, maritime pine shoots were subcultured to microboxes [8 cm base diameter per 6 cm height and green filter (XXL+) on the lid] with 20 ml solid SH medium (2 shoots per microbox). Whereas some shoots were kept uninfected, as control, others were infected with sterilized PWN suspension as described above. Sampling was performed before infection (time 0) and 1, 2, 7, 28, and 35 days after inoculation (DAI), both for *P. pinaster* shoots and for *P. pinaster* shoots inoculated with *B. xylophilus*. Culture conditions were maintained as described above. Two independent experiments were separately run and four replicates were used in each experiment. All statistical analyses were performed using Microsoft Excel 2013.

For comparison purposes, samples from greenhouse grown 1-year-old *P. pinaster* plantlets were harvested for

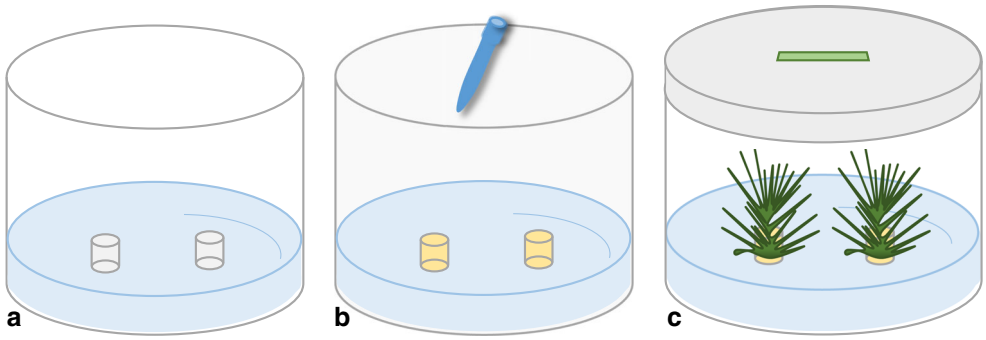

Fig. 1 Schematic representation of *P. pinaster* with *B. xylophilus* co-cultures establishment. Under asepsis, small holes were made in the culture medium (**a**), into which a 100 µl PWNs suspension (250 ± 50 PWNs) was added (**b**) together with each pine shoot (**c**)

structure and volatiles characterization. Plantlets were obtained from a mainland Portuguese nursery field (Alcácer do Sal, from seeds made available from Mata Nacional do Escaroupim, Portugal) maintained under natural 16 h light photoperiod, with average 30 °C day/18 °C night temperature and about 60 % of relative humidity. As above, two independent experiments were separately run and 4 replicates were used in each experiment. All statistical analyses were performed using Microsoft Excel 2013.

Nematode population in the co-culture

Pinewood nematodes present in the co-culture medium were counted as a measure of nematode population growth over time. Nematodes were counted by sampling 100-µl aliquots, three times, from 2 ml used to wash the co-culture medium of each microbox. To rule out nematode feeding on SH culture medium, the PWN population was compared between in vitro *cultures* with and without maritime pine shoots, at 28 DAI. PWNs were counted under an inverted microscope [Diaphot, Nikon, Japan (40×)].

At 28 DAI, the length and diameter of 30 of each randomly selected PWN females, males and juveniles (J2–J4) were measured using a stage micrometer calibrated eyepiece reticle and compared with that of PWN population grown on *Botrytis cinerea*. Permanent slides were prepared as described by Ryss (2003).

Shoots and co-cultures relative water content

Relative water content (RWC) was evaluated at 0, 1, 2, 7, 28 and 35 DAI, through the following formula:

$$\text{Relative water content } (\%) = [(\text{fresh weight} - \text{dry weight})/(\text{fresh weight})] \times 100$$

In vitro shoot fresh weight determination was performed after carefully rinsing and blotting culture medium excess with filter paper. For dry weight calculation, samples were frozen for 24 h followed by freeze-drying for 2 days, in an Alpha I-5 (Martin Christ GmbH, Osterode, Germany) apparatus, at 0.1 mbar and −42 °C.

Plantlets, shoots and co-cultures structure

In vitro shoots and co-cultures morphology and anatomy were analyzed by scanning electron microscopy (SEM) and light microscopy (LM). Sampling was performed before infection (time 0) and at the 7, 28, and 35 DAI. At each sampling time point, in vitro shoot cross-sections were processed after striping the in vitro pine needles. Samples from greenhouse grown 1-year-old *P. pinaster* plantlets were processed in a similar way.

For SEM, *P. pinaster* shoots and co-culture shoots were fixed with glutaraldehyde 2.5 % (v/v) in 0.1 M sodium phosphate buffer at pH 7.2. Samples were kept in fixative under vacuum at room temperature for 20 min, followed by 24–48 h at 4 °C. The material was then washed in the fixative buffer, dehydrated in a graded ethanol series, and critical point-dried in a Polaron E3500, according to Ascensão et al. (2005). Dried specimens were sputter coated with gold in a Polaron E5350. Observations were carried out on a JEOL T220 scanning electron microscope (JEOL Ltd., Tokyo, Japan) at 15 kV.

For LM, *P. pinaster* shoots and co-culture shoots were fixed as described for SEM, but after the washes in fixative buffer and dehydration through an ethanol series, the material was infiltrated and embedded in Leica historesin® according to Ascensão et al. (2005). To highlight the contrast between the plant tissues and PWNs, longitudinal and cross-sections (3 µm thick) were stained with periodic acid–Schiff's (PAS) reagent for polysaccharides, counterstained with Toluidine Blue O (Feder and O'Brien 1968) for general histology, and with Coomassie blue stain (Fisher 1968) for proteins. Observations were made with a Leica DM-2500 microscope (Leica Microsystems CMS GmbH, Wetzlar, Germany), images were recorded digitally

using a Leica DFC-420 camera (Leica Microsystems Ltd., Heerbrugg, Switzerland) and the Leica Application Suite software (version 2.8.1).

Plantlets, shoots and co-cultures volatiles

Pinus pinaster shoots and co-cultures volatiles were sampled at 0, 2, 7, 14, 28, 35 days after subculture and at 1 h, 8 h and 1, 2, 7, 14, 28 and 35 DAI, respectively. Isolation was performed by distillation–extraction, for 3 h, using a Likens–Nickerson type apparatus (Likens and Nickerson 1964). Distillation was run at a distillation rate of 3 ml min^{-1}, using in-lab distilled *n*-pentane (50 ml) (Honeywell Riedel-de Haën, Hanover, Germany) as organic solvent. The volatiles recovered in distilled *n*-pentane were concentrated at room temperature under reduced pressure on a rotary evaporator, collected in a vial, and concentrated to a minimum volume, again at room temperature, under nitrogen flux. In vivo pine essential oils were isolated by hydrodistillation for 3 h using a Clevenger type apparatus according to the European Pharmacopoeia (Council of Europe 2010). Hydrodistillation was run at a distillation rate of 3 ml min^{-1}. The volatile oils were stored at −20 °C until analysis. Volatiles were analyzed by gas chromatography (GC), for component quantification, and gas chromatography coupled to mass spectrometry (GC–MS) for component identification, as detailed by Faria et al. (2014).

Results and discussion

Pinus pinaster cultures establishment

Pinus pinaster in vitro shoots multiplication and elongation growth regulators requirements were optimized as reported by Calixto and Pais (1997) and Álvares et al. (2009). *P. pinaster* shoots subculture in SHm, induced meristem multiplication along the apical meristem shoot, within 4 weeks after subculture, and lead to the formation of clusters of apical needles buds (shoot clusters) (Fig. 2a). These were detached from the main multiplying shoot and subcultured monthly in the SHm. For shoot elongation, the shoot clusters were transferred from SHm to SHe medium, containing activated charcoal. Activated charcoal acts by adsorbing many organic and inorganic molecules, released from growing explants or from the culture medium. To ensure in vitro culture stability, shoots were transferred from SHm to SHe medium only after approx. 12 months in SHm culture, with routine subculture. Elongation period resulted in 3 cm shoots being obtained within about 5 months (Fig. 2b). In this period, maintenance in SHe induced shoot elongation at rates of about 0.9 mm $week^{-1}$ ($R^2 = 0.99$), that became very low after 5 months. Shoots with 5–7 months of subculture in SHe were selected for infection with the sterilized PWN.

P. pinaster with *B. xylophilus* co-cultures establishment

Within 3–4 weeks after *P. pinaster* inoculation with *B. xylophilus*, the first external signs of the PWD were observed, with several of the shoot pine needles exhibiting wilting symptoms like chlorosis and drooping when compared to control (Fig. 2c–f). These symptoms were detected in the older pine needles and progressed to the younger, towards the shoot apex, 5–6 weeks after infection the shoot was entirely brown and necrotic. In vitro infection showed to be similar to in vivo PWD phenotype as a similar symptomatology was observed in trees infected by the pine wilt disease, as reported by Kuroda et al. (1988) for *P. thunbergii* and *P. densiflora* saplings. In these species, the first symptom observed was the sudden browning of older needles that spread to younger needles accompanied by wilting and followed by host death within 1–2 months after inoculation. Symptom development in these pine species varies depending on tree age, as younger seedlings appeared to develop symptoms more rapidly than older saplings and older trees (Kuroda et al. 2007). Studying the pathogenicity of aseptic PWNs in in vitro *P. densiflora*, Zhu et al. (2012) obtained PWD symptomatology at 20 days of infection with a 250 nematode initial inoculum. Wilting and browning were observed with infection performed on the upper portion of the micro-cutting. The authors proved that aseptic PWNs maintain their pathogenicity and infection progressed to micro-cutting death. Being immature tissue, the in vitro shoots may be affected more promptly. This observation is in agreement with that herewith reported. Overall co-cultures showed similar symptoms to maritime pine under natural infection conditions.

There is an ongoing debate on the role of bacterial communities associated to the PWN on PWD. Population variations of bacterial communities generally follow those of PWN progression (Xie and Zhao 2008; Roriz et al. 2011; Nascimento et al. 2014) and evidence as pointed towards being potential triggers for disease symptomatology (Han et al. 2003; Vicente et al. 2012) and even promoters of PWN reproduction and fecundity (Zhao et al. 2006).

The data obtained in the present work for in vitro grown pine tissue, support Zhu et al. (2012), in that PWD symptomatology does not seem to be solely dependent on associated microorganism communities.

The morphometric parameters of PWN co-cultured with *P. pinaster* showed adult male body greatest diameter/length 15.6 ± 0.6 µm/685.7 ± 17.7 µm, female 20.1 ± 0.5 µm/760.5 ± 26.4 µm and juveniles (J2–J4) 12.4 ± 0.5 µm/421.9 ± 17.7 µm, were slightly smaller than those from lab-grown PWN in *Botrytis cinerea*: adult male 19.5 ± 0.4 µm/837.7 ± 12.2 µm, female 21.1 ± 0.3 µm/896.1 ± 14.8 µm and juveniles 15.7 ± 0.5 µm/555.3 ± 21.8 µm. The

Fig. 2 *Pinus pinaster* shoots under routine culture conditions grown in SH multiplication medium (SHm) (**a**) and in elongation medium (SHe) (**b**), with monthly subculturing. **c**–**f** Details of *P. pinaster* shoots (**c**, **e**) and of *P. pinaster* with *Bursaphelenchus xylophilus* co-culture 4 weeks after infection (**d**, **f**). Note, in **d** and **f**, that shoot needles exhibited wilting, that is, a *yellow-brownish* color due to chlorosis and drooping. *Scale bar* 1 cm

morphometric values (body length and greatest diameter) obtained in the present study for phytophagous PWNs are in accordance with those obtained by Penas et al. (2008) and Fonseca et al. (2008). The tendency for smaller individuals in PWNs obtained from the field, from naturally infected *P. pinaster* trees when compared with lab-grown mycophagous PWNs was also recorded by Penas et al. (2008).

Shoots and co-cultures relative water content and PWN density in co-culture medium

Being pine needle wilting one of the symptoms of nematode infection, *P. pinaster* co-cultures shoots relative water content (RWC) was assessed at 0, 1, 2, 7, 28 and 35 DAI, as a measure of PWN infection mechanism. The RWC of in vitro pine shoots varied between 72 and 85 %, not showing substantial variations (Fig. 3). Although other symptoms of PWD such as needle chlorosis were visible at latter stages of growth, the fact that no major needle desiccation was observed, during the period evaluated, may reflect the growth under in vitro-specific conditions that prevents main water loss by evaporation. Under natural conditions, interruption of the water column leads to the process of pine cavitation which diminishes water content. PWD-derived cavitation leads to discoloration in the pine needles and a decrease in

photosynthesis (Kuroda 2008) and is responsible for pine needle desiccation.

Nematode population in the co-culture medium was measured 1, 2, 7, 28 and 35 DAI. PWN inoculum of 250 ± 50 nematodes per shoot increased, having doubled by the end of the first week (Fig. 3). After 4 weeks in culture, PWNs amounted 1900 ± 204 nematodes per shoot, while solely in SH medium remained at 242 ± 60. An approx. 8× increase indicates that nematodes reproduced and completed their life cycle as in natural conditions, consuming shoot tissue to increase population numbers. PWN population numbers continued to increase and at the end of the 5th week reached 4340 ± 504 PWN per shoot. Given optimal conditions, PWN life cycle can be completed in 4 days, which is very rapid when compared with other *Bursaphelenchus* species (Futai 2013). In the present study, PWN population doubling time was approximately 1 week, which indicates that even though feeding may have occurred on the shoot basal zone in contact with culture medium, PWN population increase was still considerable.

P. pinaster plantlets, shoots and co-cultures structure

Maritime pine in vitro shoots showed the typical structure from young pine plantlets—a pith with a medullar parenchyma, a vascular ring with axial and radial resin ducts, a starch-rich cortical parenchyma with several tannin-containing cells and axial resin ducts (Figs. 4a, c, 5b). Recently developed *P. pinaster* in vitro grown shoots showed ducts with a very narrow lumen. At the cut end of the shoots, resulting of the separation from the parent shoots clusters, and facing the culture medium, a callus tissue formed, characterized by an unorganized mass of loosely arranged parenchyma cells (Fig. 2e, f). In general, depending on the plant material, medium composition and environmental conditions during culture period, callus growth characteristics may be variable in the extent and type of differentiation.

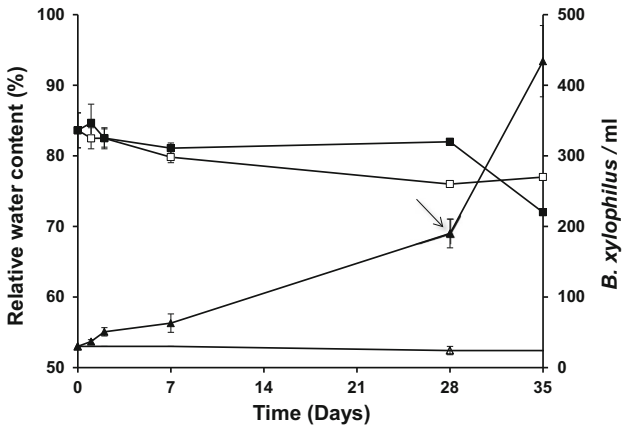

Fig. 3 Relative water content (%) of in vitro *P. pinaster* shoots (*open squares*) and of *P. pinaster* shoots with PWN co-culture (*filled squares*). Nematode population density in the microbox culture medium (*filled triangles*) at the different time points of the time-course study and at 0 and 28 days without pine shoots (*open triangles*). Two shoots were maintained per container in 20 ml of solid culture medium. *Arrow* time points when, macroscopically, pine needles started to exhibit wilting (drooping and a yellow-brownish color due to chlorosis)

Callus tissue of the pine in vitro shoot facing the culture medium showed clusters of cells giving rise to meristematic zones (Figs. 4b, 5a). This basal shoot zone in contact with the culture medium is a sink for endogenous phytohormones and lesion-derived stress compounds that stimulate tissue dedifferentiation and formation of cell meristematic centers (Washer et al. 1977; Aitken-Christie et al. 1985). These centers with starch-rich cells continued to grow throughout the culture period surrounding primordial tracheary elements (Fig. 4b, arrows). The growth and development of the meristematic centers was accompanied by the formation of fissures in the parenchyma tissue due to movement of cell masses.

One week after co-culture establishment of *P. pinaster* with PWN, numerous nematodes were found within callus parenchyma tissue gaps (Figs. 4e, 5c, d). Parasite feeding may have stimulated callus tissue development since, in addition to mechanical injury, callus tissue may be produced as a response to an invading organism. Four weeks after infection, at less than one centimetre above the cut end of the shoots, vascular bundles were already present and nematodes were observed in cavities formed in the cambium between the xylem and phloem (Fig. 4d, arrow). Above this shoot basal region no more nematodes were found, although shoots exhibited the typical anatomy of a *Pinus* species. Fully-developed resin canals were yet scarce and presented narrow lumens, being only frequent secretory ducts in early ontogenic stages.

Iwahori and Futai (1990) analyzed *calli* obtained from several susceptible and resistant pine species (*P. densiflora*, *P. thunbergii*, *P. massoniana*, *P. thunbergii* × *P massoniana*, *P. taeda*) as well as *Nicotiana tabacum* and *Medicago sativa* as a method to obtain clean PWN populations. Although high PWN growth rates were detected, probably due to an easy access to food source, *callus* tissue culture unorganized nature was not faithful to in vivo pine characteristics. Thin-walled metabolically very active cambial cells may serve for nematode feeding during the infection process. In fact, it is now well-known that nematode secretions are rich in cell wall degrading enzymes such as the β-1,4- and β-1,3-glucanases, pectate lyase and also expansins and cellulose-binding proteins (Haegeman et al. 2012; Shinya et al. 2013a, b). In the current study, nematode secretions may have influenced greatly in vitro shoot PWD symptom

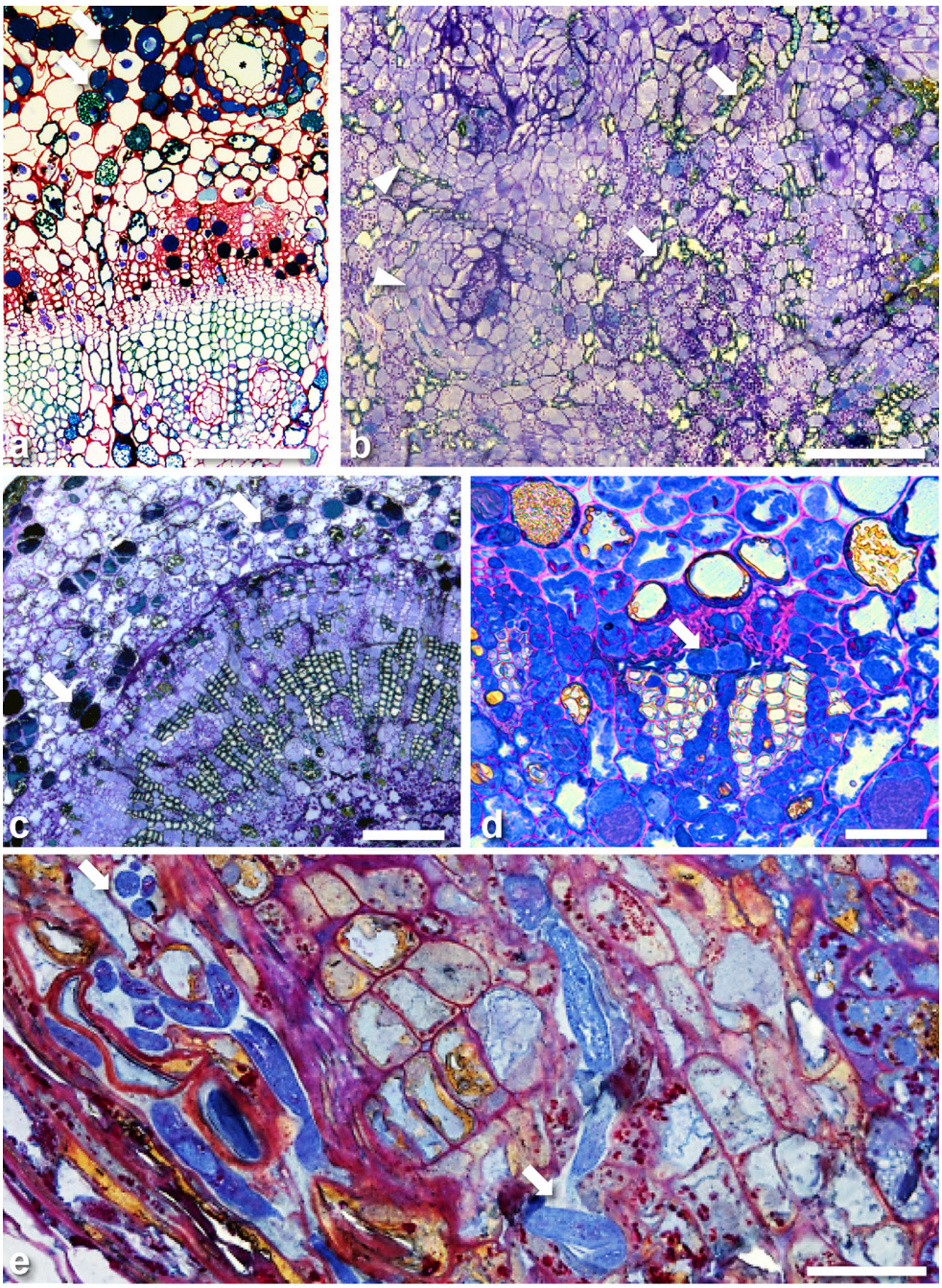

Fig. 4 Light micrographs of historesin sections of shoots from 1-year-old *Pinus pinaster* seedlings (**a**), from in vitro shoot cultures (**b**, **c**), and from shoot co-cultures with *Bursaphelenchus xylophilus* (**d**, **e**). **a** Cross-section showing the characteristic anatomy of a pine shoot. Note the presence of several tanniniferous cells (*arrows*) in the cortical parenchyma. Resin ducts (*asterisks*) are clearly seen in the cortex and xylem. **b** Callus tissue, in the zone facing the culture medium, showing the dedifferentiation centers (*arrowheads*) and tracheary elements (*arrows*). **c** A vascular ring, surrounding the pith and showing tanniniferous cells (*arrows*), is observed in shoot cross-sections some millimeters above the culture medium. **d**, **e** Nematodes were found in cavities developed in the vascular bundles between the xylem and the phloem (**d**, *arrow*) and in gaps formed in the callus tissue during the dedifferentiation process (**e**). *Scale bars* 200 μm (**a**), 50 μm (**b**), 100 μm (**c**) and 40 μm (**d**, **e**)

development, namely, macroscopically, pine shoot wilting (desiccation, chlorosis and drooping). Plant tissue degeneration was noticeable as nematode population increased, probably not solely due to nematodes feeding, as well as to parasite secretions. In fact, Melakeberhan and Webster (1992) analyzing the energy requirements of the PWN in *P. sylvestris*, concluded that food consumption is not a significant factor in the cause of pine death.

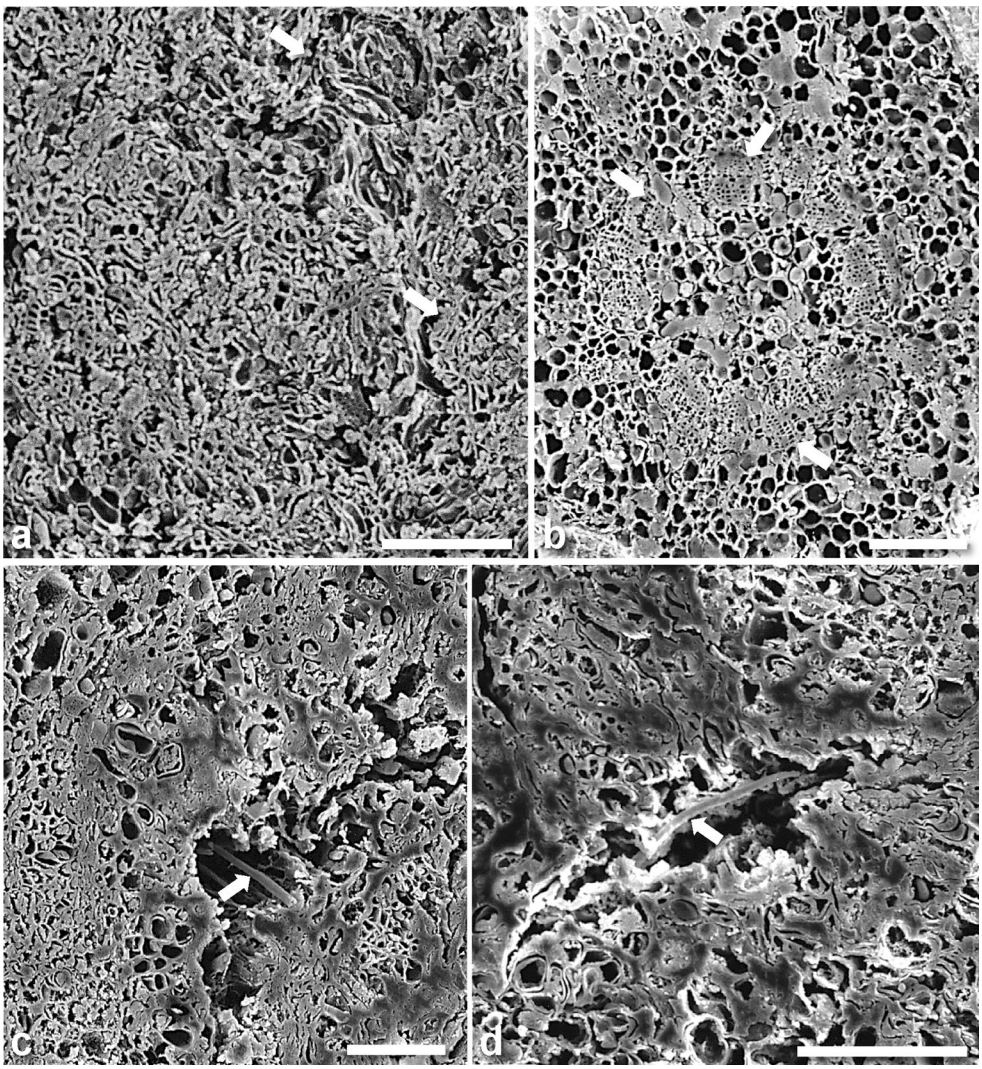

Fig. 5 Scanning electron microscopy micrographs from cross-sections of *Pinus pinaster* shoots cultures (**a**, **b**) and from *P. pinaster* shoots in co-culture with *Bursaphelenchus xylophilus* (**c**, **d**). **a** Several dedifferentiation centers (*arrows*) are observed in the callus tissue facing the culture medium. **b** A nearly continuous vascular ring (*arrows*) is clearly seen in the shoot some millimeters above the culture medium. **c**, **d** Nematodes (*arrows*) are found in callus tissue gaps forming during the dedifferentiation process. *Scale bars* 100 μm

P. pinaster plantlets, shoots and co-cultures volatiles

Volatiles isolated from in vitro grown *P. pinaster* cultures and *P. pinaster* with *B. xylophilus* co-cultures were compared with those isolated from one-year-old plantlets. Although Table 1 reports only the isolated volatiles main components (≥1 %), they were all fully chemically characterized, in a total of 80 compounds for the plantlets volatiles, 46 for *P. pinaster* cultures and for *P. pinaster* with *B. xylophilus* co-cultures.

Plantlets volatiles were β- and α-pinene rich (47 and 28 %, respectively) (Table 1). Likewise, in vitro pine cultures were also β- and α-pinene rich (38–47 % and 24–33 %, respectively), although the relative importance of several compounds differed between plantlets and pine cultures volatiles. Whereas α-terpineol (7 %) and bornyl acetate (4 %) were the third- and fourth- plantlets volatiles main components, they were always <2 and <0.5 %, respectively, in the pine shoots volatiles. Conversely, germacrene D (3–9 %), an unidentified compound (UI B Ppi, 4–7 %) and β-caryophyllene (2–5 %) that attained relatively high percentages in the pine shoots volatiles, were either <2 % (β-caryophyllene) or in trace amounts (germacrene D and UI B Ppi) (Table 1).

Co-culture of *P. pinaster* with *B. xylophilus* did not alter substantially the volatile composition compared to pine cultures volatiles. Again β- and α-pinene dominated the co-cultures volatiles (36–47 % and 24–32 %, respectively), followed by germacrene D (3–7 %), UI B Ppi (4–8 %) and β-caryophyllene (1–5 %).

Table 1 Percentage composition of the volatiles (>1 %) isolated from *P. pinaster* 1-year-old plantlets aerial parts (plantlets), from in vitro grown *P. pinaster* shoots at 0, 2, 7, 14, 28 and 35 days after subculture (shoots) and from *P. pinaster*/PWN co-cultures at 1 h, 8 h and 1, 2, 7, 14, 28 and 35 days after infection (co-cultures)

Components (>1 %)	RI	Plantlets	Shoots						Co-cultures							
			0	2	7	14	28	35	1 h	8 h	1	2	7	14	28	35
α-Pinene	930	27.9	23.7	25.5	24.4	27.1	31.5	32.8	30.9	28.9	24.2	24.5	25.4	27.0	31.6	30.5
Camphene	938	2.2	0.2	0.2	0.5	0.6	0.6	0.5	0.5	0.5	0.3	0.4	0.6	0.6	0.5	0.5
β-Pinene	963	46.6	37.7	38.0	42.4	43.9	46.8	43.8	44.4	38.6	36.3	38.9	39.7	42.3	47.3	42.3
β-Myrcene	975	0.4	1.1	1.1	1.5	1.2	1.1	1.2	1.2	0.9	2.1	1.1	1.1	2.0	1.1	1.1
β-Phellandrene	1005	0.5	1.1	1.2	1.2	1.3	1.3	1.2	1.2	1.0	0.8	1.1	1.1	1.2	1.2	1.2
Limonene	1009	2.7	1.0	1.0	1.1	1.2	1.2	1.4	1.1	0.9	0.5	0.9	1.2	1.1	1.4	1.6
Terpinolene	1064	1.4	0.1	0.7	0.4	0.5	0.6	0.5	0.2	0.3	0.2	0.3	0.3	0.3	0.7	1.0
α-Terpineol	1159	7.1	0.2	0.3	1.5	1.7	1.6	1.5	0.5	0.8	0.4	1.2	1.3	1.5	2.0	4.0
Bornyl acetate	1265	3.6	0.1	0.1	0.3	0.4	0.3	0.4	0.1	0.3	0.2	0.2	0.2	0.3	t	t
α-Copaene	1375	t	t	1.3	0.1	0.2	0.1	t	0.1	0.2	0.1	0.2	0.1	0.2	t	t
β-Caryophyllene	1414	1.4	4.6	3.7	2.9	2.1	1.5	2.0	3.2	3.1	4.6	3.4	3.2	2.5	1.3	1.7
α-Humulene	1447	0.2	0.3	1.1	0.2	0.2	0.2	0.4	0.4	0.5	0.3	0.2	0.9	0.4	t	t
Phenyl ethyl 2-methyl butanoate	1467	1.7	0.2	0.1	0.3	0.4	0.4	0.5	0.2	0.3	0.2	0.2	0.5	0.5	0.2	0.2
Phenyl ethyl isovalerate	1468	t	0.4	0.9	1.1	1.0	1.3	1.4	0.7	0.8	0.4	0.6	1.1	1.3	1.7	1.5
Germacrene D	1474	t	8.8	5.3	4.4	3.6	3.3	3.2	5.0	5.1	6.9	6.8	4.5	3.1	2.6	2.9
γ-Cadinene	1500	0.1	0.4	2.7	1.2	0.4	0.2	0.3	0.3	1.9	1.2	1.2	1.5	0.6	1.0	1.0
δ-Cadinene	1505	0.1	0.7	0.1	0.9	1.5	1.0	1.1	1.6	0.5	0.5	0.8	1.0	1.8	t	t
β-Caryophyllene oxide	1561	0.2	0.3	0.3	0.3	0.2	t	0.1	0.2	0.2	0.6	0.9	1.0	0.2	t	t
α-Cadinol	1626	t	0.9	0.1	0.2	0.2	0.2	t	0.2	0.4	0.5	1.3	0.4	0.4	t	t
Palmitic acid	1908	t	2.1	2.2	0.9	0.5	0.2	0.2	0.5	1.0	1.4	2.0	0.7	0.7	0.8	1.7
UI B Ppi[a]	2309	t	4.5	6.0	7.2	5.6	4.6	3.6	3.9	5.5	4.6	6.5	7.8	4.2	5.0	3.7
% Identification		99.8	85.7	88.1	87.9	90.0	94.5	94.1	93.2	88.6	84.1	87.8	87.7	90.3	93.2	91.0
Grouped components																
Monoterpene hydrocarbons		82.6	65.2	68.0	71.6	75.7	83.4	81.5	79.6	71.1	64.6	67.4	69.7	74.6	83.7	78.0
Oxygen-containing monoterpenes		12.9	0.3	0.6	2.1	2.5	2.0	1.9	0.8	1.2	0.7	1.6	1.6	2.1	2.0	4.0
Sesquiterpene hydrocarbons		1.8	15.4	14.9	10.3	8.7	6.7	7.3	10.7	12.1	14.3	13.1	12.0	9.4	4.9	5.5
Oxygen-containing sesquiterpenes		0.3	2.0	1.4	1.3	0.9	0.4	1.0	0.7	1.7	2.1	2.7	2.0	1.4	t	0.3
Oxygen-containing diterpenes		0.1														
Phenylpropanoids		0.4														
Fatty acids		t	2.1	2.2	0.9	0.5	0.2	0.2	0.5	1.0	1.4	2.0	0.7	0.7	0.8	1.7
Others		1.7	0.7	1.1	1.8	1.8	1.8	2.2	1.0	1.6	1.0	1.2	1.8	2.2	1.9	1.7

RI in-lab calculated retention index relative to C_9–C_{24} *n*-alkanes on the DB-1 column, *t* trace (<0.05 %)

[a] Unidentified compound detected on *Pinus pinaster* and on in vitro cultures and co-cultures (standard deviation <5 %)

Lima et al. (2010) characterized the volatiles from 2-year-old uninoculated healthy *P. pinaster* plants (HP) and from mechanically wounded uninoculated (C) and inoculated (In) individuals. As in the present study, Lima et al. (2010) did not find relevant qualitative and quantitative differences between HP, C and In isolated volatiles.

PWN shows chemotaxis to volatile terpenes, altering its behavior due to different volatile cues (Futai 2013; Zhao et al. 2014). The terpenes α-pinene, β-pinene and longifolene appear to be decisive in a fundamental step of the nematode life cycle. Zhao et al. (2007, 2014) showed that different ratios of these terpenes, observed in the host species *P. massoniana* and released by larval vector

attracted different nematode juvenile stages, and may be the cue to altering from de propagative to the dispersal form. Pine volatile response to inoculation with PWN was analyzed in six-year-old *P. thunbergii*, by Kuroda et al. (1991). In that study, volatile production was enhanced by nematode introduction, associated to the beginning of desiccation; the total volatile terpenes (e.g. α-pinene, β-pinene, β-myrcene, longifolene) showed a higher concentration when compared with those of healthy trees. Takeuchi et al. (2006) also recorded high emissions of terpenes, like α-pinene, while profiling the volatiles of infected *P. thunbergii*. This increase in volatile emissions not only attracts the vector beetle species but appears to contribute to the wilting of the tree, by weakening the tensile strength of the sap, promoting embolism in the tracheids (Kuroda 1991).

Although the present study, using in vitro cultures and co-cultures, supported earlier observations with plantlets, that showed no major qualitative differences between the volatiles from healthy and from inoculated plants, further studies on the chemical cues that promote nematode attraction would be relevant. *P. pinaster* in vitro cultures and *P. pinaster* with PWN in vitro co-cultures established and characterized in the present study may constitute a complementary biotechnological tool to investigate not only these chemical cues, but also host and parasite response to nematotoxics.

Author contribution JMSF conceived, designed research, conducted the experiment and wrote the manuscript. IS, IVS and BR conducted the experiment. PB supplied *B. xylophilus* initial inoculum and reviewed the manuscript. LA, RB, MM and ACF supervised the work and reviewed the manuscript. All the authors read and approved the manuscript.

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Conflict of interest The authors declare that they have no conflict of interest.

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