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Essential oils as anti-nematode agents and their influence on *in vitro* nematode / plant co-cultures

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“Just do it”

Nike

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Abstract

Parasitic nematodes are among the most production-limiting plant pests. In Europe, the recent introduction of *Bursaphelenchus xylophilus* [the pinewood nematode (PWN)] and some species of *Meloidogyne* has proved damaging to forest ecosystems and crop production. Due to the very laborious and environment-dependent nature of greenhouse or field assays, *in vitro* host with parasite co-cultures can be a useful biotechnological tool to evaluate potential nematotoxic essential oils (EOs). The present work intended to a) screen EOs against PWN motility and *M. chitwoodi* [Columbia root-knot nematode (CRKN)] hatching, b) establish and characterize *Pinus pinaster* (maritime pine) *in vitro* cultures and *Solanum tuberosum* (potato) hairy roots (HR) cultures as well as *P. pinaster* with PWN and *S. tuberosum* HR with CRKN co-cultures, c) determine the effect of selected nematotoxic EOs on co-cultures growth or relative water content, nematode population and volatiles production.

Ruta graveolens, *Satureja montana* and *Thymbra capitata* EOs revealed high activities against PWN. These and *Dysphania ambrosioides* and *Filipendula ulmaria* EOs also showed high inhibitory activity against CRKN. The established *P. pinaster* with PWN and *S. tuberosum* HR with CRKN co-cultures, displayed infection and developmental characteristics similar to those in nature. Of the most active EOs, those of *R. graveolens* and *S. montana* were chosen to be tested with *in vitro* co-cultures. *S. montana* EO was highly phytotoxic to both co-cultures, inhibiting potato HRs growth and inducing chlorosis and wilting in pine shoots. *R. graveolens* EO inhibited potato HRs with CRKN co-cultures growth but induced no macroscopic damages to *in vitro P. pinaster* with PWN co-cultures. In addition to constitutive compounds, biotransformation volatile compounds were detected after EOs addition to both co-cultures types.

P. pinaster with PWN and *S. tuberosum* HR with CRKN co-cultures were a good system to mimic some of the natural infection conditions, allowing an overview of the combined host / parasite reactions, and being able to assist in the evaluation of EOs phytotoxicity and nematotoxic potential.

Keywords: *Bursaphelenchus xylophilus*, hairy roots, *in vitro* shoots, *Meloidogyne chitwoodi*, nematotoxics

Resumo

As doenças resultantes de infecções por nemátodes fitoparasitas são ainda uma grande ameaça à produção vegetal, quer em culturas agronómicas quer em espécies florestais. Dotados de sofisticados mecanismos de infecção e proliferação, estes fitoparasitas provocam não só elevadas perdas económicas como graves alterações no ecossistema. Como agravante, a introdução de fitoparasitas não endémicos induz localmente aumentos populacionais exponenciais à custa de novos hospedeiros susceptíveis. No continente europeu, duas recentes introduções são exemplo desta situação. O nemátode da madeira do pinheiro (NMP), *Bursaphelenchus xylophilus*, e várias espécies do género *Meloidogyne*, nemátodes da galha da raiz (NGR). O NMP, causador da doença da murchidão do pinheiro (DMP), é um endoparasita migratório facultativo que infecta preferencialmente o género *Pinus*. O pinhal português é dominado pelo pinheiro bravo, *Pinus pinaster*, altamente susceptível à infecção, e pelo pinheiro manso, *Pinus pinea*, considerado de susceptibilidade intermédia. A transmissão do parasita é feita pelo cerambicídeo-vector *Monochamus galloprovinciallis*, que introduz o parasita ao alimentar-se de pinheiros saudáveis. Devido ao seu rápido ciclo de vida, o nemátode pode eliminar uma árvore num espaço de 1 a 4 meses. Os NGR são endoparasitas sedentários obrigatórios, dependentes da planta para completar o seu ciclo de vida. Estes parasitas mobilizam os produtos fotossintétcos para a raiz e afectam o transporte de água e nutrientes para suportar o seu desenvolvimento e reprodução. O seu efeito crónico é notório em culturas agrícolas sendo estimado que possam obstar a produção vegetal até 60% e, em casos de picos populacionais, levar à morte da cultura.

O combate destes fitoparasitas é realizado essencialmente por métodos químicos, pela aplicação de poderosos nematicidas de síntese, ou por métodos não químicos como a rotação de culturas e culturas de cobertura. Actualmente existe uma pressão crescente na despistagem de produtos naturais com elevadas capacidades nematotóxicas. Como biocidas, os óleos essenciais (OE), complexas misturas de metabolitos secundários produzidos por plantas, oferecem a vantagem de serem biodegradáveis, pouco tóxicos para mamíferos e não acumularem no meio ambiente. No entanto, os ensaios com OEs são geralmente realizados sobre o parasita negligenciando-se tanto a fitotoxicidade para o hospedeiro como a sua capacidade de biotransformar o princípio activo

nematotóxico. Além disto, os ensaios de campo estão, regra geral, muito dependentes de factores bióticos e abióticos, e também da variabilidade genética dos hospedeiros. Numa tentativa de evitar estas restrições, o uso de modelos biotecnológicos, como co-culturas *in vitro*, oferece a vantagem de a) ser uma cultura monoxénica livre de contaminantes (a fauna e flora do solo ou do interior da planta), b) permitir a manipulação de variáveis unitárias, e observar o seu efeito no sistema hospedeiro / parasita e c) permitir a obtenção de um grande número de culturas num espaço reduzido, comparativamente às condições na natureza.

Neste contexto, o presente trabalho teve como objectivo último avaliar a capacidade nematotóxica de OEs numa situação hospedeiro / parasita com recurso a co-culturas *in vitro* de planta com nemátode. Para este efeito, realizou-se uma despistagem inicial de OEs isolados de plantas da flora Portuguesa e de origem comercial, testando em ensaios de contacto directo, o seu efeito na mobilidade de NMP e na eclosão do NGR *M. chitwoodi*. Simultaneamente, foram estabelecidas as culturas *in vitro* de rebentos de *P. pinaster* e de raízes transgénicas (HR, do Inglês *hairy roots*) de *Solanum tuberosum*. Estas culturas, bem como as co-culturas de rebentos de *P. pinaster* com NMP e de HR de *S. tuberosum* com *M. chitwoodi*, seguidamente obtidas, foram caracterizadas de diversas formas. Ao nível da anatomia e morfologia foram avaliadas por microscopia óptica (MO) e microsocpia electrónica de varrimento (MEV). Avaliou-se o crescimento das culturas de HR de *S. tuberosum* e co-culturas de HR de *S. tuberosum* com *M. chitwoodi* e determinou-se o teor relativo de água nas culturas *in vitro* de rebentos de *P. pinaster* e nas co-culturas de rebentos de *P. pinaster* com NMP. A densidade populacional do fitoparasita foi determinada em diversos momentos do crescimento das culturas e a produção em voláteis determinada por cromatografia gasosa (GC) e cromatografia gasosa associada a espectrometria de massa (GC-MS). Com base na despistagem inicial de OEs, dois deles foram selecionados e aplicados às co-culturas estabelecidas, seguindo o seu efeito ao nível do crescimento, teor relativo de água, densidade populacional do fitoparasita e produção em voláteis.

A despistagem inicial da acção nematotóxica de OEs foi realizada com 84 OEs para o NMP e 56 OEs para o NGR *M. chitwoodi*. Para o NMP, os OEs isolados de *Ruta graveolens* (arruda), *Satureja montana* (segurelha) e de *Thymbra capitata* (tomilho-de-creta) mostraram a maior actividade nematotóxica; para *M. chitwoodi*, além dos anteriores, os OEs de *Dysphania*

ambrosioides e *Filipendula ulmaria* mostraram elevadas actividades anti-eclosão. O fraccionamento de alguns OEs revelou que as fracções dos compostos hidrocarbonetos e oxigenados contribuem de maneira diferente, e de forma específica para cada espécie, para a actividade nematotóxica final. Os OE de arruda e segurelha foram seleccionados para os ensaios de fitoxicidade em co-cultura devido à sua composição química e elevada actividade nematotóxica e anti-eclosão.

Após o estabelecimento das culturas *in vitro* de rebentos de *P. pinaster* e de HR de *S. tuberosum*, foram estabelecidas as respectivas co-culturas. Quatro semanas após a introdução do NMP na cultura *in vitro* de rebentos de *P. pinaster*, observaram-se os sintomas característicos da DMP, clorose foliar e procumbência das agulhas do pinheiro. Recorrendo a MO e MEV os parasitas foram detectados nos rebentos, nos interstícios de tecido caloso e nas zonas intercambiais, aumentando a sua população ao longo do tempo. Nas co-culturas de HR de batata com *M. chitwoodi* foi possível detectar as várias fases de desenvolvimento do parasita. O aumento da população *in vitro* após sub-cultura mostrou ser bifásico, com um máximo inicial devido a eclosões de NGRs da 1ª geração e um segundo máximo resultante da produção de ovos pela 2ª geração. Em termos de voláteis não foram detectadas alterações substanciais resultantes da infecção dos parasitas em ambas as co-culturas estabelecidas. Nos parâmetros analisados as co-culturas demonstraram ser adequadas à análise de pesticidas nematotóxicos naturais.

A adição dos OEs de arruda e segurelha ao meio de cultura, numa concentração de 0,5 µL/mL, induziu diferentes reacções nas co-culturas. Em co-culturas de *P. pinaster* com NMP o OE de segurelha induziu clorose foliar e procumbência dos rebentos, demonstrando fitotoxicidade para o hospedeiro, ao passo que o OE de arruda não induziu alterações detectáveis macroscopicamente e controlou efectivamente a população do parasita. Nas co-cultura de HR de batata com *M. chitwoodi*, ambos os OE induziram uma supressão do crescimento das HR quase imediata, revelando não serem nematotóxicos adequados, na concentração avaliada. Em ambas as co-culturas a adição dos OEs induziu a produção de novos compostos voláteis. Assim, dos OEs testados nas co-culturas de rebentos de *P. pinaster* com NMP, o de *Ruta graveolens* (arruda) foi o que revelou menor fitotoxicidade enquanto mantendo a acção nematotóxica.

Em conclusão, as co-culturas de rebentos de *P. pinaster* com NMP e de HR de *S. tuberosum* com

M. chitwoodi, mostraram ser um sistema que permite mimetizar algumas das condições de infecção naturais, possibilitando ter uma perspectiva das reacções combinadas hospedeiro / parasita, e ser um auxiliar na avaliação da fitotoxicidade de OEs e do seu potencial nematotóxico.

Palavras-chave: *Bursaphelenchus xylophilus*, culturas *in vitro* de plantas, *Meloidogyne chitwoodi*, nematotóxicos, raízes transgénicas

List of abbreviations

BAP	6-Benzylaminopurine
CDW	Concentrated decoction water
CHI	Corrected hatching inhibition
CRKN	Columbia root-knot nematode
DAI	Days after inoculation
DMP	Doença da murchidão do pinheiro
DW	Dry weight
EO	Essential oil
EPPO	European and Mediterranean Plant Protection Organization
FID	Flame ionization detector
FW	Fresh weight
GC	Gas Chromatography
GC-MS	Gas Chromatography coupled to Mass Spectrometry
HM	Hydrocarbon molecules fraction
HR	Hairy roots
IBA	Indole-3-butyric acid
LM	Light microscopy
MEV	Microscopia electrónica de varrimento
MO	Microscopia óptica
NGR	Nemátode das galhas da raíz
NMP	Nemátode da madeira do pinheiro
OCM	Oxygen-containing molecules fraction
OE	Óleo essencial
PAS	Periodic acid–Schiff's reagent
Ppi	*Pinus pinaster in vitro* shoot cultures
PpiBx	*P. pinaster* shoots with *Bursaphelenchus xylophilus* co-cultures
PPN	Plant parasitic nematode
PWD	Pine wilt disease
PWN	Pinewood nematode (*Bursaphelenchus xylophilus*)
Rg	*Ruta graveolens* essential oil
RI	Retention index
RKN	Root-knot nematode
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
Sm	*Satureja montana* essential oil
StHR	*Solanum tuberosum* hairy roots
t	Trace

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List of publications related to the Ph. D. Thesis

Jorge MS Faria, Pedro Barbosa, Richard N Bennett, Manuel Mota, A Cristina Figueiredo (2013) Bioactivity against *Bursaphelenchus xylophilus*: nematotoxics from essential oils, essential oils fractions and decoction waters. Phytochemistry 94:220-228.

Jorge MS Faria, Inês Sena, Carla MN Maleita, Inês Vieira da Silva, Lia Ascensão, Isabel Abrantes, Richard N Bennett, Manuel Mota, A Cristina Figueiredo (2014) *In vitro* co-culture of *Solanum tuberosum* hairy roots with *Meloidogyne chitwoodi*: structure, growth and production of volatiles. Plant Cell, Tissue and Organ Culture 118:519-530.

Jorge MS Faria, Inês Sena, Inês Vieira da Silva, Bruno Ribeiro, Pedro Barbosa, Lia Ascensão, Richard N Bennett, Manuel Mota, A Cristina Figueiredo (2015) *In vitro* co-cultures of *Pinus pinaster* with *Bursaphelenchus xylophilus*: a biotechnological approach to study pine wilt disease. Planta DOI: 10.1007/s00425-015-2257-9

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Jorge MS Faria, Cristina Moiteiro, Richard N Bennett, Manuel Mota, A Cristina Figueiredo (2015) Nematotoxic and phytotoxic activity of *Satureja montana* and *Ruta graveolens* essential oils on *Pinus pinaster* shoot cultures and *P. pinaster* with *Bursaphelenchus xylophilus in vitro* co-cultures (S*ubmitted*)

Jorge MS Faria, Inês Sena, Richard N Bennett, Manuel Mota, A Cristina Figueiredo (2015) *Ruta graveolens* and *Satureja montana* essential oils as effective nematotoxics on *Solanum tuberosum* hairy roots with *Meloidogyne chitwoodi* co-cultures (*in preparation*)

Chapter 1

Plant biotechnology in search for effective nematotoxic essential oils: pine wilt and root-knot diseases

1. Introduction

1.1. Plant parasitic nematodes

Plant diseases caused by parasitic nematodes have been reported since ancient Roman times, but a causal parasitic agent was only first reported in 1774 when John Needham exposed the presence of eelworms in galls of wheat (Needham 1774, Decker 1981). Since then, a plethora of plant parasitic nematodes (PPN), able to infect almost every plant species, has been described. The co-evolution of PPN with host plants has led to the development of sophisticated survival strategies and has resulted in a remarkable synchrony of host and nematode life cycles (Baldwin et al. 2004). But development of this interaction was not simple. According to Blaxter et al. (1998), the process of plant parasitism in the nematode phylum appears to have evolved independently at least three times.

PPN are small, worm-like parasites which usually range from 0.3 to 1 mm, with some up to 4 mm long, by 15–35 µm wide. The body is normally slender and colorless, covered by a cuticle formed of chitin, with various striations and other markings (Agrios 2005). They can be distinguished from other parasitic nematodes by the development of a stylet, fundamental to their success as plant parasites, and to a vast array of nematode secretions that affect plant cell development, many of which resulted from multiple instances of horizontal gene transfer from bacteria and fungi (Haegeman et al. 2011).

The stylet, functioning as a piercing mouthpart, is a hollow protrusible structure that functions as a “syringe”, injecting secretions into the plant tissues and retrieving nutrients from the cytoplasm of the cells fed upon by the nematode. The secretions are products of parasitism genes that are mainly expressed in the pharyngeal glands (Davies et al. 2009). PPN secretions are paramount in the host / parasite relation and are differentially secreted throughout the sophisticated cellular infection process, inducing cell wall modifications and potential interactions with signal transduction receptors in the extracellular space, with direct introduction of proteins into host cells. These can influence cellular metabolism, cell cycle, selective protein degradation, localized defense responses and have regulatory activity within the host cell nucleus (Jasmer et al. 2003,

Vanholme et al. 2004, Davis et al. 2004). Nevertheless, PPN differ remarkably from each other in morphology, ecology, biology and, especially, in behavior (Agrios 2005).

Nowadays, PPN are still a serious threat to modern agriculture and forestry. Various diseases are caused by several hundred species known to feed on living plants and cause heavy damages to marketable crop production (Nicol et al. 2013). Worldwide annual economic losses due to PPN diseases in crop cultures are estimated to be about 11-14% (approx. 70 billion euros) and 10 to 60% crop yield (crop quantity, quality, or harvest uniformity) may be reduced due to PPN infection, depending on the parasite species, host status, and environmental conditions (Trigiano et al. 2004, Agrios 2005).

Still, a direct causal effect is very difficult to ascertain since PPN-derived plant diseases have a multifactorial origin; infection with PPN is frequently accompanied with an increase in pathogenic bacteria, fungi and even the transmission of some plant pathogenic viruses, which generally lower the plant's tolerance to environmental stress conditions (Agrios 2005).

These nematodes cause diseases worldwide but generally have low impact in their native range. Globalization and industrialization of modern agriculture has led to the introduction of PPN to new areas, where the impact on susceptible plant species can be very high. Crop losses due to PPN can be much greater if species currently causing localized damage became more widespread (Singh et al. 2013). In Europe, this was the case, for e.g., for the recently introduced *Bursaphelenchus xylophilus* (Mota et al. 1999, EPPO 2014), due to its pathogenicity and rapid life cycle, and several *Meloidogyne* spp. (EPPO 2014), which show a wide host range and are less easily controlled by nematicides.

Bursaphelenchus xylophilus (the pinewood nematode, PWN) along with members of the genus *Globodera* (e.g. potato-cyst nematodes), *Heterodera* (e.g. soybean-cyst nematodes) and *Meloidogyne* (root-knot nematodes) are among the most damaging and economically devastating phytoparasites, to agriculture and forestry. Many questions remain to be answered regarding the mechanism of plant nematode invasion and proliferation, as well as on the chemical signals involved in the invasive process. Given their diverse mode of action, two different nematode species and types, *B. xylophilus* and *M. chitwoodi*, respectively, will be particularly addressed.

1.1.1 Pinewood nematode: *Bursaphelenchus xylophilus*

Bursaphelenchus xylophilus (Steiner & Buhrer) Nickle, the pinewood nematode (PWN), is a migratory non-obligate plant endoparasite that infects mainly *Pinus* species, causing pine wilt disease (PWD). It is thought to be native to North America, where it does little damage to local conifer trees, and was transported to the southern Japanese islands through infested timber at some time around the beginning of the 20th Century (Nickle et al. 1981, Mota and Vieira 2008). In Japan, conifers were highly susceptible to infection and the disease quickly spread to other Asian countries such as China and Korea. Recently it was discovered in Europe, in Portugal and Spain (Mota et al. 1999, Abelleira et al. 2011, Fonseca et al. 2012).

The phytoparasite life cycle can progress in two different ways, the reproductive and the dispersal phase, and shows different feeding habits, phytophagous and mycophagous, which are characteristic of this species (Moens and Perry 2009, Zhao et al. 2014). In each phase, its behavior, nutrition, reproduction, and distribution in the host tree are greatly influenced by cohabiting microorganisms (Futai and Mota 2008).

PWN is commonly mycophagous, feeding on the hyphae of fungi (usually *Botrytis cinerea*, *Ceratocystis* spp. and *Ophiostoma minus*) that grow on dead or decaying pine wood, rapidly multiplying and completing their life cycle (Mamiya 1983). Spreading to other feeding sites, i.e. dispersal, is performed with the aid of a vector species, predominantly cerambycid beetles of the genus *Monochamus*. Dead or decaying wood is host to oviposition of adult beetle females, which, if infected, may transmit the nematode carried in the tracheal system. The hatched beetle larvae grow and become carrier vectors, the following year. Similarly to insects, nematodes undergo several "molting" processes, progressing through four juvenile stages (J1 to J4) (Fig. 1). With food shortage, desiccation, or environmental deterioration due to overpopulation, J2 molt into a “dispersal third-stage juvenile” (J_{III}), a survival stage capable of resisting adverse conditions. Close to the time of beetle emergence nematodes molt into the special fourth-stage juvenile, called the "*dauer* stage" (J_{IV}) and enter the young callow adult beetle, being carried to new oviposition sites (Futai 2013, Zhao et al. 2014). Volatile cues may be of crucial importance in the life cycle and dispersion of the PWN as demonstrated by Zhao et al. (2007) analyzing their chemotaxis to

volatiles emitted by the host species *Pinus massoniana*, and the vector *M. alternatus*. The authors demonstrated that the ratio of the monoterpenes α-pinene and β-pinene, and the sesquiterpene longifolene, at 1:2.7:1.1, released by the larval vector strongly attracts dispersal J_{III}, whereas the different ratio (1:0.1:0.01) found in healthy xylem of *P. massoniana* attracts only the propagative stage of the PWN (J*n*). At this stage, nematodes may infect healthy pine trees, depending on their susceptibility, through damage made on young tree branches by beetle maturation feeding. PWNs exit from beetle carriers appears also to be dependent on plant and carrier volatile cues as well as the nematode neutral lipid content.

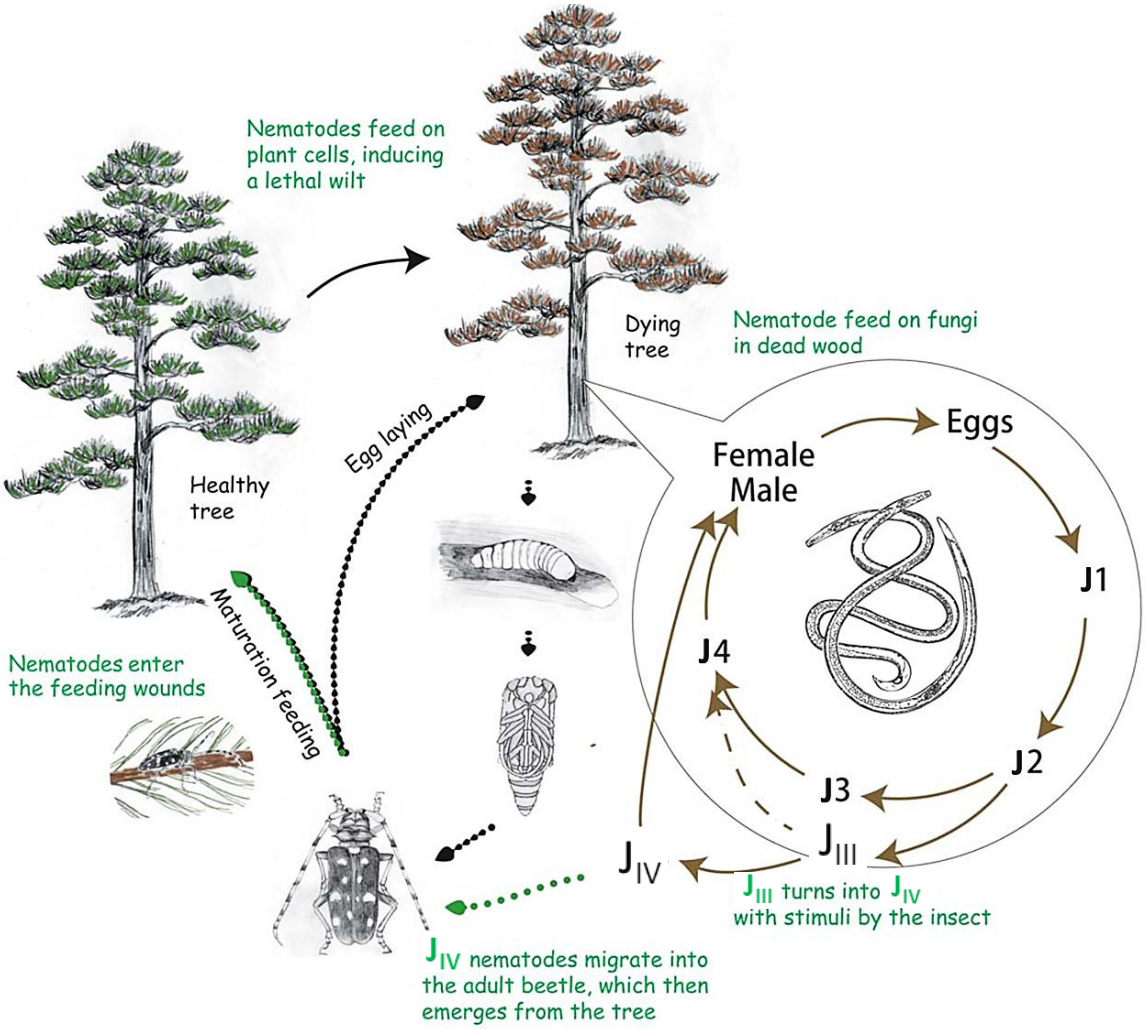

Figure 1. Pine wilt disease cycle. *Bursaphelenchus xylophilus* completes its life cycle on decaying wood where stage 4 *dauer* juveniles (J_{IV}) infect exiting *Monochamus* spp. adults. These transmit the nematode to new uninfected trees where rapid multiplication and feeding on plant tissue induces pine wilt symptoms (adapted from Kikuchi et al. 2011).

The main energy reserves of this life cycle stage are neutral lipids necessary for the histogenesis of the digestive and reproductive organs in preparation for molting to the adult stage (Stamps and Linit 2001, Zhao et al. 2014). Stamps and Linit (2001) hypothesized that nematodes with low neutral lipid content were attracted to β-myrcene, a pine volatile monoterpene hydrocarbon, while nematodes with high neutral lipid content were attracted to toluene, a beetle cuticular volatile hydrocarbon.

Attracted to pine volatiles emissions, the nematodes enter the shoots through feeding wounds (infection courts) and begin invading the resin canals, attacking the epithelial cells, causing great damage while rapidly reproducing and moving through the resin canal system. Pine wilting may be observed after approximately 3 weeks, as reduced oleoresin exudation and xylem transport damage results in embolism in the xylem column. The reduction of its defense mechanisms makes the tree attractive to adult *Monochamus* beetles. The tree may collapse within 40-60 days after infection, and can at that point contain millions of nematodes throughout the trunk, branches and roots, becoming a source for new infections (Futai and Mota 2008, Jones et al. 2008, Kuroda 2008, EPPO 2012).

The PWN has already been established for more than 100 years in Japan and East Asia and, in Portugal, despite efforts implemented by the governmental authorities to control this quarantine nematode, containment has been unsuccessful. For this reason it poses a serious threat to Portugal, as well as to the European pine wood forestry and industry.

1.1.2. Root-knot nematodes: *Meloidogyne* genus

The root-knot nematodes (RKN) belong to the genus *Meloidogyne* Göldi, 1892. These phytoparasites are among the most economically detrimental PPN genera to horticultural and field crops (Nicol et al. 2013) mainly due to their 1) pathogenic effect, 2) worldwide distribution and 3) wide host range. Being biotrophs, they require living host tissue to complete their life cycle (obligate phytoparasitism) (Agrios 2005). They reproduce sexually or parthenogenetically, many species do not have males. In the egg, embryogenesis proceeds to the first-stage juvenile, which molts into infective J2.

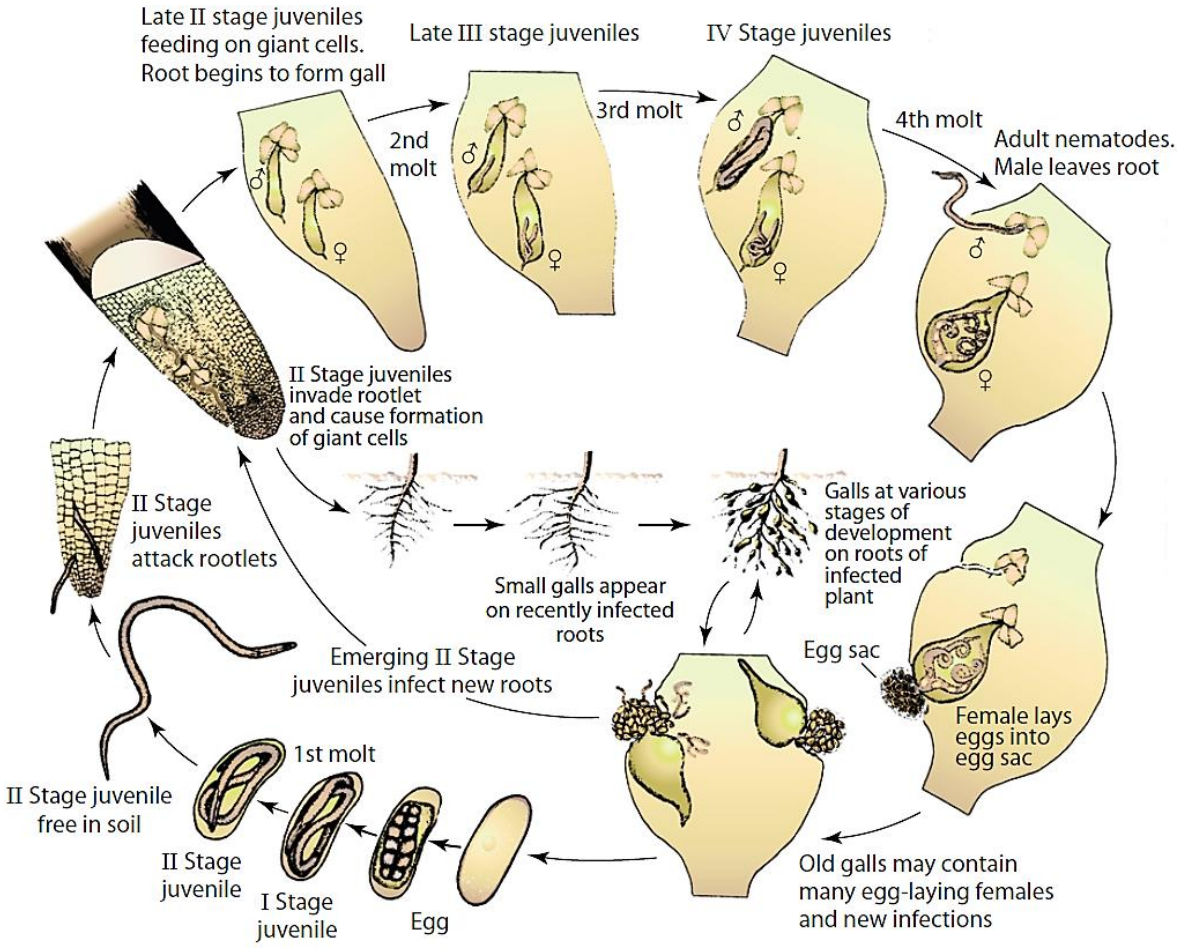

Figure 2. Root-knot disease cycle. Second-stage juveniles (J2) infect susceptible plant roots, establish the feeding site and molt into stationary pear-shaped females and motile males that exit the root. Female adult root-knot nematodes produce large numbers of eggs from where J2 hatch and exit the root to new infection sites (adapted from Agrios 2005).

Second-stage juvenile RKN hatch and move freely through the soil seeking new invasion areas, attracted to plant roots by root exudates. Root penetration and invasion, at the elongation zone, is performed with the aid of the stylet and numerous secretions, evading the plant immune response to pathogen attack (Davis et al. 2000). Within the root, juveniles move throughout, intercellularly, towards the root tip and then upward into the vascular cylinder to the differentiation zone, where the nematode feeding site is established. At this site, host cells redifferentiate into 5 to 7 hypertrophied cells, termed giant cells, through the action of different secretions, including a "cocktail" of cell wall biosynthetic and cell wall-degrading enzymes (Davis et al. 2000, Goellner et al. 2001, Abad et al. 2009) and even mobilizing plant produced enzymes (Gal et al. 2006) (Fig. 2). These specialized feeding cells are easily distinguished from the neighboring by

their increased volume, dense cytoplasm, numerous nuclei and small vacuoles. These highly metabolically active cells develop cell wall ingrowths, since they are transfer cells that are involved in rapid solute transport from the contiguous xylem elements to the nematodes, supplying them with nutrients to develop into sedentary reproductive adults (Berg et al. 2009). At this stage the J2 lose their ability to move within the root and undergo significant structural changes, molting quickly to the non-feeding J3 and J4 juvenile stages, immediately molting into an adult (male or female) sedentary stage. Adult RKN females, now with a pear-shaped swollen body, begin producing a gelatinous matrix through six rectal glands, secreted before and during egg-laying. The eggs, which may be from 500 to 2000 during the female life cycle, are retained in this matrix that provides a barrier to water loss, climate extremes and even biotic stress (Orion et al. 2001, Agrios 2005). Depending on the species, temperature and host, the life span of an adult female may extend to three months. The species composition and size of the population depend, to a considerable degree, on the conditions of the surroundings, especially the soil, climatic factors and plant cover (Sijmons et al. 1994, Orion et al. 2001). The adult male RKN is motile and exits the root.

These phytoparasites are able to move only a few meters annually on their own, but they can be spread readily through the transport of infested plants and plant products, in soil, adhering to farm implements and in irrigation water. Crop production in Europe is greatly affected by RKN parasites as new introductions continue to be reported (Kiewnick et al. 2008, EPPO 2014).

1.2. Plant parasitic nematode pest management

With the onset of industrialization in modern agriculture and the increase of monoculture mass production, the damaging effects of PPN diseases have increased dramatically requiring more effective means of pest management. Effective pest management is usually performed through non-chemical means, relying on natural host resistance or cultural controls or/and chemically, through the use of nematicides. Natural plant resistance is present only in a few crops and for a limited number of PPN species but genetic variability within field populations presents a continued threat to the durability of host resistance genes (Williamson and Hussey 1996, Mitchum et al. 2007). Cultural control (crop rotation and the use of cover crops) are the most environmentally

sustainable practice but, though frequently effective, often provide no short-term farm income and may involve further expenses in additional equipment. Unlike synthetic chemical pesticides, these approaches are mostly target-specific, environmentally safe, generally containing or leading to the formation of naturally occurring biopesticide compounds that suppress nematode population or modify nematode behavior. Still, polyphagous nematodes such as the RKN can infect a great number of plant species, from grasses to trees, which limits the usability of crop rotation (Oka et al. 2000). Furthermore, soil physical and chemical conditions, microbial populations, seasonal variation and environmental conditions influence the retention, transformation and transport of these bioactive phytochemicals introducing unpredictability in phytoparasite management that is onerous for modern crop growers (Oka et al. 2000, Kokalis-Burelle and Rodriguez-Kabana 2006).

Chemical control is performed by the application of potent synthetic chemicals that kill or disrupt the feeding or reproductive behavior of nematodes, commonly broad-spectrum fumigants and nervous system toxins, which although highly efficient (Pinkerton et al. 1986), show extremely negative environmental and public health impacts. Synthetic chemical-based pesticides are the main source for control of PPN diseases, but have been consecutively withdrawn, due to their high toxicity (generally very low LD_{50} values) and concern of becoming a major health hazard. Methyl bromide is the best example, this soil fumigant displayed outstanding properties as a pesticide, being highly effective at concentrations that many plants, vegetables and some fruits showed to be tolerant. However, the bromine in this molecule is 60 times more destructive to ozone, on an atom-per-atom basis, than the chlorine from chlorofluorocarbons (UNEP 1999), being phased out in most countries in the early 2000s, through the Montreal protocol. Another highly efficient pesticide, with extensive usage in managing soil PPN, is the carbamate aldicarb, commercialized as Temik. This cholinesterase inhibitor is still largely used against soil nematodes in potato production. Due to its high toxicity to mammals it was classified as a restricted use pesticide in the USA (EPA 2007) and banned as an active ingredient of Temik, in Europe. Synthetic chemicals available to prevent PPN infection such as avermectin, emamectin benzoate and morantel tartrate are of limited value due to poor water solubility, lack of therapeutic efficacy and/or high cost.

PPN resistance to pesticides continues to grow, and the problem of pesticide residues in food is

still a major problem. Additionally, the use of nematicides is still expensive and growing ecological concerns have risen in the past decades around the extensive application of highly bioactive synthetic chemicals as nematicides, namely in the dilapidation of natural ecosystem microorganism communities, the formation of resistance and immunity, the bioaccumulation in food products and the dangerous impact in human health (Yamashita and Viglierchio 1987, Chitwood 2003).

With the ban imposed on extremely hazardous pesticides, strong pressures on the screening of natural eco-friendly nematicides has prompted researchers to seek environmentally friendly plant-produced complex phytochemicals with high anti-nematode properties that are, at the same time, cost-effective. Finding naturally occurring nematicides or nematicidal formulations to control PPN is very complex given that they must be able to come into contact with the nematodes, which spend their lives confined to the soil or within plant organs but must also be negligibly harmful (preferably innocuous) to the host plant, besides being biocidal to the phytoparasite. Furthermore, the PPN cuticle is a poor biochemical target and is impermeable to many organic molecules. The delivery of a toxic compound by an oral route is nearly impossible because most phytoparasitic species ingest material only when feeding on plant tissue (Chitwood 2003).

The plant kingdom is very rich in secondary metabolites with high biocidal activities. Plants are able to produce a wide range of bioactive chemical compounds, namely secondary metabolites, belonging to various chemical classes. Most are impossible or very costly to produce in the laboratory through synthetic chemistry. Secondary metabolite function in plants is still not well defined but these natural products are often associated with the mediation of plant interaction with the environment, particularly in plant-insect, plant-microorganism or plant-plant interactions. Plant-derived active metabolites are a good source for environmentally safer pesticides or as model compounds for the development of chemically synthesized, easily biodegradable derivatives, that show low to negligible phytotoxicity as well as safety for humans (Chitwood 2002, Ntalli et al. 2010).

Phytochemical-based strategies for nematode control have thus become the goal of many researchers. Nematotoxic phytochemicals normally act as repellents, attractants, hatching stimulants or inhibitors and are either constitutive or may be produced as response to infection (Chitwood 2002). The use of green manure and organic amendments are effective means for the

production and/or distribution of active compounds (PiedraBuena et al. 2006). Plant extracts, aqueous or solvent extractions, are also a good source for the development of biologically based control strategies (Oka et al. 2007). Laboratory testing and research are, nevertheless, difficult to interpret biologically or biochemically, given the presence of, for example toxicants, nutrients or phytohormones that may act directly upon plant hosts (Zhou et al. 2012). Many of these bioactive secondary metabolites may be obtained from essential oils (EO), whose full chemical composition can be determined through gas chromatography (GC) and gas chromatography coupled to mass spectrometry (GC-MS). These complex secondary metabolite mixtures, which are easily isolated by steam or water distillation, offer the advantage of being natural and biodegradable but also having less strict regulatory approval mechanisms for their exploration, due to a long history of use (Isman 2006).

1.3. Essential oils

Essential oils (EOs) are commonly termed the essence of a plant. As defined by the International Organization for Standardization (ISO), the term “essential oil” is reserved for a “product obtained from natural raw material of plant origin, by steam distillation, by mechanical processes from the epicarp of citrus fruits, or by dry distillation, after separation of the aqueous phase, if any, by physical processes” (ISO 9235, 2013). They are obtained in the form of a concentrated hydrophobic liquid, at room temperature, containing volatile aroma compounds, slightly soluble in water and highly soluble in organic solvents. Chemically, EOs are comprised of terpenes, mono-, sesquiterpenes and few diterpenes, but also phenolic compounds, such as phenylpropanoids, although other groups of compounds can also occur in relevant amounts (Figueiredo et al. 2008). EOs show an extensive use in food, perfumery and pharmaceutical industries and also act as biologically active substances, revealing good properties as antioxidants but also as anti-viral, anti-microbial, fungicidal, insecticidal, insect repellent, herbicidal, acaricidal and nematicidal (Isman 2000, Chitwood 2002, Tworkoski 2002, Isman 2006, Batish et al. 2008, Koul et al. 2008).

Biological activity is commonly the result of the combined effect of compounds with activity towards the biological system but also with compounds that show no direct activity alone; these can

influence resorption, rate of reactions and bioavailability of the active compounds. Component interaction, according to their chemical nature and concentration in the EO, predominantly induce three types of effects on EO bioactivity: additive, synergistic and antagonistic. The first one occurs when the combined effect of the components is equal to the sum of the individual effects. Due to synergy, the biological activities of EOs can frequently exceed the sum of their single constituent's activities. In contrast, the antagonistic effect occurs when the activity of components in combination is inferior to when they are applied separately.

Much of the information gathered on the mode of action of EOs was obtained from studies with microorganisms, namely bacteria. In bacteria, EOs act on cell membrane permeability, disrupting ATP production, protein synthesis, pH homeostasis, altering cytoplasmic constituents and also DNA (Faleiro 2011). They can interact with the cell membranes by means of their physiochemical properties and molecular shapes, and can influence their enzymes, carriers, ion channels and receptors (Svoboda and Hampson 1999). Regarding PPN, little is known on the mode of action of EOs. Nevertheless, as complex mixtures, EOs display diverse biological activities which makes them desirable biopesticides, being able to regulate not just the targeted pest but also opportunistic species and resistant strains. This is of particular interest in phytoparasitic nematode control since the complex disease symptoms are also commonly associated with accompanying pathogenic microbiota (Back et al. 2002, Han et al. 2003, Vicente et al. 2012).

Interest in these natural phytochemicals for nematicidal purposes has been growing steadily since the early 1980's, and several plant families have revealed great potential for providing highly bioactive EOs. In the past few years, a great number of EOs have been studied as nematotoxics for PPN pest management. Studies where screenings for nematotoxic EOs are performed *in vivo* can be influenced by environmental conditions (causing variation in EO active compounds uptake, retention, transformation, degradation, etc.) (Turek and Stintzing 2013) so the bulk of the studies is performed *in vitro*, in the laboratory, using direct contact methodologies (Andrés et al. 2012, Ntalli and Caboni 2012, Barbosa et al. 2012a).

1.3.1. Activity against *Bursaphelenchus xylophilus*

PWN effective phytonematicidals should, ideally, be highly soluble in water and possess dual nematicidal and antifungal activity, thus killing not only the nematode but also the xylem-dwelling dimorphic fungi that serve as its food source (Oh et al. 2009). EOs are known to possess good antibacterial and antifungal properties (Kalemba and Kunicka 2003, Bakkali et al. 2008), and against *B. xylophilus* many have proven to be very active.

Kong et al. (2006) analyzed the activity of 88 commercial EOs against mix-stage PWNs and identified highly active *Cinnamomum zeylanicum* bark and *Coriandrum sativum* herb EOs showing $EC_{50/24h}$ of 0.12 mg/mL and 0.14 mg/mL, respectively, which proved to be higher than those obtained for some commercial synthetic nematicides (for e.g. Fenitrothion $EC_{50/24h}$ >10 mg/mL). Morphological observations indicated that EO mode of action was different from that of synthetic nematicides, inducing an extended shape rather than the usual semicircular or coiling shapes.

Again using commercial EOs, Park et al. (2007) identified highly active *Trachyspermum ammi* (rich in thymol, γ-terpinene and *p*-cymene), *Pimenta dioica* (eugenol-rich) and *Litsea cubeba* (rich in geranial, neral and limonene) EOs, with $EC_{50/24h}$ of 0.431, 0.609 and 0.504 mg/mL, respectively. The main components neral and geranial (geometrical isomers) were synthesized and likewise tested and the results indicated that position of the substituent in compound structure is very important for nematicidal activity.

Analyzing the nematotoxic potential of EOs from the Portuguese medicinal and aromatic flora, Barbosa et al. (2010) identified 5 highly active EOs against mixed-stage PWNs. Essential oils extracted from *Chamaespartium tridentatum* (rich in 1-octen-3-ol, *n*-nonanal, and linalool), *Cymbopogon citratus* (rich in geranial, neral and β-myrcene), *Origanum vulgare*, *Satureja montana* (both rich in carvacrol, γ-terpinene and *p*-cymene), *Thymbra capitata*, and *Thymus caespititius* (both with high amounts of carvacrol), showed $LC_{100/24h}$ which ranged from 0.858 to 1.984 mg/mL. This screening was furthered by Barbosa et al. (2012b), while analyzing a more suitable dilution agent for EO testing. In this work the authors concluded that the use of an organic solvent, such as acetone, appears to be more advantageous than detergent-like surfactants, like Triton X-100. Furthermore they showed that, against the PWN, the EO isolated from *Ruta graveolens*

(2-undecanone-rich) was very active, $LC_{100/24h}$ of 0.571 ± 0.046 mg/mL.

Other studies have addressed the nematotoxic potential of separate EO components. In direct contact assays against the PWN, Choi et al. (2007) evaluated the activity of different monoterpenes, using synthetic chemicals. Out of the 26 monoterpenes tested, carvacrol, thymol, geraniol, nerol, (-)-menthol, citronellol, citronellal and citral (mixture of geranial and neral) showed the highest activities against the PWN. By relating the anti-nematode activity to the chemical functional group, the authors showed that monoterpene hydrocarbons and ketones had weak or no activity. Other monoterpenes revealed a hierarchy of functional groups, phenols, aldehydes and primary alcohols being the most active followed by secondary and tertiary alcohols.

Thymol- and *p*-cymene-rich *Th. vulgaris* EOs showed a good activity in direct contact bioassays against PWN [$EC_{50/24h}$ of 1.39 mg/mL for thyme red oil and 1.64 mg/mL for white oil (which is re-distilled thyme red oil)] (Kong et al. 2007). These EOs showed to be composed of both PWN propagation stimulant- and nematicidal compounds. The authors showed that geraniol, thymol and carvacrol possessed strong nematicidal properties ($EC_{50/24h}$ of 0.47, 1.08, 1.23 mg/mL, respectively) while (-)-caryophyllene oxide, (+)-ledene, (+)-limonene, (-)-limonene, linalool oxide, β-myrcene, (-)-α-phellandrene, (+)-α-pinene and γ-terpinene were PWN propagation stimulant compounds.

The screening of PWN antagonist EOs and EO components appears to be a promising tool in discovering potential natural nematotoxics and, according to Chitwood and Meyer (2013), fractionation of nematotoxic EOs may lead to the identification of nematode-antagonistic compounds more interesting than those obtained by inferring about the nematotoxic chemical nature of the phytochemical.

1.3.2. Activity against *Meloidogyne* spp.

RKN pest management is a challenging task given that not only their life cycles show very different and distinct stages but also due to their endoparasitic ground-dwelling behavior. *In vitro* nematicide screening is generally performed in two life stages, the motile infective juvenile J2 and the egg, evaluating effects on J2 mortality and hatching, respectively. Four RKN species have been

generally used to test nematotoxics, since they are major worldwide pests (*M. arenaria*, *M. hapla*, *M. incognita* and *M. javanica*) (Moens et al. 2009). Research on nematotoxic EOs *in vitro* has been performed mainly on *M. incognita* and *M. javanica*.

The nematicidal activity of *Haplophyllum tuberculatum* and *Plectranthus cylindraceus* EOs was studied on *M. javanica* by Onifade et al. (2008). The authors showed that a 1:1 mixture of these EOs was extremely toxic to juveniles and highly toxic to hatching, at 12.5 mg/mL, after 24 h exposure. This formulation proved to be as nematicidal as carbofuran, a synthetic pesticide currently in use, at the same concentration.

Analyzing the activity of some major monoterpenoids found in aromatic plant's EOs against *M. incognita* hatching, in 72 h direct contact bioassays, Echeverrigaray et al. (2010) found that, generally, compounds with hydroxyl and carbonyl groups exhibited higher nematicidal activity than other terpenoids. *In vitro* activity was higher with borneol, carveol, citral, geraniol, and α-terpineol.

Against *M. incognita*, aromatic plants from the family *Lamiaceae* proved to be a good source for bioactive EOs. Motility bioassays were performed by Ntalli et al. (2010), using EOs from 8 Greek *Lamiaceae* plants. The EOs of *Origanum vulgare*, *O. dictamnus*, *Mentha pulegium* and *Melissa officinalis* showed the best results, with $EC_{50/96h}$ of 1.55, 1.72, 3.15, and 6.15 μL/mL, respectively. By further analyzing the activity of 13 terpenes, a hierarchy of activity was found, in decreasing order, in L-carvone, pulegone, *trans*-anethole, geraniol, eugenol, carvacrol, thymol, terpinen-4-ol (with $EC_{50/24h}$ ranging from 115 to 392 μg/mL). The EOs of *Foeniculum vulgare*, *Pimpinella anisum*, *Eucalyptus meliodora* and *Pistacia terebinthus* were analyzed by Ntalli et al. (2011a) inhibiting J2 motility, and revealed $EC_{50/96h}$ of 231, 269, 807 and 1116 μg/mL, respectively. The authors further analyzed the synergic and antagonist effects of the major nematotoxic EO components and showed that potent synergistic relations existed between *trans*-anethole/geraniol, *trans*-anethole/eugenol, carvacrol/eugenol and geraniol/carvacrol, combinations often found in EOs isolated from medicinal and aromatic plants.

The EO extracted from *Ruta chalepensis* induced paralysis in J2 nematodes of *M. incognita* and *M. javanica*. Ntalli et al. (2011b) showed that the aliphatic ketone 2-undecanone was the main responsible for nematotoxic activity ($EC_{50/24h}$ of 20.6 and 22.5 μg/mL for *M. incognita* and *M. javanica*, respectively).

Gupta et al. (2011) identified nematotoxic *Eucalyptus globulus* (1,8-cineole) and *Carum copticum* (thymol, γ-terpinene and *p*-cymene) EOs. Paralysis in J2 juveniles was recorded over time, for 72 h. At 125 µL/L, after 30 h, *Eucalyptus* essential oil induced complete mortality.

Caboni et al. (2013) found a high J2 motility suppression activity with the application of *Mentha spicata* EO ($EC_{50/72h}$ = 358 µg/mL), with high contents of the oxygen-containing monoterpene carvone. Tested solely, carvone showed $EC_{50/48h}$ of 730 µg/mL. Previously, also Abd-Elgawad and Omer (1995), had reported high hatching inhibition percentages with the application of *M. spicata* EO. Although with higher contents in carvone, *M. longifolia* showed slightly lower activity, which might indicate that minor EO components influenced EO overall activity.

Clove (*Syzygium aromaticum*) EO has also shown very good nematotoxic activities as reported by Meyer et al. (2008a) for *M. incognita*. *In vitro* J2 hatching and viability direct contact bioassays revealed EC_{50} of 0.097 and 0.145% (v/v), respectively. However, further studies on the biocidal potential of the EO detected also phytotoxicity to cucumber (*Cucumis sativus*), muskmelon (*Cucumis melo*), pepper (*Capsicum annuurn*), and tomato (*Solanum lycopersicum*) seedlings. Besides lowering seedling survival <50%, most EO concentrations decreased shoot heights and fresh shoot weights of all seedlings when applied at transplant stage (Meyer et al. 2008b).

Care must be taken when choosing an effective nematotoxic EO, while *in vitro* screening for nematotoxic phytochemicals is important, the final pesticide application will unavoidably have to deal with the plant host biology, susceptibility to toxicity and biotransformation capacity.

1.3.3. Phytotoxity and biotransformation

The production of one or more phytochemicals that have stimulatory or inhibitory effects on growth, survival, and reproduction of other plants is believed to contribute heavily in such mechanisms as plant dominance, succession, formation of communities, climax vegetation, crop productivity, and exotic plant invasion (Haig 2008). Modern organic agriculture practices use these phytochemical potentialities in weed control with the application of EOs as phytotoxics. Current use herbicidals include EOs extracted from *Pinus* spp., *Cymbopogon* spp., *Eugenia caryophyllus*, *Mentha piperita*,

Azadirachta indica, *Ricinus communis* and also from several *Eucalyptus* species (Dayan et al. 2009).

A relatively large number of highly phytotoxic phytochemicals are derived from the terpenoid pathway, yet the mode of action of only a few of these phytotoxins is well understood. To date, relatively little overlap is known between the molecular target sites of commercial herbicides and those known for natural phytotoxins. The monoterpenes 1,4-cineole and 1,8-cineole are good examples of herbicidal chemical starting structures. Despite the similarity in structure, these compounds apparently have different modes of action. Both are strong plant growth inhibitors yet 1,4-cineole causes growth abnormalities in shoots while 1,8-cineole strongly inhibits all stages of mitosis and inhibits mitochondrial respiration, similar to the action of the monoterpene camphor (Duke and Oliva 2004). The discovery of the molecular site of action of 1,4-cineole, the enzyme asparagine synthetase, lead to the development of its less volatile herbicidal analogue, cinmethylin. Also the sesquiterpene artemisinin has been quite studied for its molecular target site (Duke and Oliva 2004).

Phenolic compounds, also commonly present in EOs, exert their phytotoxicity on cell membranes, resulting in nonspecific permeability changes that alter ion fluxes and hydraulic conductivity of roots. A cascade of physiological effects follows, which include alterations in ion balance, plant-water relationships, stomatal function, and rates of photosynthesis and respiration. Phytohormones and enzymes are also affected, occurring deviations from typical patterns for biosynthesis and flow of carbon into metabolites (Einhellig 2004).

Besides the risk of being toxic, EO components may also be biotransformed by plant tissue. These processes transform exogenously supplied compounds integrating them into the endogenous metabolome or into novel compounds, products of biotransformation. These processes use the enzymes and even whole cells as biological catalysts, through which the functional groups of organic compounds are modified to a chemically different product. These reactions include reduction, oxidation, hydroxylation, acetylation, esterification, glycosylation, isomerization, methylation, demethylation, epoxidation (Giri et al. 2001, Rao and Ravishankar 2002).

In vitro cultures are often used to study precursor feeding and biotransformation processes due, not only, to the controlled environment they offer but also to the genetic stability and easy

availability. Terpene biotransformation was studied in several *in vitro* systems, for e.g. cell suspension cultures (Figueiredo et al. 1996, Zhu and Lockwood 2000, Zhu et al. 2010) or hairy roots (Faria et al. 2009, Nunes et al. 2009, Chandra and Chandra 2011).

The detailed study of these mechanisms and of those induced by the infection of the phytoparasite are very hard to determine *in vivo*, where both the nematode and the plant are subjected to many biotic and abiotic variables. Co-culturing the plant and the nematode in a single *in vitro* culture is the most direct way to establish a clean, reliable and easy to use host with phytoparasite environment.

1.4. Plant biotechnology

Research on the effect of nematicidal compounds is commonly performed on the nematode species alone and very seldom on the host-parasite system, not taking into account the cytotoxicity for the plant host or the plant's capability to metabolize or biotransform the nematicidal active substances. Studying the host / parasite system will yield only partial results if performed in each intervenient separately, on the other hand, studying this system *in vivo* or in natural conditions will be dependent on environmental and biological variations. Current pest management studies targeting to attain an effective phytochemical-based nematode pesticide require the development of a laboratory tool emulating real host with parasite environment, which can provide information on the exact response of the parasite but also of the host to nematotoxic application. The study and precise measurement of responses from both PPN and host plant can only be achieved in the laboratory, where single variables can be identified and manipulated. As stated by Bolla (1993), *if nematologists are to understand fully the mechanisms by which nematodes successfully infect plants or the mechanisms that plants use to prevent establishment of nematode infections, a simple model laboratory system must be found that mimics as closely as possible the field environment for invasion and establishment of nematode infections of natural hosts*. This simple model system may be found in current plant biotechnology methodologies, which have provided several laboratory systems for scientific research. According to Barker et al. (1994), *the principal new resource is biotechnology and its potential to advance our understanding of the molecular*

basis of nematode growth and parasitism, and host-plant susceptibility and resistance. The powerful tools of biotechnology now provide the means to solve fundamental and longstanding questions in agricultural nematology, including diagnostics.

Plant tissue culture was originally developed as a research tool to study the biochemistry and physiology of plants. It is based on the fact that individual cells of an organism are totipotent and therefore it is possible to regenerate a whole plant from a single cell. The first commercial interest in *in vitro* plant culture was the use of tissue culture for the micropropagation of plants, as factory systems they would be able to supply product throughout the year in the right quantities and of the correct quality (Scraag 1997). In research, the growth of plant tissues and cells on nutrient defined culture media has led to a widening of the methodologies available for experimental investigations. The application of these methods to phytopathology studies allowed a further and more specific study of pathogen behavior, penetration, infection and disease development as well as plant host morphological, physiological and genetic alterations, but the most substantial use was for contamination-free nematode propagation and mass culture (Maheshwari 1969, Verdejo-Lucas 1995, Punja et al. 2007). Plant biotechnology offers various *in vitro* systems which allow the precise determination of PPN interaction with plant tissue as well as the secretome, secreted organic molecules and/or inorganic elements, interplay occurring between parasite and host while providing an easily manageable study system. The growth of more than one organism or cell type in a combined culture (*in vitro* co-cultures) has the advantage of simulating the host-pathogen conditions and eliminating variables due to the environmental *in vivo* conditions. *In vitro* plant / nematode co-cultures have been commonly used since mid-1900's to increase and maintain nematodes on the lab and are very useful systems to study host / parasite interactions (Bonga and Durzan 1982, Maheshwari 1969). In monoxenic cultures the host-pathogen system is free from contaminants such as microbial flora and fauna which characterize the natural conditions (Bonga and Durzan 1982, Mitkowski and Abawi 2002). Moreover, in a controlled environment of an *in vitro* culture, single variables can be manipulated and plant / nematode responses can be observed directly, which is very difficult to achieve on greenhouse or in field (Winterhagen et al. 2007). Also, *in vitro* cultures have the advantage of having a higher quantity of plant material with fewer resources (Georgiev et al. 2009).

1.4.1. *In vitro* shoot cultures as model hosts for pinewood nematode

Applied to forestry, *in vitro* culture systems have the potential for rapidly multiplying high value genotypes for reforestation. Unlike hairy root systems, *in vitro* shoot culture is obtained by the exposure of plant material to exogenous plant growth regulators, supplied in the culture medium, generally high cytokinin and low auxin concentrations (Fig. 3). This plant biotechnology technique requires the isolation of meristematic tissue from *in vivo* plant and, in asepsis, its exposure to specific concentrations of compounds that promote cell division and cytokinesis, generally natural or synthetic cytokinins. The prolonged exposure promotes meristem multiplication. With the appropriate stimulus (plant growth regulator concentration, activated charcoal, etc.) meristem tissue can be elongated to a shoot to produce identical genetic clones of the mother-plant (Fig. 3) (Hall 1999).

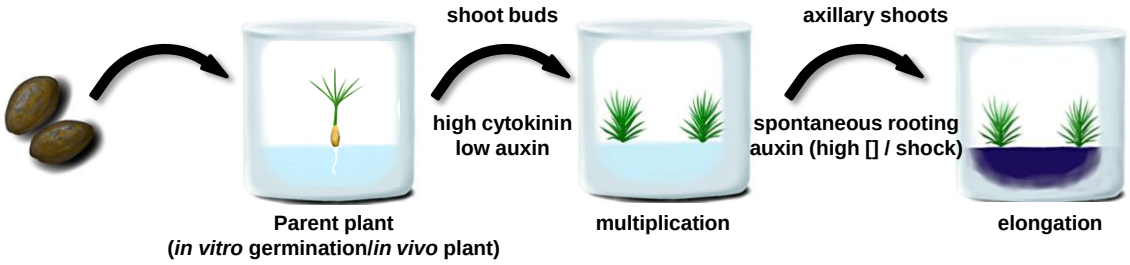

Figure 3. *In vitro* shoot culture process. Shoot buds isolated from full grown *in vivo* plants or from aseptic *in vitro* germinants are subjected to specific growth regulators for shoot multiplication induction. With appropriate stimulae, these can be elongated to produce genetically identical *in vitro* shoots (illustration by J. Salvado).

In vitro shoot culture system is advantageous given that it allows the production of many plants a) that are a population of genetically identical individuals, b) where many forest traits, which can be lost through sexual propagation, can be analyzed, c) free from diseases, contaminants and microbiota, d) that are recalcitrant to other techniques (small amount and/or non-viable seeds), e) that grow in a controlled environment and f) using fewer resources *per* quantity of plant material, which is of extreme importance when dealing with large woody species that make greenhouse studies difficult and time-consuming (Hall 1999, Jain and Häggman 2007).

Nevertheless, this process is still very expensive, requiring specialized labor, and *in vitro* shoots might not always become true-to-type after tissue culture. Migratory nematodes of trees are rarely

studied in *in vitro* culture conditions. Most studies use *in vitro* cultures as a means to establish clonal regenerants to test genetic stability. Goto et al. (1998) have established *in vitro* micropagated *Pinus thunbergii* that showed resistance against the PWN. A single nematode-resistant mother plant was chosen for *in vitro* culture and cultures were established, maintained and increased in numbers for over ten years. *In vitro* material was tested for genetic stability, required for the establishment of resistant clonal propagation *in vivo*. Infection with the nematode was not tested *in vitro*, in a co-culture system, but the authors envisioned obtaining plantlets for outplanting.

Using *in vitro* cultures of *Prunus persica* resistant to *M. incognita*, Hashmi et al. (1995) analyzed genetic variation due to *in vitro* conditions. In most genotypes resistance was maintained. *In vitro* shoots and rooted regenerants were infected with the nematode but co-culture conditions were not established and maintained.

The use of micropropagated large woody species in phytopathology allows testing of nematotoxic EOs without the need of a great volume of both nematicide and diseased plant material. This makes possible a great number of studies that could not be performed *in vivo* and most studies less laborious and time consuming. Nevertheless ascertaining the adequacy of the co-culture is of utmost importance so that the studies performed may mimic what is observed *in vivo*.

1.4.2. *Hairy roots* as model hosts for root-knot nematodes

Hairy roots (HR) take advantage of the natural infection mechanism of the bacteria *Rhizobium rhizogenes* [according to new taxonomic revisions (Bull et al. 2010), *Rhizobium rhizogenes* (Riker et al. 1930) Young et al. 2001a is the most recent synonym of *Agrobacterium rhizogenes* (Riker et al. 1930) Conn 1942] on several plant species, particularly dicotyledonous. This soil-dwelling gram negative bacterium is able to penetrate wounds in the plant and induce, at the infection site, the abnormal growth of root tissue with primary growth characteristics, with high root hair density.

These morphologic alterations are induced by the stable integration of the bacterial Ri (Root inducing) plasmid into the plant genome (Fig. 4a).

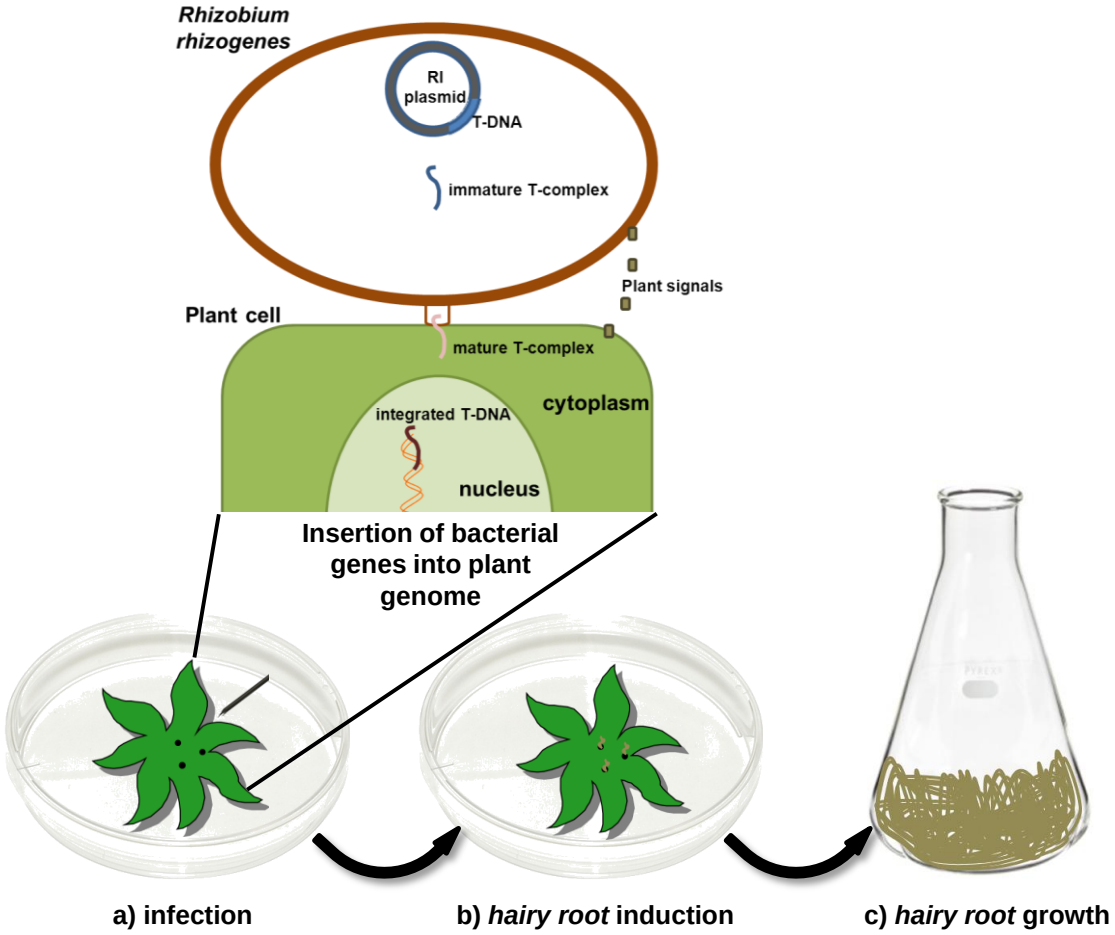

Figure 4. *Rhizobium rhizogenes* cellular infection mechanism leads to the production of transgenic hairy roots in plant tissue that can be cultured *in vitro*. a) Plant derived signals promote transference of the T-DNA from the bacterial Ri plasmid to the plant genome, resulting in altered phytohormone production. b) Phytohormone unbalance leads to the growth of transgenic roots with the *hairy root* phenotype. c) In defined growth culture media root growth can be maintained without exogenous plant growth regulator supply.

Transcription of these genes leads to the production of enzymes involved in the biosynthesis of plant hormones, namely auxins and cytokinins that regulate secondary root formation and generate the hairy root phenotype. Mediation is not performed solely through increase of internal phytohormone levels but also to a higher sensibility to these morphogenes. Genes from the Ri plasmid are also responsible for the production of low weight carbon compounds named opines, which function as carbon and/or nitrogen source and can induce plasmid transference between bacteria trough conjugation (Gelvin 1990, Komari et al. 2004).

In the laboratory, aseptic plant material is generally maintained in co-culture with *R. rhizogenes* to ascertain infection and gene transfer. With infection, plant cells release phenolic compounds

(for e.g. acetosyringone), inducing bacteria virulence genes that are responsible for T-DNA integration into the plant genome. Within a few days the plant sections can be transferred to culture medium supplemented with antibiotics to eliminate the bacteria. The formation of transgenic roots may be as fast as a week or as long as a month, depending on the plant species (Fig. 4b). Once in axenic culture, HR can be maintained in plant growth regulator-free culture medium indefinitely (Fig. 4c) (Hamill and Lidgett 1997, Hu and Du 2006).

Besides being differentiated tissue, required for RKN cell cycle completion, the advantage of using HR can be observed at many levels, mainly 1) their high growth rate in the absence of plant growth regulators, which have been proven to influence nematode infection and development (Huettel and Hammerschlag 1986, Akhkha et al. 2002, Gutierrez et al. 2009), 2) a high ratio of biomass production per unit time, 3) their cellular integrity and longevity, which determine a higher genetic and biochemical stability and 4) the accumulation of specific secondary metabolites in high yields (Figueiredo et al. 2006). These characteristics make HR *in vitro* cultures a suitable biotechnological model system to analyze RKN infection.

HR have been used in several nematode phytopathology studies. Mugnier (1988) used HR cultures to analyze the effect of transport of the nematicide oxamyl on infection with *M. incognita* and *Heterodera schachtii* J2. In HR of the hosts *Beta vulgaris*, *Brassica napus*, *Daucus carota*, *Medicago sativa*, *S. lycopersicum*, and *Vigna unguiculata* the J2s were attracted and initiated infection by thrusting their stylets repeatedly into the apical cells, while in oxamyl treated HR, transport of the nematicide to the root tips immobilized J2 juveniles.

RKN reproduction was studied on several HR systems under monoxenic conditions by Verdejo et al. (1988). *M. javanica* infection was tested on HR of *S. tuberosum*, *S. lycopersicum*, *Convolvulus sepium*, *D. carota*, *Phaseolus lunatus*, the first two were the most successful. Co-culture growth dynamics revealed to be very important, as phytoparasite and host growth/development had to be timely for HR growth not to surpass nematode development or for nematode infection not completely consume the HR culture.

So the authors established that 1) HR that grew at moderate rates into the agar and produced many secondary roots supported the highest reproduction, 2) nematode inoculum is very important, both nematode number and the nematode life stage, being second-stage juveniles preferred

because quantity and viability of inoculum can easily be assessed and 3) nematode infectivity should be tested *in vivo* to ascertain its maintenance. The most successful cultures (tomato and potato) were further tested by Verdejo and Jaffe (1988) to analyze the effect of introducing a third intervenient into the co-culture, the bacteria *Pasteuria penetrans* as a means of biological control. The authors found substantial variations in eggs / culture and eggs / healthy female ratios.

Kumar and Forest (1990) also studied nematode reproduction on *S. tuberosum* HR cultures. The potato cyst nematode, *Globodera rostochiensis*, developed and within 7-8 weeks had completed its life cycle in the transgenic roots. Co-culture stability showed to be suitable for large-scale culture and storage of potato cyst nematode pathotypes under sterile conditions and for *in vitro* screening of large numbers of susceptible and resistant mutant lines.

Envisioning the production and storage of *Heterodera glycines*, the soybean cyst nematode, Savka et al. (1990) established *Glycine max* hairy roots and analyzed nematode reproduction and development. The authors successfully established a subculture methodology to obtain indeterminate growth of the co-cultured host with phytoparasite system.

HR have also been used as source for nematicidal compounds. Kyo et al. (1990) established α-tertienyl-rich *Tagetes patula* HR that showed a high nematicidal potential against the nematodes *Caenorhabditis elegans* and *Pratylenchus penetrans*. The high activity was associated with nematicidal α-tertienyl (terthiophene) but also with other compounds present in the HR.

Being, usually, easily established, HR *in vitro* cultures can be adapted to the nematode pest of interest, taking into account the specific plant material and the conditions of the site were the pest is active. The external (temperature, photoperiod, etc.) and internal (medium composition, pH, etc.) conditions can be improved according to the ones observed for the natural infestation conditions, allowing a number of optimization steps towards localized bioactive phytochemical nematicides.

1.4.3. *In vitro* co-cultures simulate the host / phytoparasite interactions

Co-culture adequacy to phytopathology studies must be analyzed, having in mind the limitations of *in vitro* culture to reproduce *in vivo* natural conditions. Co-culture response to testing should be

equivalent to that observed *in vivo*, so by using the same techniques similar responses should be observed. Phytoparasite penetration, infection and multiplication in the *in vitro* grown tissue must be followed and compared to that which is detailed in previous agricultural studies performed *in vivo*. Establishment of "true-to-type" co-cultures permits the experimentation of nematicidal compounds and formulations with a degree of confidence similar to that of the *in vivo* host / phytoparasite system.

Co-culture suitability to phytopathology studies is seldom analyzed. Studying *R. rhizogenes* transformed tomato cultures and non-transformed root tomato, onion and dandelion cultures, Mitkowski and Abawi (2002) analyzed the aptness of monoxenic hosts for the maintenance of the root-knot nematode (*Meloidogyne hapla*). Tomato hairy root systems yielded considerably higher levels of nematode reproduction than inoculated onion and dandelion root cultures.

Wubben et al. 2009 established cotton (*Gossypium hirsutum*) HR from *M. incognita* resistant and susceptible plants. These cultures were infected with the nematode and the co-culture morphology, growth and genetic structure were followed. Nematode life cycle was completed and plant with nematode co-cultures were maintained. According to the authors the co-cultures established can serve as a model system for studying molecular cotton–nematode interactions, being advantageous for the maintenance of monoxenic RKN as well as be useful in evaluating the effect of manipulated host gene expression on nematode resistance in cotton.

Although in its early stage, co-culture establishment directed to nematicidal screening appears to be a very advantageous biotechnological technique to be applied in the pesticide industry to enhance and validate natural products as nematode pesticides.

2. Objectives

Plant / nematode interactions has been the object of extensive studies. Great efforts and labor have gone into research on this field, with testing on experimental fields and greenhouses, and in the laboratory, trying to contain the different variables that this pest is subjected to in the field, trying to effectively reproduce the natural conditions of this kind of phytopathology. Yet, there is still a need to implement an adequate testing tool that allows an easy manipulation as well as confident

stimulus-response relationship to study PPN disease development. Biotechnology may prove to be an adequate methodology since it offers a set of *in vitro* techniques capable of supporting nematode infection and development. As obligate plant endoparasites, RKN require a plant growth regulator-free differentiated tissue to complete their life cycle, so can easily be cultured in *in vitro* HR cultures. For the PWN, which require plant shoot tissue for the phytoparasite phase of its life cycle, forest tree *in vitro* shoot culture can produce genetically identical clones capable of harboring nematode populations. Fundamental for laboratory use of these *in vitro* host / parasite co-cultures is the assurance of their adequacy, by the use of more than one methodology. Once created these co-cultures allow the batch testing of nematicidal compounds or molecules, in a much swift manner. With the pressure on the use of natural and eco-friendly pesticides, EO experimentation would highly benefit from such systems. Regarding EO testing, *in vitro* co-cultures would allow the evaluation of EO effect simultaneously in the parasite and also the host plant. In effect, a single *in vitro* culture container would simulate what occurs in an agricultural field or in a forest that is affected by PPN diseases. For the pesticide industry these systems could allow development of innovative testing systems as well as advance the discovery of new nematicides that are most effective locally, due to the possibility of parameter variation to emulate local seasonal variations.

Given this, the current work had the following main goals:

a) Evaluation, through direct contact assays, of the nematotoxic potential of essential oils, essential oil fractions and/or decoction waters (remaining hydrodistillation water) against the PWN motility and CRKN hatching.
b) Establishment of *S. tuberosum* HR, and *S. tuberosum* HR with *M. chitwoodi* co-cultures and also *in vitro P. pinaster* and *in vitro P. pinaster* with *B. xylophilus* co-cultures to characterize culture structure and growth, quantify nematodes in *in vitro* co-cultures medium and evaluate the constitutive and induced production of volatiles.
c) Determination of the effect of the selected putative nematotoxic phytochemicals on the host with parasite co-cultures established by analyzing growth, nematode population in *in vitro* co-cultures medium and production of induced volatiles.
d) Proposal of *in vitro* cultures as biotechnological models for pest control of phytoparasitic nematodes using phytochemicals.

2.1. Thesis outline

The PhD thesis work was organized in 8 chapters, 6 of these chapters consisting of scientific articles, all with introduction, methodology, results and discussion and independent bibliography, with the corresponding indication of whether they are published, accepted or submitted for publication.

Chapter 1 introduces the plant parasitic *Bursaphelenchus xylophilus* and *Meloidogyne* spp. and pest management practices focusing on studies of EOs as nematotoxics. Also, plant biotechnology laboratory models most adequate to establish host with parasite co-cultures are reviewed.

The following chapters were organized according to the studies performed on each of the two nematodes types, that is, Chapters 2 to 4 deal with the work developed with *B. xylophilus* and Chapters 5 to 7 deal with the work developed with *M. chitwoodi*. **Chapter 2** and **Chapter 5** deal with the direct contact surveys of nematotoxics to pinpoint potential nematotoxic pesticides. **Chapter 2** addresses the work with the highly infectious PWN which has become a major threat to the European forest ecosystem, for causing pine wilt disease (PWD). **Chapter 5** focuses on the work performed on nematotoxic EOs against CRKN hatching, which had not been reported before. From this screening a set of EOs was selected as putative nematotoxic pesticides to be tested for activity in host with parasite co-cultures as biotechnological lab models.

Chapter 3 and **Chapter 6** discuss the successful establishment and analysis of these models. The first describes the establishment of *in vitro Pinus pinaster* shoots with *B. xylophilus* in a co-culture system and comparison of its structure and volatiles to that of one-year old seedlings. The second reports on the establishment of co-cultures of *Solanum tuberosum* hairy roots with *Meloidogyne chitwoodi* as a model system and on co-culture volatiles profiling as well as root gall structure throughout culture time.

Finally, **Chapter 4** and **Chapter 7** focus on the application of two nematotoxic EOs in the host / parasite environment of the co-cultures established and on the analysis of their immediate effects in terms of growth, nematode population in *in vitro* co-cultures medium and production of induced volatiles.

On the whole, these chapters try to demonstrate that the use of biotechnological models is a more expeditious form to quickly screen effective PPN antagonists.

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The pine wilt disease

Chapter 2

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

Jorge MS Faria, Pedro Barbosa, Richard N Bennett, Manuel Mota, A Cristina Figueiredo (2013) Bioactivity against *Bursaphelenchus xylophilus*: nematotoxics from essential oils, essential oils fractions and decoction waters. Phytochemistry 94:220-228

Abstract

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

Keywords: *Bursaphelenchus xylophilus*, carvacrol, decoction waters, essential oils, hydrocarbon fraction, nematotoxic, oxygen-containing fraction, *Pinus pinaster*, γ-terpinene, 2-undecanone

1. Introduction

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

Phytoparasitic nematode control is very complex, generally relying upon synthetic chemicals, as broad-spectrum nematicides which are extremely damaging (Chitwood 2003). The PWN is commonly controlled by controlling the insect vector through aerial application of synthetic insecticides, by fumigation of infected trees, pine tree-free strips, use of vector natural enemies, or by controlling the nematode through trunk injection of nematicidal compounds such as abamectins (Lee et al. 2003, Takai et al. 2003, Mota and Vieira 2008). Nevertheless, the use of synthetic pesticides is associated with environment pollution and undesirable influences on human health or against non-target organisms (Zhao 2008).

The use of plant-derived natural products for pest control is not recent, but has gained ground in modern pest management due to growing ecological concerns about the use of synthetic pesticides, and their consequent phasing out in the market and banning. Essential oils (EOs) are complex mixtures of volatiles, mainly products of plant secondary metabolism. Commonly, they are comprised of terpenes, mono- and sesquiterpenes, and phenolic compounds, such as phenylpropanoids, although other groups of compounds can also occur in relevant amounts. They are generally biodegradable, have low toxicity to mammals and do not accumulate in the environment (Figueiredo et al. 2008). The biological activities of EOs can frequently exceed the sum of their single constituent's activities, due to synergy. As complex mixtures, EOs may display several biological activities which makes them desirable biopesticides, being able to control not just the targeted pest but also opportunistic species and resistant strains. This is of particular interest in phytoparasitic nematode control since complex disease symptoms are also commonly associated

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

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

2. Material and methods

2.1. Plant material

Collective and/or individual samples, from cultivated and wild-growing medicinal and aromatic plants, were collected from mainland Portugal and at the Azores archipelago (Portugal) (Table 1). Dried aerial parts from commercially available products sold in local herbal shops were also analyzed. Thirteen families were sampled, from a total of 84 samples. For all plants collected from the wild state a voucher specimen of each plant species was deposited in the Herbarium of the Botanical Garden of Lisbon University, Lisbon, Portugal. For commercially obtained plant material, a reference sample from each plant is retained in our laboratory and is available on request.

2.2. Essential oil extraction

Essential oils (EOs) 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. EOs were stored in the dark at -20ºC, until analysis.

2.3. Essential oil fractionation

Fractions containing hydrocarbons (HM) or oxygen-containing molecules (OCM) were separated from each EO sample on a silica gel column [22 g of Silica gel 60 (Merck 9385) on a 8.5 mm internal diameter, 380 mm length column] by elution with 20 mL of distilled *n*-pentane (Riedel-de Haën, Sigma-Aldrich, Germany) followed by 20 mL diethyl ether (Panreac Química S.A.U., Barcelona, Spain), per mL of essential oil. A total of 5 mL of EO was fractionated. The hydrocarbon fraction was obtained after distilled *n*-pentane elution, and diethyl ether eluted the EO oxygen-containing components.

Both fractions were concentrated, separately, at room temperature under reduced pressure on a rotary evaporator (Yamato, Hitec RE-51), collected in a vial, and concentrated to a minimum volume, again at room temperature, under nitrogen flux. Fractions were then stored in the dark at -20ºC until analysis.

2.4. Essential oil and fractions composition analysis

Essential oils and the corresponding HM or OCM fractions were analyzed by gas chromatography (GC), for component quantification, and gas chromatography coupled to mass spectrometry (GC-MS) for component identification. Gas chromatographic analyses were performed using a Perkin Elmer Autosystem XL gas chromatograph (Perkin Elmer, Shelton, CT, USA) equipped with two flame ionization detectors (FIDs), a data handling system, and a vaporizing injector port into which two columns of different polarities were installed: a DB-1 fused-silica column (30 m × 0.25 mm i.d., film thickness 0.25 µm; J & W Scientific Inc., Rancho Cordova, CA, USA) and a DB-17HT fused-silica column (30 m × 0.25 mm i.d., film thickness 0.15 µm; J & W Scientific Inc., Rancho

Cordova, CA, USA). Oven temperature was programmed to increase from 45 to 175°C, at 3°C/min increments, then up to 300°C at 15°C/min increments, and finally held isothermal for 10 min. Gas chromatographic settings were as follows: injector and detectors temperatures, 280°C and 300°C, respectively; carrier gas, hydrogen, adjusted to a linear velocity of 30 cm/s. The samples were injected using a split sampling technique, ratio 1:50. The volume of injection was 0.1 µL of a pentane-oil solution (1:1). The percentage composition of the oils was computed by the normalization method from the GC peak areas, calculated as a mean value of two injections from each volatile oil, without response factors.

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

The percentage composition of the isolated EOs was used to determine the relationship between the different samples by cluster analysis using Numerical Taxonomy Multivariate Analysis System (NTSYS-pc software, version 2.2, Exeter Software, Setauket, New York) (Rohlf 2000). For cluster analysis, correlation coefficient was selected as a measure of similarity among all accessions, and the Unweighted Pair Group Method with Arithmetical Averages (UPGMA) was used for cluster definition. The degree of correlation was evaluated according to Pestana and Gageiro (2000) in very high (0.9-1), high (0.7-0.89), moderate (0.4-0.69), low (0.2-0.39) and very low (<0.2).

2.5. Isolation of decoction waters

After hydrodistillation, each of the 45 decoction waters (remaining hydrodistillation water) was separated from the plant material through coarse sieving using filter paper. The decoction waters were separately concentrated to a minimum volume, at 60ºC under reduced pressure, in a rotary evaporator. The concentrated decoction water (CDW), was stored in the dark at -20ºC until use.

2.6. Nematode collection and rearing

Pinewood nematodes (PWNs) were obtained according to Barbosa et al. (2010). Axenic cultures of *Botrytis cinerea* (de Bary) Whetzel were grown for 7 days, at 25±1ºC, on steam-sterilized hydrated commercial barley grains (*Hordeum vulgare* L.). An aliquot of 100 µL, containing 100-200 mixed-stage PWNs, in ultrapure water, was then added to these cultures.

After 7-10 days, in darkness, at 25±1ºC, the PWN population, grown by consuming the fungus, was isolated by a modified Baermann funnel technique (Viglierchio and Schmitt 1983). Live nematodes, which naturally descended to the bottom of the apparatus, were collected after 24 h into a 20 µm mesh sieve and rinsed thoroughly with ultrapure water. Nematode solutions were used for further inoculations or stored at 4ºC. PWN mortality assessment was performed using an inverted microscope [Diaphot, Nikon, Japan (40x)].

2.7. Direct contact bioassays

Essential oils, HM or OCM fractions and concentrated decoction waters (CDW) were assayed in newly extracted nematode suspensions as detailed in Barbosa et al. (2010). EOs and fractions stock solutions were prepared in methanol (Panreac Química S.A.U., Barcelona, Spain), at 40 µL/mL. A previous study has shown that the use of water-miscible solvents such as acetone (Barbosa et al. 2012) are more advantageous than the widely used detergents, such as Triton X-100, when performing direct contact assays employing EOs. Methanol was presently chosen due to its high polarity and high solvent capacity.

To obtain a final concentration of 2 µL/mL, 5 µL of these solutions were added to 95 µL of

nematode suspensions with 50 to 100 mixed stage nematodes. Stock solutions for 1, 0.5 and 0.25 µL/mL were obtained by serial dilutions with a dilution factor of two. The EOs which showed corrected mortalities below 96% were not further assayed at lower concentrations. Control trials were performed with methanol 5% (v/v, methanol/nematode suspension). In order to check for methanol induced mortality ultrapure water was used as corresponding control.

Stock solutions for the CDW were obtained by centrifuging a 1:1 mixture of CDW in ultrapure water (2500 G), to remove high weight debris. The supernatant was assayed at the final concentration, in the test suspension, of 25 µL of CDW per mL of nematode suspension. CDWs were assessed for the nematode's complete mortality only; if live nematodes were detected CDWs were not further assayed at lower concentrations. CDWs which showed complete mortality at one given concentration were tested at a lower one, 12.5 and 6.3 µL of CDW per mL of nematode suspension.

All bioassays were performed in flat bottom 96-well microtiter plates (Carl Roth GmbH + Co. KG, Karlsruhe, Germany), covered with plastic film, to diminish volatilization, and aluminum foil to establish total darkness. The plates were maintained at 25±1ºC in an orbital shaker at 90 r.p.m., for 24 h. Dead and live nematodes were counted under an inverted microscope (40x). Nematodes were considered dead if they did not move even when physically stimulated. A minimum of 10 assays were performed for each sample, in, at least, two separate trials.

2.8. Lethal concentration (LC_{100}) determination

The determination of the lowest concentration at which 100% death is observed (LC_{100}), was based on mean corrected mortality values. Mean corrected mortality calculated in each trial, was obtained by comparing the percentage mortality due to essential oil treatment to the percentage mortality in the methanol control, using the Schneider-Orelli formula (Putenter 1981):

Corrected mortality % = [(Mortality % in treatment - mortality % in control) / (100 – mortality % in control)] x 100

The application of this formula normalizes the mortality values, allowing the comparison of different runs with slightly different mortalities.

Nematotoxic activity was evaluated according to Kong et al. (2006) by classifying mortality as strong (>80%), moderate (80-61%), weak (60-40%) and low or inactive (<40%).

To estimate the half maximal effective concentration (EC_{50}), mean corrected mortality values were subjected to non-linear regression analysis using a dose-response log-logistic equation proposed by Seefeldt et al. (1995):

$$y = C + (D - C) / 1 + \exp \{b [\log (x) - \log (EC_{50})]\}$$

which relates the average response y to dose x, and where C and D are, respectively, the lower- and the upper limit of the sigmoidal dose-response curve, b is the slope and EC_{50} is the EO or fraction concentration which induces a response halfway between the lower- and the upper limit. This analysis was performed using GraphPad Prism® version 5.00 for Windows, San Diego California USA (www.graphpad.com), setting C to 0% and D to 100% with variable slope (b). LC_{100} values were calculated using the GraphPad 5.0 software QuickCalcs (http://www.graphpad.com/quickcalcs/Ecanything1.cfm).

3. Results and discussion

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

The essential oils isolated from 59 plant species (13 families), revealed yields that ranged from <0.05% to 9% (v/w). The highest yields were obtained from *Syzygium aromaticum* (9%), *Eucalyptus radiata* (6%), *E. dives* (3%) and *Cymbopogon citratus* (3%) (Table 1). All 84 EOs isolated were fully chemically characterized (detailed relative amounts of all the identified components are listed in the Supplementary Table, Annex 1), although Table 1 reports only their main components (≥10%).

For some species, duly identified in Table 1, the EO composition was previously reported (Barbosa et al. 2010, Barbosa et al. 2012, Faria et al. 2011). Some species can show several EO chemotypes, such as *Thymus caespititius* carvacrol, thymol or α-terpineol-rich chemotypes (Table 1), which may provide different biological properties. These chemotypes were separately

assessed for their nematotoxic activity.

Some EOs were further chosen for fractionation, aiming to assess the independent input of the HM or OCM against PWN. The main components (≥10%) in each separate EO fraction are featured in Table 2 (detailed relative amounts are listed in the Supplementary Table, Annex 1).

Table 1. Plant species scientific names, arranged in alphabetic order of the corresponding plant family, sampling year, plant part used for hydrodistillation, plant source, essential oil yield and EO main components (≥10%) of each of the 84 EOs analyzed.

Family / species	Code	Sampling date	Plant part	Collection place / source	Yield (%, v/w)	Main components (%)
Apiaceae / Umbelliferae						
Angelica lignescens Reduron et Danton [a,b]	Al	2008	FV	Flores, Azores	0.08	limonene 65, β-phellandrene 13, β-myrcene 12
Apium graveolens L. [b]	Ag	2010	FV	Lisbon	0.05	limonene 92
Chaerophyllum azoricum Trel. [a,b]	Ca	2008	FV	Flores, Azores	0.25	terpinolene 30, myristicin 26, acorenone B 17
Foeniculum vulgare Mill. [a,b]	Fv	2008	FF	Graciosa, Azores	0.33	*trans*-anethole 73, α-pinene 13
Petroselinum crispum (Mill.) Nym. [b]	Pc	2009	FV	Lisbon	0.09	1,3,8-*p*-menthatriene 50, β-myrcene 13, apiole 11
Asteraceae / Compositae						
Achillea millefolium L.	Am	2010	DF	herbal shop	0.85	β-thujone 33, *trans*-chrisantenyl acetate* 19
Inula viscosa (L.) Aiton [b]	Iv	2009	FV	Lisbon	<0.05	1,8-cineole 30
Cupressaceae						
Cryptomeria japonica (Thunb. ex L.f.) D. Don [a,b]	Cj	2008	Ffruit	Flores, Azores	0.41	terpinen-4-ol 24, α-pinene 23, sabinene 17
Juniperus brevifolia (Seub.) Antoine 1 [a,b]	Jb1	2008	Ffruit	Flores, Azores	0.06	limonene 63, α-pinene 18
Juniperus brevifolia (Seub.) Antoine 2 [a]	Jb2	2008	FV	Flores, Azores	0.45	limonene 82, α-pinene 11
Fabaceae / Leguminosae						
Genista tridentata L.	Gt	2010	DV	herbal shop	<0.05	*cis*-theaspirane 27, *trans*-theaspirane 22
Geraniaceae						
Pelargonium graveolens L'Hér. [a,b]	Pg	2009	FV	Lisbon	0.19	citronellol 34, guaia-6,9-diene 15, citronellyl formate* 14
Lamiaceae / Labiatae						
Calamintha nepeta (L.) Savi [b]	Cn	2009	FF	Castelo Branco	1.43	isomenthone 52, isomenthol 19, 1,8-cineole 11

Family / species	Code	Sampling date	Plant part	Collection place / source	Yield (%, v/w)	Main components (%)
Melissa officinalis L. [a,b]	Mo	2009	FF	herbal shop	0.04	geranial 38, citronellol 32, geraniol 18
Mentha arvensis L.	Ma	2009	FV	Lisbon	0.06	piperitenone oxide 56
Mentha cervina L. 1 [b]	Mc1	2009	DV	Castelo Branco	0.80	pulegone 80
Mentha cervina L. 2 [b]	Mc2	2009	DF	Castelo Branco	1.10	pulegone 86
Mentha x piperita L. 1 [b]	Mp1	2009	FV	Lisbon	0.11	menthol 31, menthone19
Mentha x piperita L. 2	Mp2	2009	FV	Lisbon	0.73	menthone 56, pulegone 13
Mentha pulegium L.	Mpu	2008	DV	Lisbon	0.35	pulegone 49, piperitenone 10
Mentha spicata L. 1 [b]	Ms1	2009	FV	Lisbon	0.07	carvone 54
Mentha spicata L. 2 [a]	Ms2	2009	FV	Beja	0.25	carvone 70
Nepeta cataria L. [a]	Nc	2009	FF	herbal shop	0.18	4aα, 7α, 7aα-nepetalactone 89
Origanum majorana L.	Om	2010	FV	Coimbra	0.06	*cis*-sabinene hydrate 33, terpinen-4-ol 13
Origanum vulgare L. 1	Ov1	2010	FV	Coimbra	<0.05	carvacrol 14, *cis*-sabinene hydrate 14, γ-terpinene 10
Origanum vulgare L. 2	Ov2	2010	DL	herbal shop	1.00	α-terpineol 16, thymol 15, γ-terpinene 15, carvacrol 10
Origanum vulgare subsp. *virens* (Hoffmanns. & Link) Bonnier & Layens	Ovi	2010	DV	herbal shop	0.83	α-terpineol 40, linalool 16, thymol 12
Rosmarinus officinalis L. [a,b]	Ro	2009	DL	herbal shop	1.95	β-myrcene 29, α-pinene 15
Salvia officinalis L. [a,b]	So	2009	FV	Lisbon	0.54	α-thujone 29, 1,8-cineole 26, β-thujone 10
Satureja montana L. 1 [a,b]	Sm1	2009	DV	herbal shop	0.55	carvacrol 40, *p*-cymene 20, thymol 15
Satureja montana L. 2 [b]	Sm2	2010	DF	herbal shop	1.31	carvacrol 64, γ-terpinene 18
Thymbra capitata (L.) Cav.	Tc	2010	FF	Algarve	1.40	carvacrol 68, γ-terpinene 11
Thymus caespititius Brot. 1 [a,b]	Thc1	2008	FF	Flores, Azores	0.06	carvacrol 35, *p*-cymene 19
Thymus caespititius Brot. 2 [a]	Thc2	2008	FF	Corvo, Azores	0.22	carvacrol 47, carvacryl acetate 12, T-cadinol 12
Thymus caespititius Brot. 3 [a,b]	Thc3	2008	FF	Gerês	0.35	α-terpineol 36, *p*-cymene 13, γ-terpinene 13
Thymus caespititius Brot. 4 [a]	Thc4	2008	FF	Graciosa, Azores	0.38	α-terpineol 62
Thymus caespititius Brot. 5 [b]	Thc5	2009	FF	Terceira, Azores	0.33	thymol 42, thymyl acetate15, *p*-cymene 14
Thymus caespititius Brot. 6	Thc6	2004-2009	FF	Azores	[c]	carvacrol 54, carvacryl acetate 10
Thymus caespititius Brot. 7	Thc7	2010	FF	Coimbra	0.48	carvacrol 59, *p*-cymene 11
Thymus camphoratus Hoffmans. & Link [a,b]	Thca	2008	FF	Algarve	0.21	linalool 26, linalyl acetate 18

Family / species	Code	Sampling date	Plant part	Collection place / source	Yield (%, v/w)	Main components (%)
Thymus mastichina (L.) L. [b]	Thm	2010	FV	Coimbra	1.17	1,8-cineole 46, limonene 23
Thymus villosus subsp. *lusitanicus* (Boiss.) Coutinho [a]	Thvl	2008	FF	Leiria	1.25	linalool 69
Thymus vulgaris L.	Thv	2010	FV	Coimbra	0.08	thymol 48, *p*-cymene 20, γ-terpinene 12
Thymus zygis L. [b]	Thz	2010	FV	Coimbra	0.30	thymol 50, *p*-cymene 15
Thymus zygis subsp. *silvestris* (Hoffmanns. & Link) Coutinho 1 [a,b]	Thzs1	2008	FF	Leiria	0.23	α-terpineol 32, γ-terpinene 16, linalool 11
Thymus zygis subsp. *silvestris* (Hoffmanns. & Link) Coutinho 2 [b]	Thzs2	2008	FF	Santarém	0.94	α-terpineol 60
Lauraceae						
Cinnamomum camphora (L.) Sieb. [b]	Cc	2009	FF	Coimbra	0.47	camphor 49, α-pinene 10
Laurus azorica (Seub.) J. Franco [a,b]	La	2008	FV	Flores, Azores	0.25	α-pinene 35, β-pinene 16, *trans*-α-bisabolene 15
Laurus nobilis L. [b]	Ln	2009	DL	herbal shop	0.95	1,8-cineole 35, α-terpenyl acetate 13
Myrtaceae						
Eucalyptus bosistoana F. Muell. [b,d]	Eb	2009	FV	Santarém	1.80	1,8-cineole 59, α-pinene 14
Eucalyptus botryoides Sm. [b,d]	Ebo	2009	FV	Santarém	1.20	α-pinene 43, 1,8-cineole 35
Eucalyptus camaldulensis Dehnh. [b,d]	Ec	2009	FV	Santarém	0.13	1,8-cineole 51, α-pinene 32
Eucalyptus cinerea F. Muell. [d]	Eci	2009	FF	Santarém	1.60	1,8-cineole 67, α-terpinyl acetate 10
Eucalyptus citriodora Hook. [b,d]	Ect	2009	FV	Santarém	0.86	citronellal 36, isopulegol 13, citronellol 12, 1,8-cineole 11
Eucalyptus cordieri Trabut [d]	Eco	2009	FV	Santarém	1.12	1,8-cineole 72
Eucalyptus dives Schauer [d]	Ed	2009	FV	Santarém	3.30	piperitone 40, α-phellandrene 19, *p*-cymene 19
Eucalyptus ficifolia F. Muell. [b,d]	Ef	2009	FV	Santarém	0.35	α-pinene 44, limonene 41
Eucalyptus globulus Labill. [d]	Eg	2009	FV	Santarém	1.33	1,8-cineole 70, α-pinene 16
Eucalyptus pauciflora Sieber ex Spreng [d]	Ep	2009	FF	Santarém	0.84	α-pinene 82
Eucalyptus polyanthemos Schauer [b,d]	Epo	2009	FV	Santarém	0.55	1,8-cineole 27
Eucalyptus radiata Sieber [d]	Er	2009	FV	Santarém	5.55	1,8-cineole 48, *p*-cymene 13
Eucalyptus saligna Sm. [b,d]	Es	2009	FV	Santarém	1.00	1,8-cineole 48, α-pinene 40
Eucalyptus smithii R.T. Baker [d]	Esm	2009	FV	Santarém	2.80	1,8-cineole 83
Eucalyptus urophylla S. T. Blake [d]	Eu	2009	FV	Santarém	0.86	α-phellandrene 45, 1,8-cineole 23
Eucalyptus viminalis Labill. [d]	Ev	2009	FV	Santarém	1.10	1,8-cineole 46, α-pinene 13, γ-terpinene 12
Myrtus communis L. [b]	Mco	2008	FF	Algarve	0.30	1,8-cineole 37, α-pinene 24, limonene 13

Family / species	Code	Sampling date	Plant part	Collection place / source	Yield (%, v/w)	Main components (%)
Syzygium aromaticum (L.) Merrill & Perry	Sa	2010	Dfb	herbal shop	9.00	eugenol 92
Pittosporaceae						
Pittosporum undulatum Vent. 1 [a]	Pu1	2008	Ffruit	Graciosa, Azores	0.21	sabinene 31, terpinen-4-ol 21, limonene 14, γ-terpinene 10
Pittosporum undulatum Vent. 2 [a,b]	Pu2	2008	FL	Graciosa, Azores	0.08	limonene 22, sabinene 18, terpinen-4-ol 15, γ-terpinene 12
Poaceae / Gramineae						
Cymbopogon citratus (DC) Stapf. 1 [a]	Cci1	2008	FL	Faro	0.80	geranial 43, neral 29, β-myrcene 25
Cymbopogon citratus (DC) Stapf. 2	Cci2	2010	DL	herbal shop	1.16	β-myrcene 38, geranial 23, neral 20, geraniol 14
Cymbopogon citratus (DC) Stapf. 3	Cci3	2010	DL	herbal shop	3.04	geranial 34, neral 22, β-myrcene 20, geraniol 18
Rutaceae						
Citrus limon (L.) Burm. f. 1 [b]	Cl1	2009	FF	Lisbon	0.22	β-pinene 34, limonene 32
Citrus limon (L.) Burm. f. 2 [b]	Cl2	2009	Fex	Lisbon	0.32	limonene 52, 1,8-cineole 17, β-pinene 14
Citrus limon (L.) Burm. f. Var. Meyer 3	Cl3	2009	Fex	Algarve	0.25	limonene 45, 1,8-cineole 15, β-pinene 14
Citrus sinensis (L.) Osbeck 1 [b]	Cs1	2009	Ffl	Lisbon	0.14	sabinene 47, limonene 10
Citrus sinensis (L.) Osbeck 2 [b]	Cs2	2009	FV	Lisbon	0.26	sabinene 64
Citrus sinensis (L.) Osbeck 3 [b]	Cs3	2009	Fex	Algarve	0.34	limonene 81, β-phellandrene 14
Citrus sinensis (L.) Osbeck var Valencia Late 4	Cs4	2009	Fex	Algarve	0.45	limonene 78, β-phellandrene 13
Ruta graveolens L. 1 [a]	Rg1	2009	FV	Évora	2.60	2-undecanone 94
Ruta graveolens L. 2 [a]	Rg2	2009	DF	herbal shop	0.90	2-undecanone 93
Ruta graveolens L. 3	Rg3	2010	DV	herbal shop	0.51	2-undecanone 91
Verbenaceae						
Aloysia citriodora Gómez Ortega & Palau [a,b]	Ac	2009	DV	herbal shop	0.19	geranial12, limonene 11, neral 10
Zingiberaceae						
Zingiber officinale Roscoe [b]	Zo	2008	Frhiz	herbal shop	0.16	geranial 29, β-phellandrene 17, citronellol 14, camphene 14

DF – Dry, flowering phase aerial parts, Dfb - Dry flower buds, DL - Dry leaves, DV – dry, vegetative phase aerial parts, Fex - Fresh exocarp, FF - fresh, flowering phase aerial parts, Ffl - Fresh flowers, Ffruit - Fresh fruit, FL - Fresh leaves, Frhiz - Fresh rhizome, FV - fresh, vegetative phase aerial parts, a - EOs previously tested (Barbosa et al. 2010, Barbosa et al. 2012), b - evaluated decoction waters, c - EO resulted from the combination of several EOs from the same chemotype collected in Azores from 2004 to 2009, d - EOs composition previously reported (Faria et al. 2011, Annex 2), * - Identification based on mass spectra only.

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

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

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

Table 2. Main components (≥10%) and mean corrected mortality at 2 µL/mL (mean±s.e., in %) of the EOs fractions.

Plant species*	EOs hydrocarbon molecules fraction main components	Corrected mortality (%)	EOs oxygen-containing molecules fraction main components	Corrected mortality (%)
Cymbopogon citratus 3	β-myrcene 72	58±6	geranial 45, neral 36	100±0
Origanum vulgare 2	γ-terpinene 36, *p*-cymene 11	14±11	α-terpineol 26, thymol 23, terpinen-4-ol 16, carvacrol 15, linalool 14	100±0
Satureja montana 2	γ-terpinene 44, *p*-cymene 19	53±13	carvacrol 96	100±0
Thymbra capitata	γ-terpinene 36, *p*-cymene 23	39±38	carvacrol 93	100±0
Thymus caespititius 6	*p*-cymene 29, γ-terpinene 16, *trans*-dehydroagarofuran 12	36±21	carvacrol 66, carvacryl acetate 14	100±0

* The EOs that were chosen for fractionation showed corrected mortalities ≥96% at 2 µL/mL.

3.2. PWN mortality and LC_{100} assessment

3.2.1. Essential oils

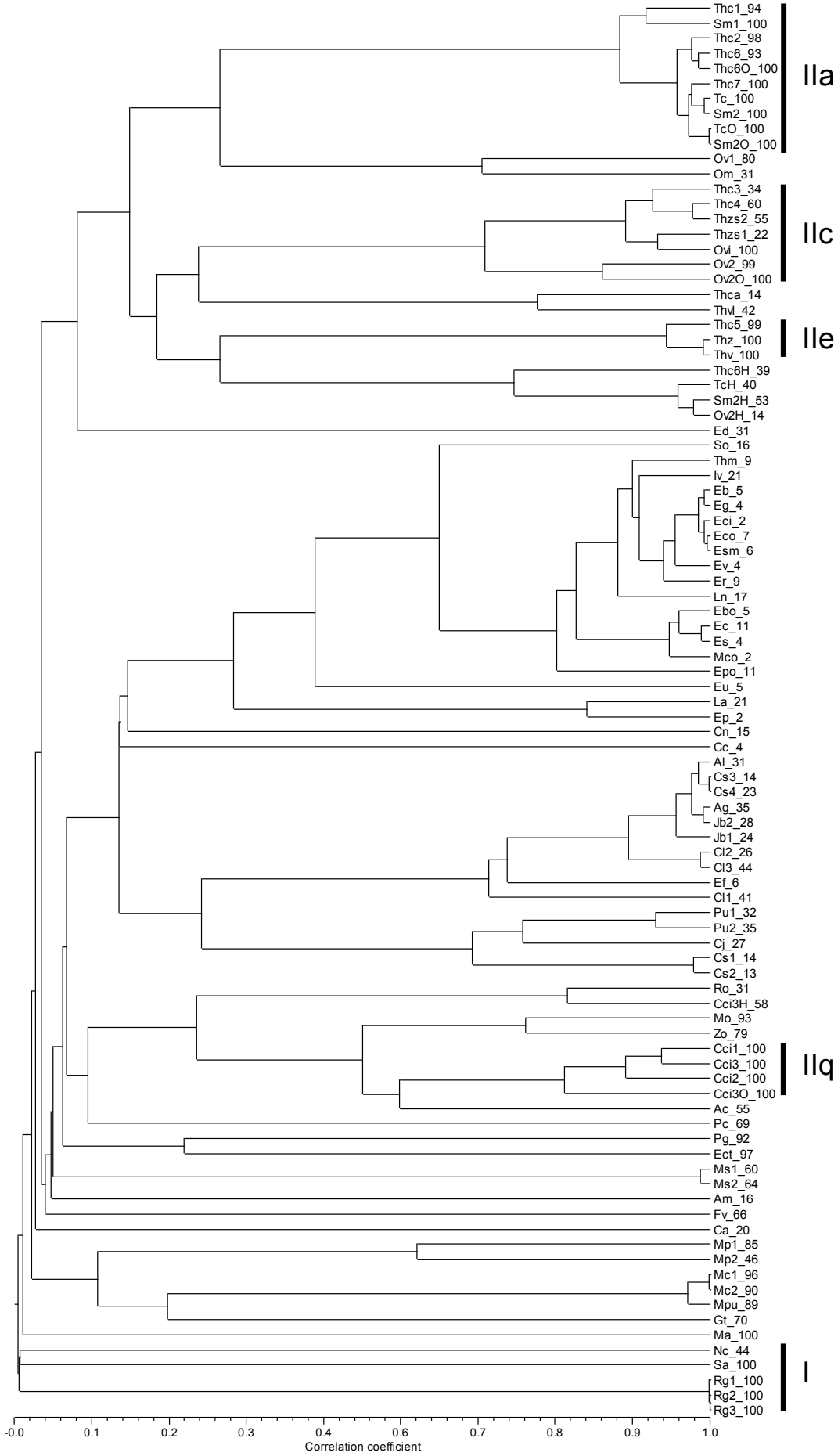
Essential oils were tested for activity against *B. xylophilus* through direct contact bioassays. Assays, performed with ultrapure water, showed an average mortality of 8 ± 4%, considered to be natural mortality. The mortality due to methanol, used as the EO solvent, was 10 ± 6%, which can be considered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

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

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

Sub-cluster IIa included EOs, or OCMs, with ≥93% corrected mortality, which were chemically characterized by dominant contents of carvacrol (35-96%), *p*-cymene (traces-20%), γ-terpinene (traces-18%) and thymol (not detected-15%). Only three out of the seven samples from sub-cluster IIc showed corrected mortalities ≥96%. These differed from the remaining members of the same cluster by showing high amounts of thymol (12-23%) and carvacrol (6-15%). Sub-cluster IIe integrated EOs rich in thymol (42-50%), *p*-cymene (14-20%), thymyl acetate (traces-15%) and γ-terpinene (6-12%), which showed corrected mortalities ≥99%. *Th. caespititius* chemotypes rich in carvacrol and/or thymol showed high nematotoxic activities while α-terpineol-rich chemotypes showed corrected mortalities ≤60%.

Figure 1. Dendrogram obtained by cluster analysis of the percentage composition of essential oils from the 84 samples and 10 fractions evaluated, based on correlation and using unweighted pair-group method with arithmetic average (UPGMA). For each EO sample abbreviation, see Table 1. EOs fractions abbreviations begin with the sample code followed by uppercase H for fractions containing hydrocarbon molecules or uppercase O for or oxygen-containing molecules. Values after underscore are the mean corrected mortality percentages obtained with each EO or fraction at 2 µL/mL.


Thc1_94
Sm1_100
Thc2_98
Thc6_93
Thc6O_100
Thc7_100
Tc_100
Sm2_100
TcO_100
Sm2O_100
Ov1_80
Om_31
Thc3_34
Thc4_60
Thzs2_55
Thzs1_22
Ovi_100
Ov2_99
Ov2O_100
Thca_14
Thvl_42
Thc5_99
Thz_100
Thv_100
Thc6H_39
TcH_40
Sm2H_53
Ov2H_14
Ed_31
So_16
Thm_9
Iv_21
Eb_5
Eg_4
Eci_2
Eco_7
Esm_6
Ev_4
Er_9
Ln_17
Ebo_5
Ec_11
Es_4
Mco_2
Epo_11
Eu_5
La_21
Ep_2
Cn_15
Cc_4
Al_31
Cs3_14
Cs4_23
Ag_35
Jb2_28
Jb1_24
Cl2_26
Cl3_44
Ef_6
Cl1_41
Pu1_32
Pu2_35
Cj_27
Cs1_14
Cs2_13
Ro_31
Cci3H_58
Mo_93
Zo_79
Cci1_100
Cci3_100
Cci2_100
Cci3O_100
Ac_55
Pc_69
Pg_92
Ect_97
Ms1_60
Ms2_64
Am_16
Fv_66
Ca_20
Mp1_85
Mp2_46
Mc1_96
Mc2_90
Mpu_89
Gt_70
Ma_100
Nc_44
Sa_100
Rg1_100
Rg2_100
Rg3_100
IIa
IIc
IIe
IIq
I
-0.0
0.1
0.2
0.3
0.4
0.6
0.7
0.8
0.9
1.0
Correlation coefficient

The occurence of chemotypes must be taken into account when choosing a nematotoxic EO bearing-species, since EO particular chemotype proved to be determinant in nematotoxic activity. High PWN mortality (100%) was also observed with *C. citratus* EOs, and the related OCM. These EOs grouped in sub-cluster IIq and were dominated by geranial (23-45%), β-myrcene (traces-38%), neral (20-36%), and geraniol (1-18%).

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

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

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

At the lowest concentration tested, 0.25 µL/mL, *R. graveolens*, *S. montana*, and *T. capitata* EOs revealed to be the most active. The lethal doses (LC_{100}) of these EOs were calculated, since the PWN has a high reproductive capability and can proliferate even from a very small population. LC_{100} ranged from 0.358-0.544 µL/mL for *R. graveolens* EOs, 0.374 µL/mL for *S. montana* 2 EO and 0.375 µL/mL for *T. capitata* EO (Table 3).

Previous studies (Barbosa et al. 2010, 2012) have shown similar high nematicidal potential, which is low LC_{100}, for *O. vulgare*, *S. montana*, *T. capitata*, *Th. caespititius* (all rich in carvacrol, γ-terpinene and *p*-cymene) and 2-undecanone-rich *R. graveolens* EOs.

R. graveolens EOs herewith studied showed very good *B. xylophilus* anti-nematodal activity. *Ruta chalepensis* EOs, also 2-undecanone rich, showed also high activity against the root-knot

nematodes *Meloidogyne incognita* and *M. javanica* (Ntalli et al. 2011a).

Other thymol and carvacrol-rich essential oils have also been reported to have high *B. xylophilus* anti-nematodal activity. Thymol-rich *Th. vulgaris* EOs showed a good activity in direct contact assays against PWN (Kong et al. 2007). These EOs showed to be composed of both PWN propagation stimulant- and nematicidal compounds. Kong et al. (2007) showed that geraniol, thymol, carvacrol and terpinen-4-ol possessed strong nematicidal properties and (-)-caryophyllene oxide, (+)-ledene, (+)-limonene, (-)-limonene, linalool oxide, β-myrcene, (-)-α-phellandrene, (+)-α-pinene and γ-terpinene were PWN propagation stimulant compounds.

Table 3. Lethal concentrations (LC_{100}, μL/mL) of EOs and related fractions with oxygen-containing molecules against the PWN. EC_{50} and slope values are given for comparison purposes.

EOs / Fractions with oxygen-containing molecules	code	LC_{100}	EC_{50}	slope (b*)
Cymbopogon citratus 3	Cci3	1.059	0.456	10.930
Cymbopogon citratus 3 O	Cci3O	1.801	0.454	6.685
Origanum vulgare 2	Ov2	2.120	0.754	8.909
Origanum vulgare 2 O	Ov2O	1.606	0.811	13.480
Ruta graveolens 1	Rg1	0.359	0.232	21.080
Ruta graveolens 2	Rg2	0.544	0.184	8.490
Ruta graveolens 3	Rg3	0.358	0.230	20.780
Satureja montana 2	Sm2	0.374	0.261	25.630
Satureja montana 2 O	Sm2O	0.374	0.262	25.850
Thymbra capitata	Tc	0.375	0.265	26.660
Thymbra capitata O	TcO	0.387	0.275	26.950
Thymus caespititius 6	Thc6	1.464	0.972	22.470
Thymus caespititius 6 O	Thc6O	0.721	0.471	21.610

O – EO oxygen-containing molecules fraction, * - b in $y = C + (D – C) / 1 + \exp \{b [\log (x) – \log (EC_{50})]\}$.

Satureja montana 2 and *T. capitata* EOs, evaluated in the present study, are good candidates for EO fractionation since they contain several of the above cited compounds and, in addition, show high relative amounts of carvacrol and γ-terpinene.

To our knowledge this is the first report on several species EOs against the PWN. *Calamintha nepeta*, *Inula viscosa* and *O. majorana* EOs showed weak-, whereas *Genista tridentata* EO revealed moderate- and *O. vulgare* subsp. *virens* and *Mentha arvensis* EOs showed strong nematotoxic activities. From the *Eucalyptus* genus, eleven species and respective EOs were

herewith tested for the first time against *B. xylophilus*, namely *E. bosistoana*, *E. botryoides*, *E. camaldulensis*, *E. cinerea*, *E. cordieri*, *E. ficifolia*, *E. pauciflora*, *E. polyanthemos*, *E. saligna*, *E. urophylla* and *E. viminalis*.

Eucalyptus citriodora EO showed weak activity against the PWN in a previous study (Park et al. 2005). Nevertheless, the fact that Park et al. (2005) do not detail this EO composition does not allow a direct comparison between studies.

3.2.2. Essential oils and fractions containing hydrocarbons or oxygen-containing molecules

Given each specific EO composition, the PWN mortality and LC_{100} results, some essential oils were further chosen for fractionation, to evaluate the separate contribution of the HM or OCM against the PWN.

Despite the high PWN mortality and LC_{100} results, *R. graveolens* EOs were not fractionated due to already high 2-undecanone content (Table 1). Thus, only *C. citratus* 3, *O. vulgare* 2, *S. montana* 2, *T. capitata* and *Th. caespititius* 6 EOs were fractionated (Table 2). These OCMs showed 100% corrected mortality at 2 µL/mL (Fig. 1, Table 2). At the same concentration, the corresponding HM fractions showed corrected mortalities ≤58% (Fig. 1, Table 2).

Cluster analysis (Fig. 1) showed that all OCMs grouped close to their corresponding EOs ($S_{corr} \geq 0.8$), because of the dominance of oxygen-containing compounds, both in the fraction and in the original EO. On the contrary, with the exception of *C. citratus* 3, the HMs clustered together ($S_{corr} \geq 0.7$), showing similar chemical compositions (Fig. 1).

Given the results above, only the OCMs LC_{100} values were determined (Table 3). *S. montana* 2 and *T. capitata* OCMs revealed quite similar LC_{100} values to those of their corresponding EOs. On the other hand, *C. citratus* 3 OCM showed higher lethal dose value, than that of the related EO, even though the oxygen-containing compounds were present in higher proportions in the fraction. This suggests that in addition to the oxygen-containing compounds, the hydrocarbon fraction also plays an important role in the overall PWN toxicity of these EOs, probably by additive and/or synergic interactions between EO fractions or compounds.

Unlike previous fractions, *O. vulgare* 2 and *Th. caespititius* 6 OCMs showed lower LC_{100} values comparatively to the related EOs. The diverse results obtained suggest that *B. xylophilus* toxicity may be EO specific, and no general conclusions should be drawn solely from the EOs main components.

Other studies have addressed the nematotoxic potential of separate EO components. In direct contact assays against PWN, Choi et al. (2007b) evaluated the activity of different monoterpenes, using synthetic chemicals. Out of the 26 monoterpenes tested, carvacrol, thymol, geraniol, nerol, (-)-menthol, citronellol, citronellal and citral (mixture of geranial and neral) showed the highest activities against PWN. By relating the anti-nematode activity to the chemical functional group, Choi et al. (2007b) showed that monoterpene hydrocarbons and ketones had weak or no activity. Other monoterpenes revealed a hierarchy of functional groups, phenols, aldehydes and primary alcohols being the most active followed by secondary and tertiary alcohols. Ntalli et al. (2011b) analyzed the synergistic and antagonistic interactions between components from EOs active against *Meloidogyne incognita*, showing that combinations of nematicidal EO components, such has carvacrol/thymol or carvacrol/geraniol, had a synergistic activity on *M. incognita* J2 juvenile paralysis.

Although in the present study the nematotoxic activity of separate components was not assessed, the evaluation of the isolated HM or OCM against PWN showed that the activity of an EO reflects the contribution of its different components, in distinct ways. Synergistic or antagonistic interactions between the EO components will influence the EO overall toxicity against PWN.

3.3. Decoction waters

Nine decoction waters (remaining hydrodistillation waters), out of the 45 evaluated (Table 1), showed strong nematotoxic activity, at 12.5 µL/mL. Only *Angelica lignescens*, *Citrus sinensis* (flowers and fresh vegetative aerial parts), *F. vulgare*, *Laurus nobilis*, *Melissa officinalis, M. spicata*, *Salvia officinalis* and *Zingiber officinale* decoction waters revealed 100% PWN mortality.

Interestingly, with the exception of *M. officinalis* EO, the EOs isolated from the remaining samples with successful decoction waters, showed corrected mortalities ≤80%, at 2 µL/mL.

Further studies are required to assess the active chemical constituents of these decoction waters. Decoction waters generally retain water-soluble, heat-stable, nonvolatile phytochemicals, such as high-weight terpenes and phenols and alkaloids (Gonçalves et al. 2009, Tiwari et al. 2011), which have been shown to possess strong nematicidal activities (Chitwood 2002, Zhao 1999).

4. Conclusion

Essential oils and decoction waters, isolated from 84 samples, were evaluated through direct contact assays against PWN. Twenty highly nematicidal EOs were obtained from the initial screening. Of these *Ruta graveolens*, *Satureja montana* and *Thymbra capitata* EOs were the most active. Nematotoxic activity from fractions containing hydrocarbons or oxygen-containing molecules from the most successful EOs were further assessed, separately. For some EOs, fractionation may prove to be a good way to improve nematotoxic activity.

The use of EOs for pest management against the PWN must take into consideration the unique characteristics of each essential oil, as not every EO component contributes similarly against the PWN. The results obtained suggest that the EO fractions with oxygen-containing molecules, and some components, namely 2-undecanone, carvacrol, thymol, *p*-cymene and/or γ-terpinene may be responsible for EO nematotoxic activity. Nevertheless, despite the overall low activity of the EOs hydrocarbon fraction, this type of components also seems to contribute, in several cases, to the EOs PWN total nematotoxic activity, probably by additive and/or synergic interactions between EO fractions or compounds. PWN nematotoxic phytochemicals will be further evaluated to determine highly active formulations of EOs, EO fractions, EO individual components, and/or decoction waters aimed at an integrated action against Pine Wilt Disease.

PWD control strategies can be accomplished in several ways and many times the strategies of management should be combined. Keeping in mind the expensive and labour-intensive work, as well as the possibility of chemical injury, the most effective compounds should be tested by trunk injection, in a preliminary assessment, both in healthy trees, as a preventive measure, as well as in already affected trees to evaluate the host and the pathogen response under field conditions.

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Chapter 3

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

Jorge MS Faria, Inês Sena, Inês Vieira da Silva, Bruno Ribeiro, Pedro Barbosa, Lia Ascensão, Richard N Bennett, Manuel Mota, A Cristina Figueiredo (2015) *In vitro* co-cultures of *Pinus pinaster* with *Bursaphelenchus xylophilus*: a biotechnological approach to study pine wilt disease. Planta DOI: 10.1007/s00425-015-2257-9

Abstract

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* one 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 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

1. 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 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}) enter 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 Oka 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 one-year-old plantlets, pine *in vitro* cultures and pine / PWN co-culture structure and volatile production was also determined. The present work proposes maritime pine with PWN co-cultures as an adequate biotechnological tool to study the PWD, capable of simulating many conditions of the *ex vitro* nematode infection.

2. Materials and methods

2.1. *In vitro* cultures establishment

2.1.1. *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, seeds were surface sterilized by immersion in ethanol 96%, in an ultrasound bath for 10 min, as before. In asepsis, the seeds were rinsed, 3 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 4ºC for 2 days and sown in sterile wetted filter paper in covered glass jars. Seedlings were maintained in darkness, at 24±1ºC, for one week and then transferred to a 16 h light photoperiod [cool fluorescent lamps (32 $\mu E/m^2/s$)].

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 sucrose, supplemented with 0.5 mg/L 6-benzylaminopurine (BAP) and 0.1 mg/L 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 121ºC 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), 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 was statistically analyzed using Microsoft Excel 2013.

2.1.2. *Pinus pinaster* with *Bursaphelenchus xylophilus* co-cultures (co-cultures)

Bursaphelenchus xylophilus (isolate BxPt51T, retained at NemaLab (University of Évora, Portugal) and available on request) was obtained as described in 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, ressuspended in 1 mL sterile water. PWNs sterilization was tested on potato dextrose agar plates for 4 days at 25ºC. 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 ressuspended in 1 mL sterile water and used as described above.

2.2. 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 4 replicates were used in each experiment. All statistical analyses were performed using Microsoft Excel 2013. For comparison purposes, samples from greenhouse grown one-year-old *P. pinaster* plantlets were harvested for 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.

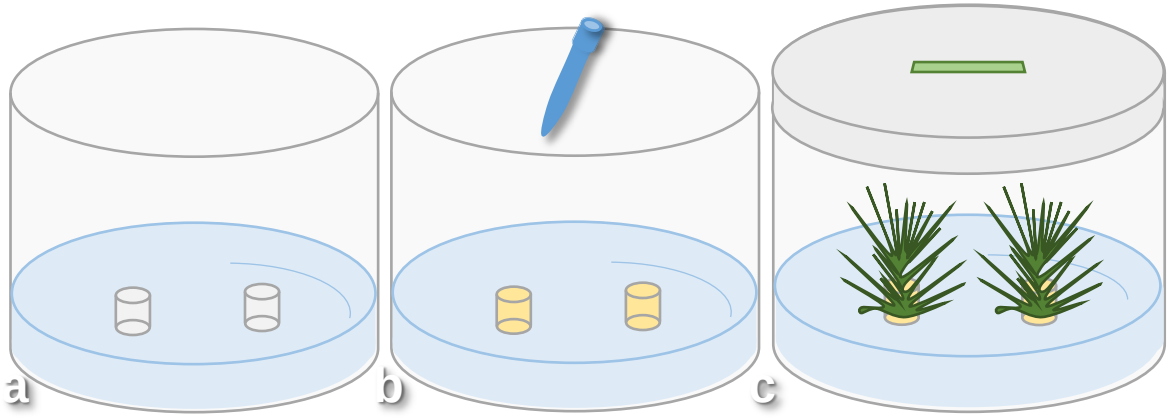


Figure 1. Schematic representation of *P. pinaster* with *B. xylophilus* co-cultures establishment. Under asepsis, small holes were made in the culture medium (a), into which a 100 µL PWNs suspension (250±50 PWNs) was added (b) together with each pine shoot (c).

2.2.1. Nematode population in the co-culture

PWNs 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, 3 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 (40x)].

At 28 DAI, the length and diameter of 30 of each randomly selected PWN females, males and juveniles (J2 to 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).

2.2.2. 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:

Relative water content (%) = [(fresh weight – dry weight) / (fresh weight)] x 100

In vitro shoots 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 -42ºC.

2.2.3. 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 one-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 4ºC. The material was then washed in the fixative buffer, dehydrated in a graded ethanol series, and critical point-dried in a Polaron E 3500, 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, counter-stained 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).

2.2.4. 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, 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. The volatile oils were stored at -20ºC 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 in Faria et al. (2014).

3. Results and discussion

3.1. *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 Álvarez 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. In order 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 (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.

3.2. *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.

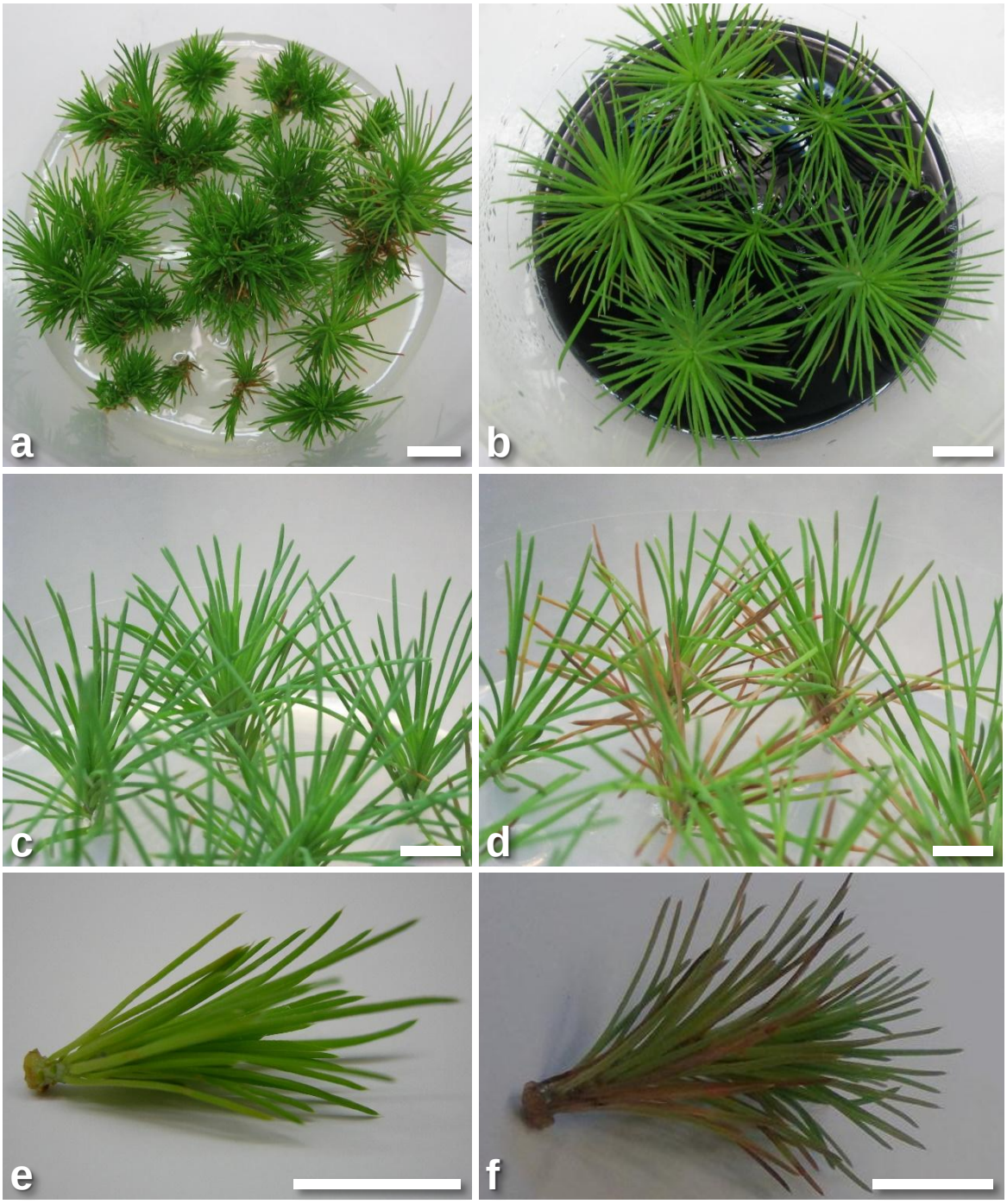


Figure 2. a-b *Pinus pinaster* shoots under routine culture conditions grown in SH multiplication medium (SHm) (a) and in elongation medium (SHe) (b), with monthly subculturing. c-f. Details of *P. pinaster* shoots (c, e) and of *P. pinaster* with *Bursaphelenchus xylophilus* co-culture 4 weeks after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

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 microcutting. The authors proved that aseptic PWNs maintain their pathogenicity and infection progressed to microcutting 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 et al. 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, supports 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 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).

3.3. 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.

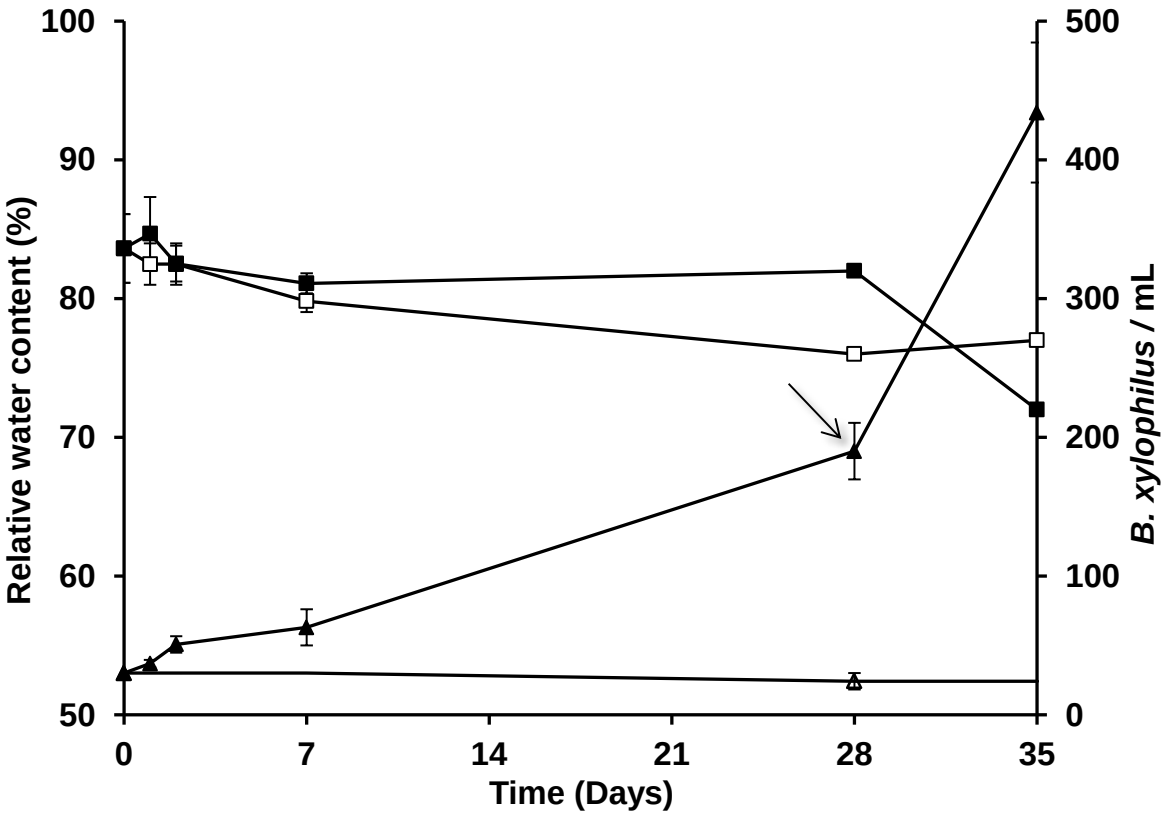


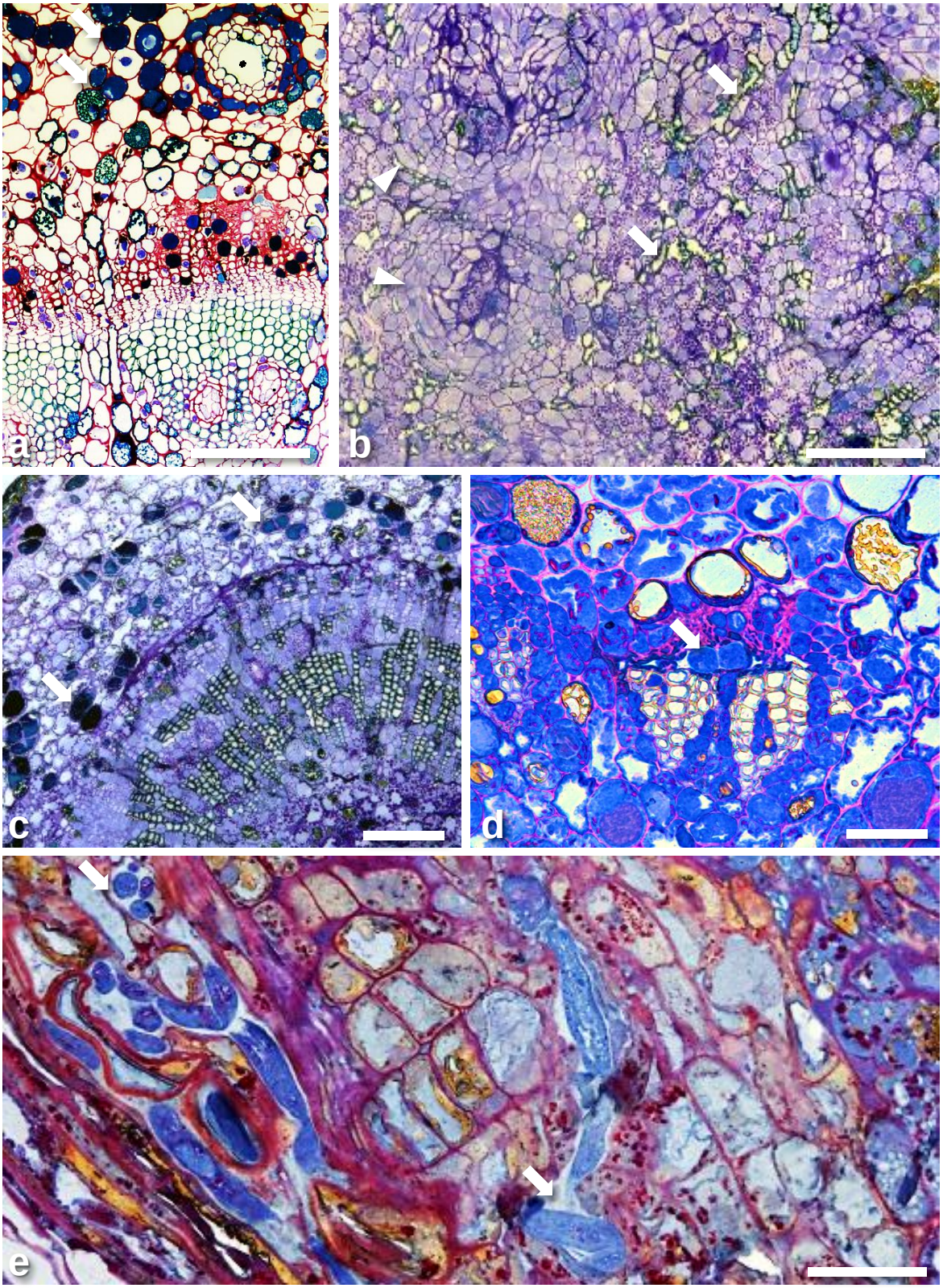
Figure 3. Relative water content (%) of *in vitro P. pinaster* shoots (□) and of *P. pinaster* shoots with PWN co-culture (■). Nematode population density in the microbox culture medium (▲) at the different time points of the time-course study and at 0 and 28 days without pine shoots (Δ). Two shoots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

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. 8x 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 one 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.

3.4. *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 (Fig. 4a, c and 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.

Callus tissue of the pine *in vitro* shoot facing the culture medium showed clusters of cells giving rise to meristematic zones (Fig. 4b, 5a).


a
b
c
d
e

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 (Fig. 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. Full-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* x *P massoniana*, *P. taeda*) as well as *Nicotiana tabacum* and *Medicago sativa* as a method to obtain clean PWN populations.

Figure 4. Light micrographs of historesin sections of shoots from one-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).

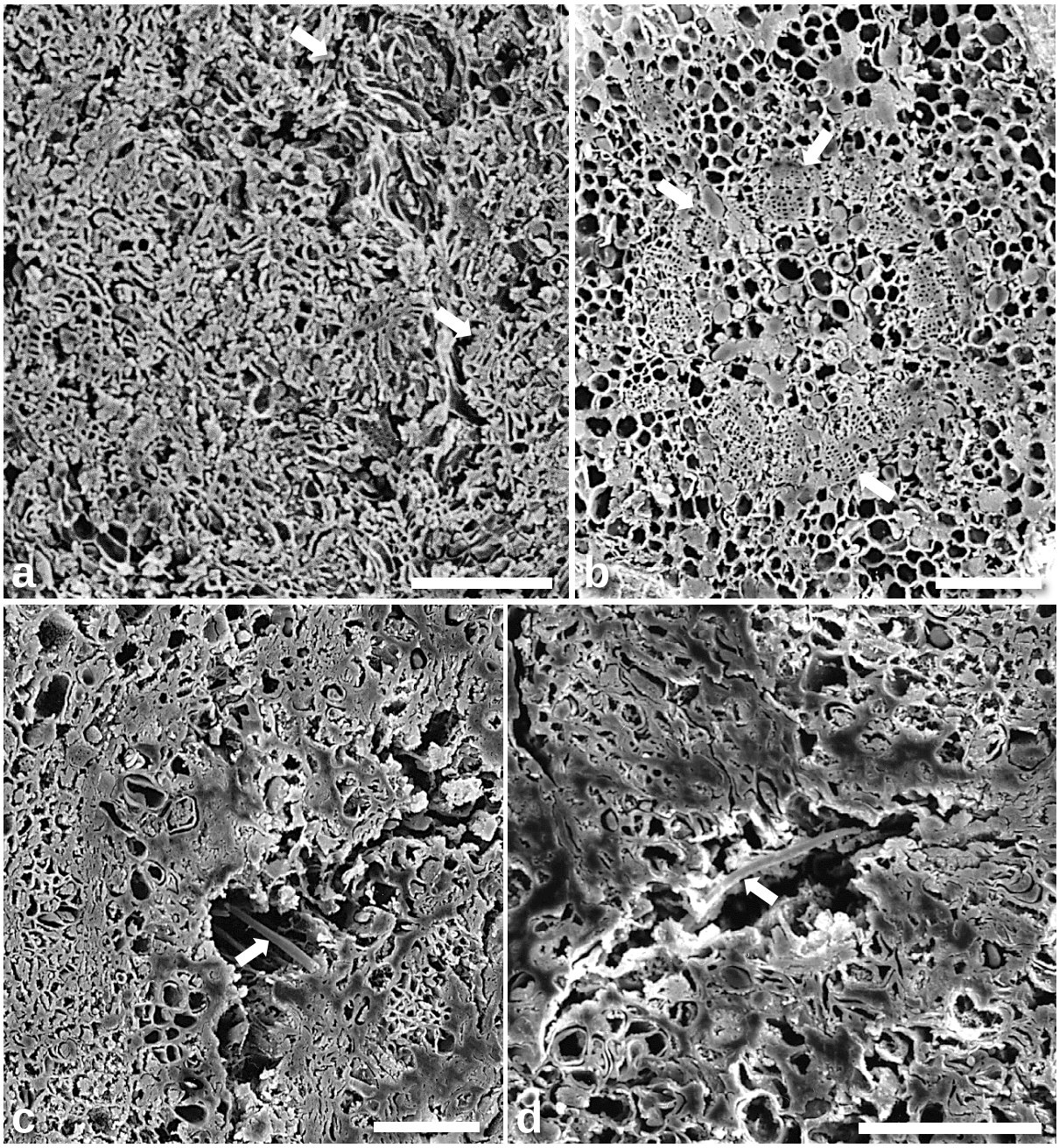


Figure 5. Scanning electron microscopy micrographs from cross sections of *Pinus pinaster* shoots cultures (a, b) and from *P. pinaster* shoots in co-culture with *Bursaphelenchus xylophilus* (c, d). a. Several dedifferentiation centers (arrows) are observed in the callus tissue facing the culture medium. 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.

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, 2013b). In the current study, nematode secretions may have influenced greatly *in vitro* shoot PWD symptom development, namely, macroscopically, pine shoot wilting (desiccation, chlorosis and drooping).

Plant tissue degeneration was noticeable as nematode population increased, probably due not solely 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.

3.5. *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* one-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). RI: In-lab calculated retention index relative to C_9-C_{24} *n*-alkanes on the DB-1 column. t: trace (<0.05%).

		Plantlets	Shoots						Co-cultures							
Components (>1%)	**RI**		**0**	**2**	**7**	**14**	**28**	**35**	**1h**	**8h**	**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*	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

* Unidentified compound detected on *P. pinaster* plantlets, shoots and co-cultures. [Standard deviation <5%].

Lima et al. (2010) characterized the volatiles from two 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.

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Chapter 4

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

Jorge MS Faria, Inês Sena, Cristina Moiteiro, Richard N Bennett, Manuel Mota, A Cristina Figueiredo (2015) Nematotoxic and phytotoxic activity of *Satureja montana* and *Ruta graveolens* essential oils on *Pinus pinaster* shoot cultures and *P. pinaster* with *Bursaphelenchus xylophilus in vitro* co cultures (*submitted*)

Abstract

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

Keywords: carvacrol, monoxenic culture, pine wilt disease, pinewood nematode, volatiles, 2-undecanone

1. Introduction

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

The present work is the first report on the use of plant with nematode co-cultures as models for screening effective nematotoxic EOs. Using previously established (Faria et al. 2015), *Pinus pinaster in vitro* shoot cultures and *P. pinaster* shoots with PWN co-cultures, the present study aims at evaluating the nematotoxic and phytotoxic activities of winter savory and rue EOs by assessing the nematode density in the co-cultures, the relative water content and volatile profiles of both *in vitro* cultures types.

2. Material and methods

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

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

2.2. *Ruta graveolens* and *Satureja montana* essential oils nematotoxic and phytotoxic activity

The effect of *Ruta graveolens* (Rg) and *Satureja montana* (Sm) EOs, at 0.5 µL/mL, was assessed both on a) Ppi cultures and b) PpiBx co-cultures (Fig. 1). The EOs nematotoxic effect was evaluated on *B. xylophilus* population density in PpiBx co-cultures, and the phytotoxic activity to the host was assessed both on Ppi cultures and on PpiBx co-cultures. For Ppi cultures, after 7 days of growth in regular SH solid medium (Phase 1), the cultures were transferred, for 7 days, to SH solid medium without, or with EO (Phase 2), and then again transferred to regular SH solid medium, for 7 days (Phase 3, recovery time), Fig. 1. PpiBx co-cultures were established by adding a 100 µL suspension (250±50 PWNs) into a small hole made in the culture medium into which the cut end of *P. pinaster* shoot (Ppi) was inserted, and maintained for 7 days for nematode infection (Phase 1). PpiBx co-cultures Phase 2 and 3 were run as detailed for Ppi cultures. Sampling was performed at the beginning (time 0), at days 1, 2 and 7 of Phase 2 and at the end of recovery time (day 7 of Phase 3). In both cases, control cultures (without addition of EO) were maintained simultaneously, under the same growth conditions. To prepare SH solid medium with EO, a solution of EO in methanol (1:1, v/v) was added, in asepsis with agitation, to previously autoclaved medium (121ºC for 15 min) after reaching room temperature, in such a way as to give 0.5 µL EO/mL culture medium.

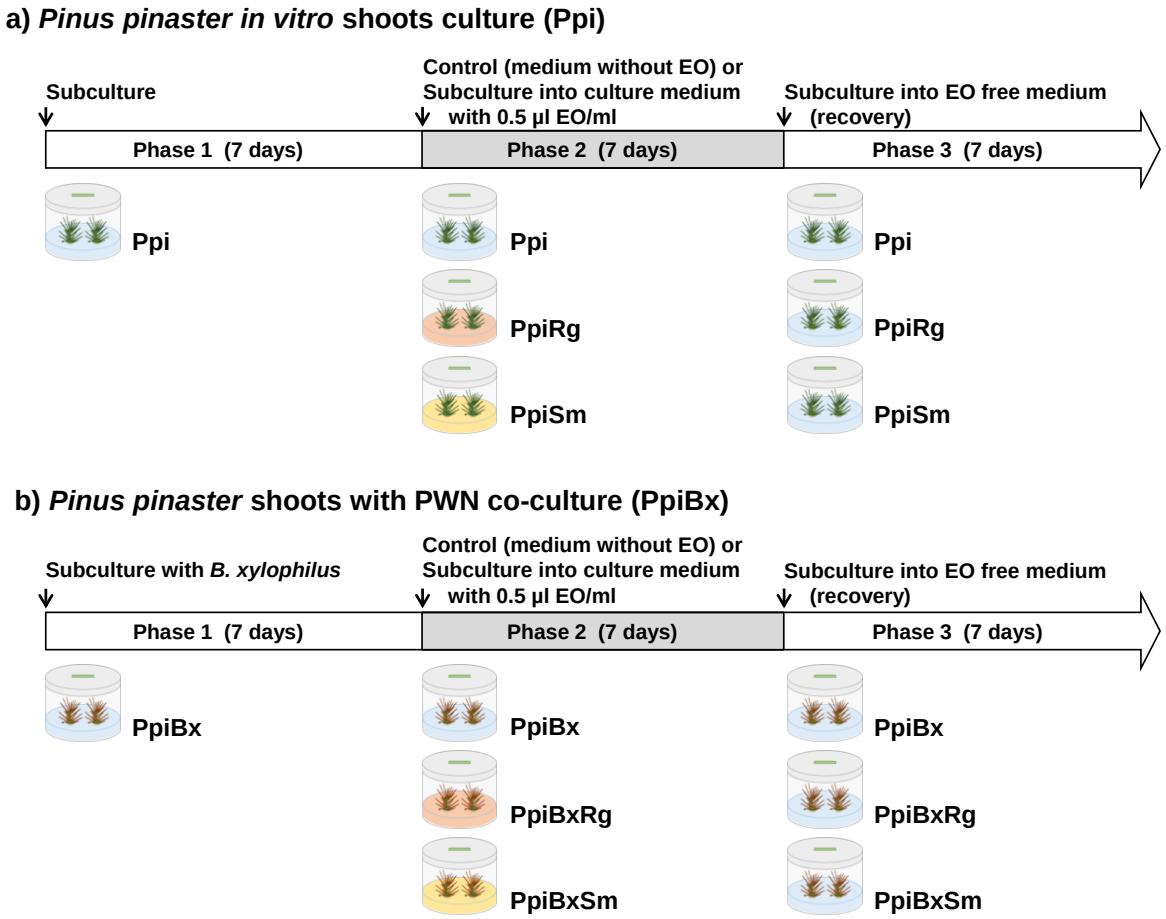

Figure 1. Schematic representation of experimental design (for details see experimental section). The effect of *Ruta graveolens* (Rg) and *Satureja montana* (Sm) EOs was assessed both on a) *Pinus pinaster in vitro* shoot cultures (Ppi) and b) *P. pinaster* shoots with PWN co-cultures (PpiBx).

EO evaporation and decomposition control experiments were performed by adding the same amount of EO to microboxes containing only regular culture medium, and keeping them in the same conditions as the experimental ones. Two independent experiments were separately run and 4 replicates per experimental time-point were used in each experiment. The data shown were calculated as mean values of all experiments. All statistical analyses were performed using Microsoft Excel 2013. The effect of adding winter savory and rue EOs to Ppi cultures and PpiBx co-cultures was followed by measuring relative water content (RWC), nematode density in the culture medium and *in vitro* volatiles production as in Faria et al. (2015).

2.3. *Ruta graveolens* and *Satureja montana* essential oils and isolation and identification of 8-phenyl-2-octanone

Satureja montana and *Ruta graveolens* EOs were isolated from the dried aerial parts sold in local herbal shops and the chemical profiling was performed as detailed in Faria et al. (2013). Given the presence of an unidentified compound >7%, in *R. graveolens* EO, this was further fractionated for compound isolation and identification. Hydrocarbon molecules (HM) and oxygen-containing molecules (OCM) were fractionated according to Faria et al. (2013). EOs were fractionated on a silica gel column by successive elution with distilled *n*-pentane and diethyl ether. The fractions were analyzed by gas chromatography coupled to mass spectrometry (GC-MS) and purified according to Figueiredo et al. (1992). OCM fractions (approximately 0.2 ml each in a total of 1.4 ml) were separated twice on silica gel plates (60 F_{254}, Merck, 20 x 20 cm, 1 mm layer thickness) using *n*-hexane : ethyl acetate (95:5) as eluent. The fraction obtained (7.4 mg) was characterized and identified by NMR spectroscopy and GC-MS spectrometry. 1D NMR (^{1}H, ^{13}C and APT) and 2D NMR (HSQC, HMBC and COSY) spectra were recorded on Bruker spectrometer CXP400 operating at 400.13 MHz (^{1}H) and 100.61 MHz (^{13}C). All chemical shifts are given at ppm and using CD_2Cl_2 signals as reference (δ = 5.30 ppm). Identification was as follows: ^{1}H RMN (400 MHz, CD_2Cl_2) δ 7.29 – 7.21 (m, 2H, H-11, H-11'), 7.20 – 7.12 (m, 3H, H-10, H-10', H-12), 2.59 (t, 2H, *J* = 7.6 Hz, CH_2-8), 2.39 (t, 2H, *J* = 7.6 Hz, CH_2-3), 2.08 (s, 3H, CH_3-1), 1.65 – 1.48 (m, 4H, CH_2-7 and CH_2-4), 1.36 – 1.27 (m, 4H, CH_2-5 and CH_2-6); ^{13}C RMN (101 MHz, CD_2Cl_2) δ 209.5 (C-2), 143.5 (C-9), 128.9 (C-10, C-10'), 126.1 (C-12), 128.7 (C-11, C-11'), 44.1 (C-3), 36.4 (C-8), 30.1 (C-1), 32.0, 24.3 (C-7 and C-4), 29.6, 29.5 (C-5 and C-6); MS (EI, 70 eV) *m/z* ($C_{14}H_{20}O$): 204 $(M)^+$ (0), 186 $(M\text{-}H_2O)^+$ (30), 130 (10), 105 $(C_6H_5CO)^+$ (20), 104 $(C_7H_7)^+$ (98), 91 $(C_7H_7)^+$ (100), 82 (14), 71 (30), 65 (15), 58 (10), 43 (60).

3. Results and discussion

3.1. Phytotoxicity to *Pinus pinaster* shoot cultures

Pinus pinaster shoots (Ppi) showed both the typical *in vitro* pine macroscopic aspect, green upright shoots with straight pine needles (Fig. 2a), and similar volatiles composition (Table 1), to that previously reported (Faria et al. 2015).

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

The isolated constitutive volatiles showed no substantial differences in composition throughout experimental time. Although Table 1 reports only the main volatile components (≥1%), they were all fully chemically characterized, in a total of 46 compounds. Volatiles were dominated by β-pinene (40-46%) and α-pinene (24-29%), an unidentified compound (UI B Ppi) (4-7%), germacrene D (4-7%) and β-caryophyllene (2-3%).

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

		Ppi Shoots					PpiBx Co-cultures				
Components (>1%)	**RI**	**0**	**1**	**2**	**7**	**R**	**0**	**1**	**2**	**7**	**R**
α-Pinene	930	24.4	26.6	25.9	28.7	24.6	25.4	28.2	17.7	29.4	20.5
β-Pinene	963	42.4	44.1	41.3	45.7	40.2	39.7	45.5	35.3	47.2	34.7
β-Myrcene	975	1.5	1.3	1.0	1.1	1.1	1.1	1.0	1.1	1.1	1.0
β-Phellandrene	1005	1.2	1.3	1.4	1.3	1.6	1.1	1.2	1.6	1.2	1.9
Limonene	1009	1.1	1.2	1.3	1.3	1.3	1.2	1.5	1.3	1.2	1.3
α-Terpineol	1159	1.5	1.1	1.7	0.7	1.5	1.3	1.8	2.6	0.9	2.2
β-Caryophyllene	1414	2.9	2.4	2.0	1.9	2.3	3.2	2.4	3.1	1.4	2.6
Phenyl ethyl 2-methyl butanoate	1467	0.3	0.3	0.1	0.3	0.2	0.5	0.6	1.0	0.3	t
Phenyl ethyl isovalerate	1468	1.1	0.9	0.6	0.7	0.6	1.1	1.6	2.0	1.0	0.4
Germacrene-D	1474	4.4	4.2	6.6	4.1	6.7	4.5	3.5	8.7	3.1	9.3
γ-Cadinene	1500	1.2	1.0	0.7	0.7	0.4	1.5	0.7	1.1	0.7	t
δ-Cadinene	1505	0.9	0.9	1.9	0.8	1.4	1.0	1.7	1.8	0.5	1.9
β-Caryophyllene oxide	1561	0.3	1.1	1.8	1.9	2.5	1.0	0.4	2.5	2.8	3.0
α-Muurolol	1618	0.3	0.2	0.2	0.1	0.8	0.3	0.2	0.1	t	1.4
α-Cadinol	1626	0.2	0.2	0.5	0.3	2.0	0.4	0.3	0.8	0.4	3.8
Palmitic acid	1908	0.9	0.8	0.5	0.6	1.0	0.7	0.6	0.9	0.6	1.4
UI B Ppi*	2309	7.2	5.6	4.2	4.0	6.8	7.8	4.1	8.2	2.6	9.5
% Identification		88.0	90.7	90.4	93.5	90.2	87.7	93.9	86.2	95.7	87.0
Grouped components											
Monoterpene hydrocarbons		71.7	75.6	72.3	79.4	69.5	69.6	78.7	57.9	81.6	59.6
Oxygen-containing monoterpenes		2.1	1.7	2.2	1.3	1.9	1.6	2.2	3.9	2.1	2.5
Sesquiterpene hydrocarbons		10.3	9.1	11.5	7.8	10.8	12.0	8.7	15.9	6.4	13.8
Oxygen-containing sesquiterpenes		1.3	2.0	2.5	2.6	5.4	2.0	1.6	4.3	3.5	8.2
Others		2.6	2.4	1.9	2.3	2.6	2.5	2.8	4.3	2.1	2.9

RI: In-lab calculated retention indices relative to C_9-C_{24} *n*-alkanes on the DB-1 column, t: trace (<0.05%), * UI: Unidentified compound

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

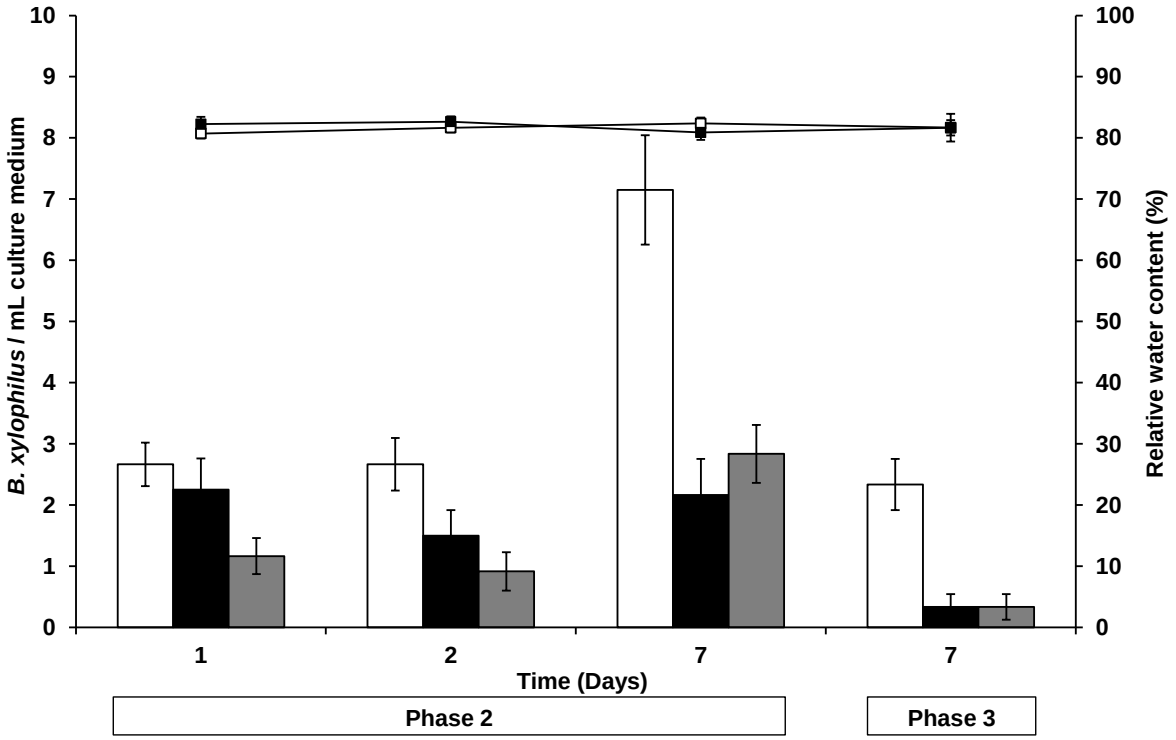

Figure 3. PWN population density in the culture medium of *P. pinaster* shoots with PWN co-cultures (PpiBx), without- (Control, white column), and with *S. montana* (black column) and *R. graveolens* (gray column) added EOs, at 0.5 µL EO/mL culture medium, and relative water content average of all *P.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

In addition to winter savory EO compounds, and to Ppi shoots constitutive volatiles, also new volatiles were detected in Ppi shoots winter savory EO added cultures. Of the main *S. montana* EO components (carvacrol 64% and γ-terpinene 18%) (Faria et al. 2013), only carvacrol was detected in high relative amounts, increasing to the end of the experimental time (67 to 80%) (Fig. 4a). γ-Terpinene maximum detected was 0.1%, at 0, 1 and 2 days of Phase 2, and remained at trace amounts thereafter. Part of this decrease can be due to volatilization, as this was also detected in

controls of EO evaporation and decomposition. Nevertheless, since *in vitro* cultures are known to have biotransformation capacity (Giri et al. 2001, Faria et al. 2009, Nunes et al. 2009), the conversion of γ-terpinene into a non-volatile glycosylated form, can also partly explain the difference between γ-terpinene percentage in winter savory EO and in winter savory EO added cultures (PpiSm).

Despite the transference of the shoots to EO-free medium, at the end of phase 3 (recovery time), carvacrol was still detected in high relative amount (19%). As a probable phytotoxic effect, and opposite of constitutive volatiles from control Ppi shoots, the PpiSm monoterpenes percentage was lower than that of sesquiterpenes, palmitic acid, or the unidentified compound B. Moreover, two new volatiles were detected in PpiSm shoots, 2-undecane, detected in trace amounts, and pentadecanal, <2% throughout the experimental time.

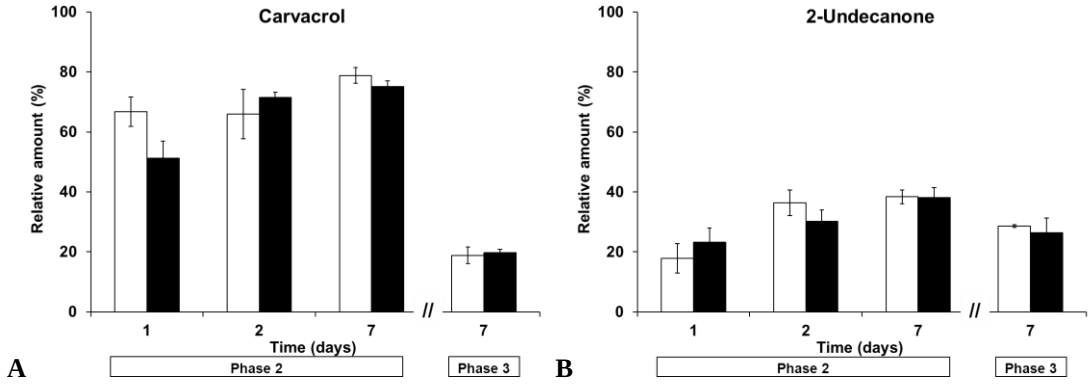

Figure 4. Variation in the percentage composition of the main components of the EOs added to the culture media. a) Carvacrol from *Satureja montana* and b) 2-undecanone from *Ruta graveolens* EOs added, at 0.5 µL EO/mL culture medium, to *P. pinaster* shoots cultures (Ppi) (white columns) and to *P.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

Winter savory EOs are commonly dominated by carvacrol, γ-terpinene and *p*-cymene which have been associated to its phytotoxic activity. In assays analyzing the activity of EOs in weeds and crops seeds germination, winter savory EO showed to be damaging to both, making it a poor choice for an herbicidal pesticide. Angelini et al. (2003) tested the EO at 0.5 mg/mL, and also its main compound carvacrol (57%), and found it to be highly inhibitory against *in vitro* seed germination. Grosso et al. (2010) also obtained high phytotoxic activity applying carvacrol-rich

S. montana EO (52%) to 4 crops and 3 weed seeds and seedlings *in vitro*. Both germination and seedling root/shoot growth were affected negatively making this herbicidal EO only appropriate for uncultivated fields. Albuquerque et al. (2012) tested both carvacrol and its isomer thymol, at 0.4 µL EO/mL culture medium, on *in vitro* shoot cultures of *Heliconia psittacorum* x *Heliconia spathocircinata* var. Golden Torch. Their highly damaging effect was due to general destruction of the cell membranes and coagulation of the cytoplasm, detected through transmission electron microscopy. *S. montana* EOs appear not to be a sound choice for application as nematode biopesticides given their very negative effect on plant growth and development.

No macroscopic negative effect was detected after transferring Ppi shoots into rue EO-added culture medium (PpiRg) at phase 2 (Fig. 2c). As for winter savory EO, also in this case, in addition to rue EO volatiles, and to Ppi shoots constitutive volatiles, also new volatiles were detected in PpiRg shoots. 2-Undecanone, the major component in *R. graveolens* EO (91%) (Faria et al. 2013), increased throughout Phase 2 (18-38%) (Fig. 4b), decreasing after transference to Phase 3 (29%). During this study, the identity of a previously unidentified component from *R. graveolens* EO (Faria et al. 2013), was ascertained as 8-phenyl-2-octanone by NMR (Fig. 4d, 5). There was no major variation in the relative amount of this compound during Phase 2 and 3 (3-5%). Four new, as yet unidentified, volatiles (0-3%) were detected in PpiRg shoots, whose relative amount either declined, increased or remained relatively stable throughout time.

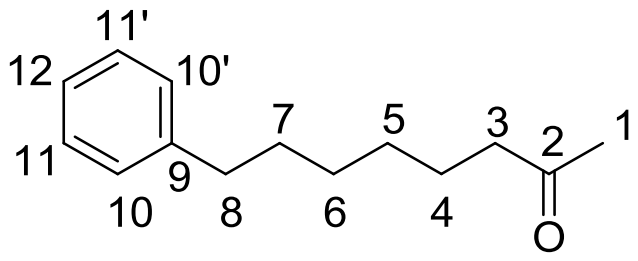

Figure 5. Chemical structure of 8-phenyl-2-octanone as determined by NMR.

de Feo et al. (2002) tested *in vitro* phytotoxic activity of rue essential oil and some of its constituents on *Raphanus sativus* germination and radicle growth and found activity only in the EO and some minor constituents but not with the EO major components, 2-undecanone (47%) or

2-nonanone (19%). While showing no detectable phytotoxic activity, rue EO used in the present work differed from that used by de Feo et al. (2002) by displaying a higher amount of 2-undecanone and the presence of the ketone 8-phenyl-2-octanone.

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

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

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

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

with high concerns due to its activity as a mammalian toxicant (European Commission 2012).

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

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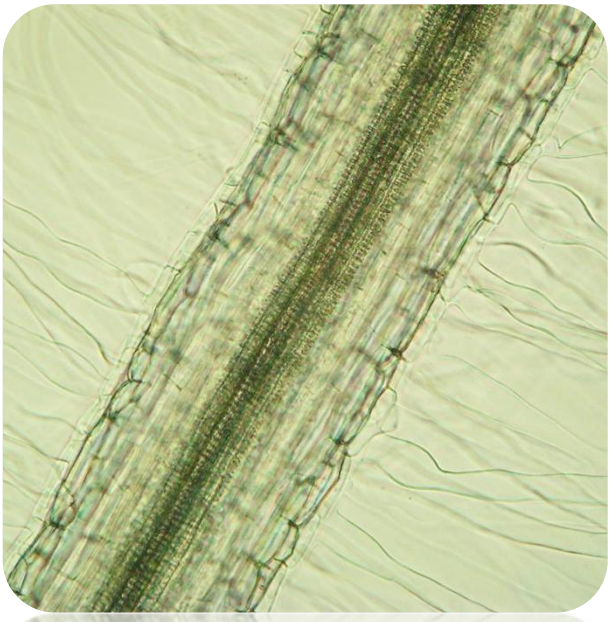
The root-knot disease



Chapter 5

First report on *Meloidogyne chitwoodi* hatching inhibition activity of essential oils and essential oils fractions

Jorge MS Faria, Inês Sena, Bruno Ribeiro, Ana Margarida Rodrigues, Carla MN Maleita, Isabel Abrantes, Richard N Bennett, Manuel Mota, A Cristina Figueiredo (2015) First report on *Meloidogyne chitwoodi* hatching inhibition activity of essential oils and essential oils fractions. Journal of pest science (*in press*)

Abstract

The Columbia root-knot nematode (CRKN), *Meloidogyne chitwoodi*, is an EPPO A2 type quarantine pest since 1998. This nematode causes severe damage in economically important crops such as potato and tomato, making agricultural products unacceptable for the fresh market and food processing. Commonly used nematicidal synthetic chemicals are often environmentally unsafe. Essential oils (EOs) may constitute safer alternatives against RKN. EOs, isolated from 56 plant samples, were tested against CRKN hatching, in direct contact bioassays. Some of the most successful EOs were fractionated and the hydrocarbon molecules (HM) and oxygen-containing molecules (OCM) fractions tested separately. Twenty-four EOs displayed very strong hatching inhibitions (≥90%) at 2 µL/mL and were further tested at lower concentrations. *Dysphania ambrosioides*, *Filipendula ulmaria*, *Ruta graveolens*, *Satureja montana* and *Thymbra capitata* EOs revealed the lowest EC_{50} values (< 0.15 µL/mL). The main compounds of these EOs, namely 2-undecanone, ascaridol, carvacrol, isoascaridol, methyl salicylate, *p*-cymene and/or γ-terpinene, were putatively considered responsible for CRKN hatching inhibition. *S. montana* and *T. capitata* OCM fractions showed hatching inhibitions higher than HM fractions. The comparison of EO and corresponding fractions EC_{50} values suggests interactions between OCM and HM fractions against CRKN hatching. These species EOs showed to be potential environmentally friendly CRKN hatching inhibitors, nonetheless, bioactivity should be considered globally, since its HM and OCM fractions may contribute, diversely, to the full anti-hatching activity.

Keywords: Columbia root-knot nematode, *Dysphania ambrosioides*, *Filipendula ulmaria*, *Ruta graveolens*, *Satureja montana*, *Thymbra capitata*

1. Introduction

Root-knot disease is caused by plant parasitic nematodes of the genus *Meloidogyne* and is characterized by the presence of galls or knots in the roots below ground and stunted growth, yellowing of the leaves, lack of vigour, a tendency to wilt under moisture stress and collapse of individual plants above ground that are similar to other root diseases. These symptoms are due to infection which mobilizes the plant's photosynthates from shoots to roots and affects water and nutrient absorption and translocation in the root system to support nematode development and reproduction (EPPO 2012). The Columbia root-knot nematode (CRKN), *Meloidogyne chitwoodi* Golden, O'Bannon, Santo and Finley, 1980, a sedentary and obligate plant endoparasite, is responsible for large economic losses in several horticultural and field crops and has been classified, in 1998, as an A2 type quarantine pest by the European Plant Protection Organization (EPPO 2012). Since its first description in the Pacific Northwest, USA, several reports have been made in South Africa, Belgium, Germany, the Netherlands, Portugal, Mexico and Argentina (Golden et al. 1980, Conceição et al. 2009, OEPP/EPPO 2009, Wesemael et al. 2011). In potato, tuber infection is characterized by the presence of galls, small raised swellings on the surface above the developing nematodes, and necrosis and browning of the internal tissue below the gall. When 5% or more of the tubers are tarnished, the crop is usually unmarketable (EPPO 2012). Being the world’s fourth-largest food crop (FAO 2009), potato production employs large quantities of pesticides, mainly synthetic chemicals applied by soil fumigation (e.g. 1,3-dichloropropene, methyl bromide, dazomet or nervous system toxins such as oxamyl and fenamiphos) (Mitkowski and Abawi 2003). Although highly efficient in controlling this soil pest (Pinkerton et al. 1986), fumigation has a negative environmental impact, making the continued availability and use of soil fumigants uncertain. In face of the recent EU environmental restrictions, it is necessary to develop environmentally safer control techniques based upon natural products. Essential oils (EOs) may prove to be sound alternatives to synthetic nematicides. They are complex mixtures of volatiles, mainly products from the plant secondary metabolism, comprised of terpenes, mostly mono-, sesqui- and diterpenes, and phenolic compounds, such as phenylpropanoids, although other groups of compounds can also occur in relevant amounts.

Generally biodegradable, EOs have low toxicity to mammals and do not accumulate in the environment (Figueiredo et al. 2008). Moreover, the biological activities of EOs can often exceed the sum of their single constituent's activities, due to synergy (Ntalli et al. 2011a, Kumrungsee et al. 2014). As complex mixtures, EOs may display several biological activities which make them desirable biopesticides (Batish et al. 2008) able of controlling not only the targeted pest but also opportunistic species and resistant strains.

No studies on the effect of EOs against *M. chitwoodi* have been conducted, but a strong anti-nematode activity, against other *Meloidogyne* species, was found in several EOs, such as those of *Allium sativum*, *Carum capticum, C. carvi*, *Chrysanthemum coronarium*, *Eucalyptus globulus*, *Foeniculum vulgare*, *Mentha rotundifolia*, *M. spicata*, *Origanum majorana*, *Pimpinella anisum*, *Syzygium aromaticum,* among others (Oka et al. 2000, Pérez et al. 2003, Ibrahim et al. 2006, Meyer et al. 2008, Douda et al. 2010, Gupta et al. 2011, Ntalli et al. 2011a, Andrés et al. 2012).

In an attempt to clarify the nematotoxic potential of essential oils against different types of nematodes and their value to sustainable pest control, a previous study addressed the nematotoxicity of several EOs against a different nematode type, the pinewood nematode (PWN), *Bursaphelenchus xylophilus* (Faria et al. 2013). To our best knowledge no previous study has addressed the activity of EOs against CRKN hatching. In view of the increasing potato demand and the need for environmentally safer anti-nematode compounds, the present study aimed at performing a comparative screening of EOs to a) determine, through direct contact bioassays, those that show high hatching inhibition and b) assess the relative importance of EOs hydrocarbon- and oxygen-containing molecules fractions in *M. chitwoodi* hatching inhibition.

2. Material and methods

2.1. Nematodes

CRKN eggs, used in the bioassays, were obtained from previously established *Solanum tuberosum* hairy roots with *M. chitwoodi* co-cultures (Faria et al. 2014). Subculture was performed monthly by

refreshment of the culture medium. Approximately 5 g (fresh weight) of co-culture was transferred to 200 mL SH liquid medium (Schenk and Hildebrandt 1972), supplemented with 30 g/L sucrose, pH=5.6, maintained in darkness at 24±1ºC on orbital shakers (80 rpm). After 3 months of subculture, galled hairy roots were excised and the CRKN eggs extracted by a 5 min immersion in a 0.52% (v/v) sodium hypochlorite (NaOCl) solution, with vigorous agitation (Hussey and Barker 1973). Eggs were collected in a 20 µm mesh sieve, rinsed thoroughly with ultrapure water, to remove NaOCl traces, quantified and used directly in the bioassays. Nematode and egg counting was performed using an inverted microscope [Diaphot, Nikon, Japan (40x)].

2.2. Plant material, essential oils and essential oil fractions

Collective and/or individual samples, from cultivated and wild-growing medicinal and aromatic plants, were collected from mainland Portugal and at the Azores archipelago (Portugal) (Table 1). Dried aerial parts from commercially available products sold in local herbal shops were also analysed. A total of 56 samples from sixteen families were tested. A voucher specimen of each plant species, collected from wild state condition, was deposited in the Herbarium of the Botanical Garden of Lisbon University, Lisbon, Portugal. For commercial plant material, a reference sample from each plant is retained at the CBV laboratory and is available upon request.

EOs 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 and EOs stored in the dark at -20ºC, until analysis. Fractions containing hydrocarbons molecules (HM) or oxygen-containing molecules (OCM) were separated from each EO sample on a silica gel column by elution with distilled *n*-pentane and diethyl ether, respectively, as previously detailed (Faria et al. 2013).

2.3. Analysis of volatiles

Volatiles were analyzed by gas chromatography (GC), for component quantification, and gas chromatography coupled to mass spectrometry (GC-MS) for component identification.

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

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

The percentage composition of the isolated EOs was used to determine the relationship among the samples by cluster analysis using Numerical Taxonomy Multivariate Analysis System (NTSYS-pc software, version 2.2, Exeter Software, Setauket, New York) (Rohlf 2000). For cluster analysis, correlation coefficient was selected as a measure of similarity among all accessions and the Unweighted Pair Group Method with Arithmetical Averages (UPGMA) was used for

cluster definition. The degree of correlation was evaluated, according to Pestana and Gageiro (2000), as very high (0.9-1), high (0.7-0.89), moderate (0.4-0.69), low (0.2-0.39) and very low (<0.2).

2.4. Bioassays

All bioassays were performed in flat bottom 96 well microtiter plates (Carl Roth GmbH + Co. KG, Karlsruhe, Germany). EOs, hydrocarbon molecules (HM) and oxygen-containing molecules (OCM) fractions were assayed in suspensions of newly extracted mixed-developmental stage *M. chitwoodi* eggs, using a methodology adapted from Faria et al. (2013). Egg suspensions were chosen for experimentation, instead of egg masses, to ensure that EO concentration was homogenous for all eggs. Aliquots with 99 µL of a suspension of eggs (80 to 100) were introduced to each well and 1 µL of EOs or fractions stock solutions prepared in methanol (Panreac Química S.A.U., Barcelona, Spain), at 200 µL/mL, was added, being the highest concentration tested 2 µL/mL. Stock solutions for 1, 0.5, 0.25 and 0.125 µL/mL were obtained by serial dilutions with a dilution factor of two. The EOs and fractions which showed hatching inhibitions < 90% were not further assayed at lower concentrations. Controls were performed with methanol, 1% (v/v, methanol/egg suspension) and ultrapure water was used to check the hatching inhibition induced by methanol. The plates were covered to diminish EO volatilization, wrapped with aluminium foil to establish total darkness and maintained at 27±1ºC. Hatched second-stage juvenile nematodes (J2) were counted every 24 h during three days (72 h). A minimum of 10 replicates was performed for each sample, in, at least, two separate assays.

2.5. Determination of hatching inhibition percentages and EC_{50} values

Hatching rates (J2/day) were obtained by fitting a linear regression to the cumulative time-course hatching data. Slope (m) values, corresponding to hatching rates, were used to determine the corrected hatching inhibition (CHI) through an adaptation of the Abbott formula (Abbott 1925), corrected hatching inhibition (CHI)% = $[1– (m_{treatment}/m_{control})] \times 100$.

Classification of the EOs and fractions hatching inhibition activity was adapted from Dias et al. (2012) in very strong (≥90%) strong (60-89%), moderate (37-59%), weak (11-36%) and low or inactive (<10%).

Effective doses which resulted in 50% hatching inhibition (EC_{50}) were determined using the mean corrected hatching inhibition percentage values. This data was subjected to non-linear regression analysis using a dose-response log-logistic equation (Seefeldt et al. 1995):

$y = C + (D – C) / 1 + \exp \{b [\log (x) – \log (EC_{50})]\}$

which relates the average response y to dose x, and where C and D are, respectively, the lower- and the upper limit of the sigmoidal dose-response curve and b is the slope. This analysis was performed using GraphPad Prism® version 5.00 for Windows, San Diego California USA (www.graphpad.com), setting C to 0% and D to 100% with variable slope (b).

3. Results

3.1. Essential oils CRKN hatching inhibition

CRKN hatching inhibition percentages were evaluated through direct contact bioassays. Control assays were performed with ultrapure water and pure methanol, used as EO solvent. The average hatching rate of the controls ultrapure water and methanol was 5.0±0.4 J2/day and a 4.5±0.3 J2/day, respectively. Hatching inhibition due to methanol, in the concentration 1% (v/v), was considered negligible.

Herewith, 42 of the EOs previously tested against *B. xylophilus* motility (Faria et al. 2013) plus 14 new EOs were comparatively assessed against *M. chitwoodi* hatching. All 56 EOs were fully chemically characterized, although Table 1 reports only their main components (≥10%). The full chemical composition of 42 samples was previously reported in Faria et al. (2013) Supplementary Table (Annex 1), and the 14 new EOs full compositions are detailed in the current Supplementary Table, Annex 3. Cluster analysis was performed on the EOs and EOs fractions full composition to identify groups of similar EO volatile patterns with very strong anti-hatching activities (Fig. 1). Samples were grouped into two main unrelated clusters ($S_{corr} < 0.2$) (Fig. 1).

Cluster I included EOs with specific volatile composition, namely those of *Filipendula ulmaria*, *Nepeta cataria*, *Ruta graveolens* and *Syzygium aromaticum* (Table 1). Cluster II grouped the remaining EOs and related HM and OCM fractions. This cluster grouped terpene-rich EOs and was sub-divided in several sub-clusters (Fig. 1).

Ineffective EOs, showing ≤10% activity at the highest concentration (2 µL/mL), were dominated by e.g. the monoterpenes, α-pinene, sabinene, camphor and/or terpinen-4-ol (Table 1, Fig. 1). A total of 24 EOs were the most successful with a CHI ≥ 90% at 2 µL/mL.

Table 1. Plant family and species, sampling year, plant part used for hydrodistillation, plant source, essential oil (EO) yield and main components (≥10%).

Family / Species	Code	Sampling date	Plant part[a]	Collection place / source	Yield (%, v/w)	Main components (%)
Amaranthaceae						
Dysphania ambrosioides (L.) Mosyakin & Clemants	Da	2013	FF	Évora	0.56	Isoascaridol 51, ascaridol 16
Apiaceae (Umbelliferae)						
Foeniculum vulgare Mill. 1[b]	Fv1	2008	FF	Graciosa, Azores	0.33	*trans*-Anethole 73, α-pinene 13
Foeniculum vulgare Mill. 2	Fv2	2013	Seeds	Herbal shop	1.16	Methyl chavicol 79, limonene 12
Petroselinum crispum (Mill.) Nym.[b]	Pc	2009	FV	Lisbon	0.09	1,3,8-*p*-Menthatriene 50, β-myrcene 13, apiole 11
Asteraceae (Compositae)						
Achillea millefolium L.[b]	Am	2010	DF	Herbal shop	0.85	β-Thujone 33, *trans*-chrisantenyl acetate[e] 19
Solidago virgaurea L.	Sv	2013	FF	Setúbal	0.72	β-Pinene 22, α-pinene 21, germacrene D 15, limonene 12
Cupressaceae						
Cryptomeria japonica (Thunb. ex L.f.) D. Don[b]	Cj	2008	Ffruit	Flores, Azores	0.41	Terpinen-4-ol 24, α-pinene 23, sabinene 17
Fabaceae (Leguminosae)						
Genista tridentata L.[b]	Gt	2010	DV	Herbal shop	<0.05	*cis*-Theaspirane 27, *trans*-theaspirane 22
Geraniaceae						

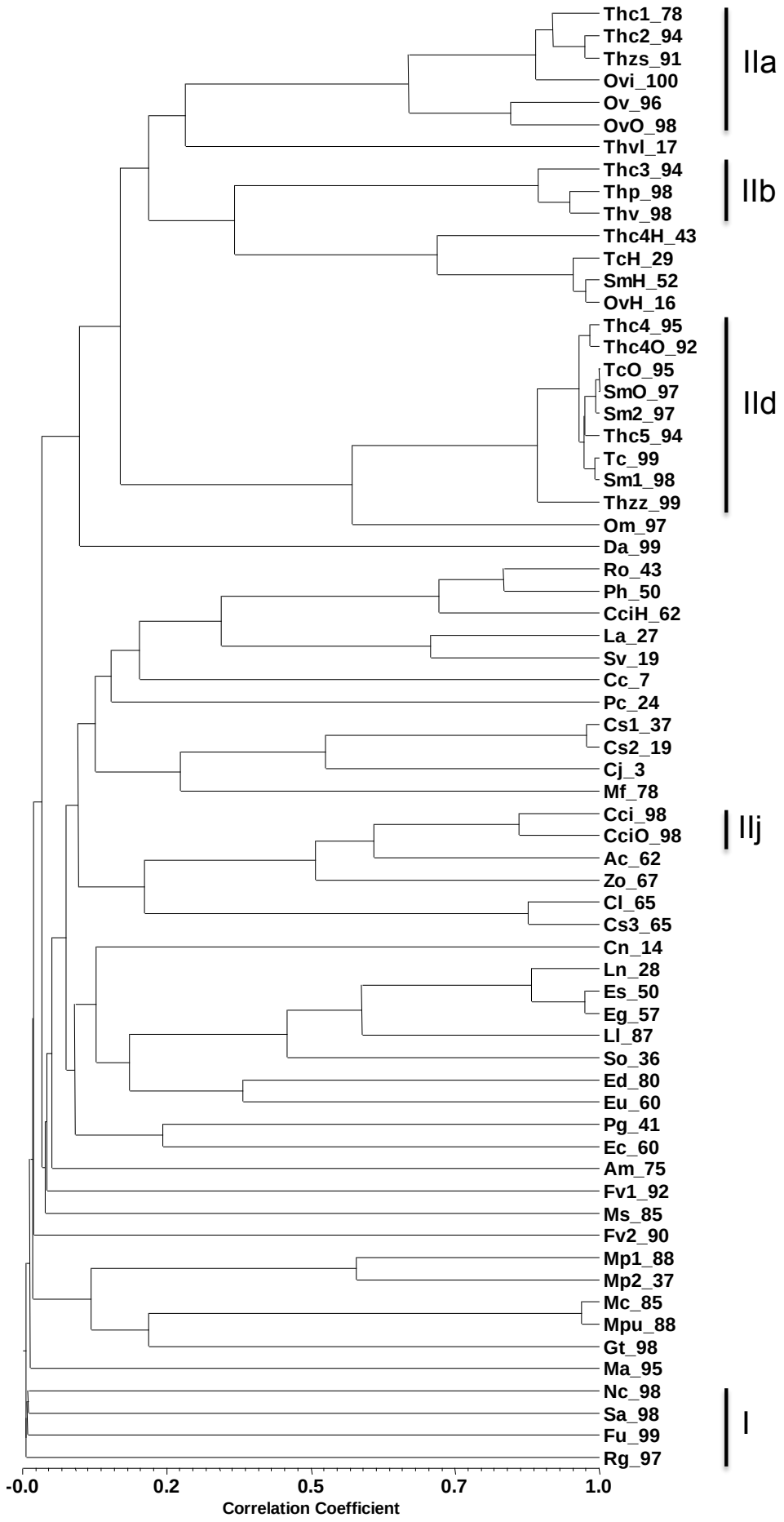
Family / Species	Code	Sampling date	Plant part[a]	Collection place / source	Yield (%, v/w)	Main components (%)
Pelargonium graveolens L'Hér.[b]	Pg	2009	FV	Lisbon	0.19	Citronellol 34, guaia-6,9-diene 15, citronellyl formate[e] 14
Lamiaceae (Labiatae)						
Calamintha nepeta (L.) Savi[b]	Cn	2009	FF	Castelo Branco	1.43	Isomenthone 52, isomenthol 19, 1,8-cineole 11
Lavandula luisieri (Rozeira) Rivas Mart.[c]	Ll	2013	DF	Herbal shop	0.44	5-Methylene-2,3,4,4-tetramethylcyclopent-2-enone 18, 1,8-cineole 16
Mentha arvensis L.[b]	Ma	2009	FV	Lisbon	0.06	Piperitenone oxide 56
Mentha cervina L.[b]	Mc	2009	DV	Castelo Branco	0.80	Pulegone 80
Mentha x piperita L. 1[b]	Mp1	2009	FV	Lisbon	0.11	Menthol 31, menthone 19
Mentha x piperita L. 2[b]	Mp2	2009	FV	Lisbon	0.73	Menthone 56, pulegone 13
Mentha pulegium L.[b]	Mpu	2008	DV	Lisbon	0.35	Pulegone 49, piperitenone 10
Mentha spicata L.[b]	Ms	2009	FV	Lisbon	0.07	Carvone 54
Nepeta cataria L.[b]	Nc	2009	FF	Herbal shop	0.18	4aα, 7α, 7aα-Nepetalactone 89
Origanum majorana L.	Om	2013	DV	Herbal shop	0.90	Terpinen-4-ol 18, carvacrol 17, γ-terpinene 13, methyl carvacrol 13
Origanum vulgare L.[b]	Ov	2010	DL	Herbal shop	1.00	α-Terpineol 16, thymol 15, γ-terpinene 15, carvacrol 10
Origanum vulgare subsp. *virens* (Hoffmanns. & Link) Bonnier & Layens[b]	Ovi	2010	DV	Herbal shop	0.83	α-Terpineol 40, linalool 16, thymol 12
Rosmarinus officinalis L.[b]	Ro	2009	DL	Herbal shop	1.95	β-Myrcene 29, α-pinene 15
Salvia officinalis L.	So	2010	FV	Herbal shop	1.20	1,8-Cineole 13, borneol 12, α-humulene 12
Satureja montana L. 1[b]	Sm1	2010	DF	Herbal shop	1.31	Carvacrol 64, γ-terpinene 18
Satureja montana L. 2	Sm2	2013	DV	Herbal shop	1.48	Carvacrol 77
Thymbra capitata (L.) Cav.[b]	Tc	2010	FF	Algarve	1.40	Carvacrol 68, γ-terpinene 11
Thymus caespititius Brot. 1[b]	Thc1	2008	FF	Gerês	0.35	α-Terpineol 36, *p*-cymene 13, γ-terpinene 13
Thymus caespititius Brot. 2[b]	Thc2	2008	FF	Graciosa, Azores	0.38	α-Terpineol 62
Thymus caespititius Brot. 3[b]	Thc3	2009	FF	Terceira, Azores	0.33	Thymol 42, thymyl acetate15, *p*-cymene 14
Thymus caespititius Brot. 4[b]	Thc4	2004/09	FF	Azores	[d]	Carvacrol 54, carvacryl acetate 10
Thymus caespititius Brot. 5[b]	Thc5	2010	FF	Coimbra	0.48	Carvacrol 59, *p*-cymene 11
Thymus pulegioides L.	Thp	2013	DV	Herbal shop	0.49	Thymol 32, *p*-cymene 22
Thymus villosus subsp. *lusitanicus* (Boiss.) Coutinho[b]	Thvl	2008	FF	Leiria	1.25	Linalool 69

Family / Species	Code	Sampling date	Plant part[a]	Collection place / source	Yield (%, v/w)	Main components (%)
Thymus vulgaris L.	Thv	2013	DV	Herbal shop	1.20	Thymol 45, *p*-cymene 21, γ-terpinene 16
Thymus zygis subsp. *silvestris* (Hoffmanns. & Link) Coutinho [b]	Thzs	2008	FF	Santarém	0.94	α-Terpineol 60
Thymus zygis Loefl. ex L. subsp. *zygis*	Thzz	2013	FF	Bragança	0.71	Carvacrol 45, *p*-cymene 22, γ-terpinene 17
Lauraceae						
Cinnamomum camphora (L.) Sieb.[b]	Cc	2009	FF	Coimbra	0.47	Camphor 49, α-pinene 10
Laurus azorica (Seub.) J. Franco[b]	La	2008	FV	Flores, Azores	0.25	α-Pinene 35, β-pinene 16, *trans*-α-bisabolene 15
Laurus nobilis L. [b]	Ln	2009	DL	Herbal shop	0.95	1,8-Cineole 35, α-terpenyl acetate 13
Myristicaceae						
Myristica fragrans Houtt.	Mf	2013	Macis	Herbal shop	2.60	Safrole 41, terpinen-4-ol 11, sabinene 10
Myrtaceae						
Eucalyptus citriodora Hook.[b]	Ect	2009	FV	Santarém	0.86	Citronellal 36, isopulegol 13, citronellol 12, 1,8-cineole 11
Eucalyptus dives Schauer [b]	Ed	2009	FV	Santarém	3.30	Piperitone 40, α-phellandrene 19, *p*-cymene 19
Eucalyptus globulus Labill.	Eg	2009	FV	Lisbon	3.02	1,8-Cineole 65, α-pinene 20
Eucalyptus smithii R.T. Baker [b]	Esm	2009	FV	Santarém	2.80	1,8-Cineole 83
Eucalyptus urophylla S. T. Blake[b]	Eu	2009	FV	Santarém	0.86	α-Phellandrene 45, 1,8-cineole 23
Syzygium aromaticum (L.) Merrill & Perry [b]	Sa	2010	Dfb	Herbal shop	9.00	Eugenol 92
Pinaceae						
Pinus halepensis Mill.	Ph	2010	FV	Algarve	0.21	α-Pinene 32, β-myrcene 29
Poaceae (Gramineae)						
Cymbopogon citratus (DC) Stapf. [b]	Cci	2010	DL	Herbal shop	3.04	Geranial 34, neral 22, β-myrcene 20, geraniol 18
Rosaceae						
Filipendula ulmaria (L.) Maxim.	Fu	2013	DV	Herbal shop	0.10	Methyl salicylate 85

Family / Species	Code	Sampling date	Plant part[a]	Collection place / source	Yield (%, v/w)	Main components (%)
Rutaceae						
Citrus limon (L.) Burm. f. Var. Meyer [b]	Cl	2009	Fex	Algarve	0.25	Limonene 45, 1,8-cineole 15, β-pinene 14
Citrus sinensis (L.) Osbeck 1[b]	Cs1	2009	Ffl	Lisbon	0.14	Sabinene 47, limonene 10
Citrus sinensis (L.) Osbeck 2 [b]	Cs2	2009	FV	Lisbon	0.26	Sabinene 64
Citrus sinensis (L.) Osbeck var Valencia Late 3[b]	Cs3	2009	Fex	Algarve	0.45	Limonene 78, β-phellandrene 13
Ruta graveolens L. [b]	Rg	2010	DV	Herbal shop	0.51	2-Undecanone 91
Verbenaceae						
Aloysia citrodora Gómez Ortega & Palau [b]	Ac	2009	DV	Herbal shop	0.19	Geranial 12, limonene 11, neral 10
Zingiberaceae						
Zingiber officinale Roscoe [b]	Zo	2008	Frhiz	Herbal shop	0.16	Geranial 29, β-phellandrene 17, citronellol 14, camphene 14

[a] DF - Dry, flowering phase aerial parts, Dfb - Dry flower buds, DL - Dry leaves, DV - Dry, vegetative phase aerial parts, Fex - Fresh exocarp, FF - Fresh, flowering phase aerial parts, Ffl - Fresh flowers, Ffruit - Fresh fruit, FL - Fresh leaves, Frhiz - Fresh rhizome, FV - Fresh, vegetative phase aerial parts. [b] Detailed composition of EOs reported in Faria et al. (2013), Annex 1. [c] Commercialized as *Lavandula stoechas* L. [d] EO resulted from the combination of several EOs from the same chemotype collected in Azores from 2004 to 2009, [e] Identification based on mass spectra only.

Those isolated from *Dysphania ambrosioides* (isoascaridol 51%, ascaridol 16%), *Filipendula ulmaria* (methyl salicylate 85%), *Foeniculum vulgare* 1 (*trans*-anethole 73%) and 2 (methyl chavicol 79%, limonene 12%), *Genista tridentata* (*cis*-theaspirane 27% and *trans*-theaspirane 22%), *Mentha arvensis* (piperitenone oxide 56%), *N. cataria* (4aα, 7α, 7aα-nepetalactone 89%), *R. graveolens* (2-undecanone 91%) and *S. aromaticum* (eugenol 92%) had a low correlation with other EOs ($S_{corr} < 0.4$) (Fig. 1). Very strong inhibition percentages (≥ 90%) at 2 µL/mL were also obtained for EOs showing highly correlated compositions ($S_{corr} > 0.7$). These were gathered in sub-clusters IIa ($S_{corr} > 0.7$), IIb ($S_{corr} > 0.9$), IId ($S_{corr} > 0.9$) and IIj ($S_{corr} > 0.8$) (Fig. 1). α-Terpineol (16-62%), thymol (traces-23%), linalool (traces-16%)], terpinen-4-ol (1-16%), γ-terpinene (traces-15%), carvacrol (traces-15%) and *p*-cymene (traces-13%) dominated the EO composition of the samples grouped in the first cluster.


Thc1_78
Thc2_94
Thzs_91
Ovi_100
Ov_96
OvO_98
Thvl_17
Thc3_94
Thp_98
Thv_98
Thc4H_43
TcH_29
SmH_52
OvH_16
Thc4_95
Thc4O_92
TcO_95
SmO_97
Sm2_97
Thc5_94
Tc_99
Sm1_98
Thzz_99
Om_97
Da_99
Ro_43
Ph_50
CciH_62
La_27
Sv_19
Cc_7
Pc_24
Cs1_37
Cs2_19
Cj_3
Mf_78
Cci_98
CciO_98
Ac_62
Zo_67
Cl_65
Cs3_65
Cn_14
Ln_28
Es_50
Eg_57
Ll_87
So_36
Ed_80
Eu_60
Pg_41
Ec_60
Am_75
Fv1_92
Ms_85
Fv2_90
Mp1_88
Mp2_37
Mc_85
Mpu_88
Gt_98
Ma_95
Nc_98
Sa_98
Fu_99
Rg_97
IIa
IIb
IId
IIj
I
-0.0
0.2
0.5
0.7
1.0
Correlation Coefficient

Corrected hatching inhibitions ≥ 94%, at 2 µL/mL, were obtained in sub-cluster IIb whose EOs showed thymol (32-45%), *p*-cymene (14-22%), γ-terpinene (6-16%) and thymyl acetate (0-15%) as major components.

Sub-cluster IId grouped samples with CHI ≥ 92%, at 2 µL/mL, which revealed to be rich in carvacrol (45-96%), *p*-cymene [not detected (nd)-22%], γ-terpinene (nd-18%) and carvacryl acetate (nd-14%). The highly hatching inhibitor *Cymbopogon citratus* EO grouped in sub-cluster IIj, with the corresponding OCM fraction, due to their richness in geranial (34-45%), neral (22-36%), β-myrcene (traces-20%) and geraniol (5-18%).

Very strong EOs that caused CHI ≥90%, at 2 µL/mL, namely those of *D. ambrosioides*, *C. citratus*, *F. ulmaria*, *F. vulgare* 1, 2, *G. tridentata*, *M. arvensis*, *N. cataria*, *Origanum majorana, O. vulgare* subsp. *virens*, *O. vulgare*, *R. graveolens*, *Satureja montana* 1, 2, *S. aromaticum*, *Thymbra capitata*, *Th. caespititius* 2, 3, 4, 5, *Th. pulegioides*, *Th. vulgaris*, *Th. zygis* subsp. *silvestris* and *Th. zygis* subsp. *zygis* (Table 1, Fig. 1) were tested at lower concentrations. At the lowest concentration, 0.125 µL/mL, only the EOs extracted from *D. ambrosioides*, *F. ulmaria*, *R. graveolens*, *S. montana* 1, 2 and *T. capitata* exhibited a moderate to strong inhibitory activity. Half maximal effective concentrations (EC_{50}) for these EOs were calculated by fitting a dose-response log-logistic curve to the percentage CHI data. EC_{50} values obtained were 0.041 µL/mL for *D. ambrosioides*, 0.032 µL/mL for *F. ulmaria*, 0.061 and 0.033 µL/mL for *S. montana* 1 and 2, respectively, 0.121 µL/mL for *R. graveolens* and 0.140 µL/mL for *T. capitata* EOs (Table 2).

3.2. Hydrocarbons or oxygen-containing molecules fractions CRKN hatching inhibition

Due to their specific EO composition, a balanced percentage of hydrocarbons and

Figure 1. Dendrogram obtained by cluster analysis of the full percentage composition of essential oils (EOs) from the 56 samples and 10 fractions based on correlation and using unweighted pair-group method with arithmetic average (UPGMA). For each EO sample abbreviation, see Table 1. EO fractions abbreviations begin with the sample code followed by uppercase H for fractions containing hydrocarbon molecules or uppercase O for oxygen-containing molecules. Values after underscore are the mean hatching inhibition percentages obtained with an EO concentration of 2 µL/mL

oxygen-containing molecules, and to CHI ≥90%, the EOs of *C. citratus, O. vulgare, S. montana*, *T. capitata* and *Th. caespititius* 4 (Table 3) were chosen for fractionation, to evaluate the separate contribution of the HM and OCM fractions against hatching. OCM fractions of these EOs revealed hatching inhibitions ≥ 92% at 2 µL/mL (Fig. 1, Table 3).

Table 2. EC_{50} values (µL/mL) of the most active essential oils (EOs) and related oxygen-containing molecules (OCM) fractions against *Meloidogyne chitwoodi* hatching. The R^2 values and the 95% confidence limits (CI95%) are given for toxicity comparison.

EOs/OCM	Code	EC_{50}	$CI_{95\%}$	R^2
Dysphania ambrosioides	Da	0.041	0.016 - 0.108	0.94
Filipendula ulmaria	Fu	0.032	0.013 - 0.083	0.96
Ruta graveolens	Rg	0.121	0.107 - 0.136	0.99
Satureja montana 1	Sm1	0.061	0.028 - 0.133	0.94
Satureja montana 1 O	Sm1O	0.099	0.075 - 0.132	0.98
Satureja montana 2	Sm2	0.033	0.019 - 0.058	0.98
Thymbra capitata	Tc	0.140	0.125 - 0.157	0.99
Thymbra capitata O	TcO	0.120	0.114 - 0.126	0.99

O – EO oxygen-containing molecules fraction.

Table 3. Corrected hatching inhibition (CHI) percentages of the essential oils (EOs) and corresponding fractions, at 2 µL/mL (mean±s.e., in %) and main components (≥10%) of the EOs hydrocarbon molecules (HM) and oxygen-containing molecules (OCM) fractions. Values are means of 10 replicates.

	CHI (%)			EOs fractions main composition (%)[a]	
Plant species[b]	EOs	HM	OCM	HM	OCM
Cymbopogon citratus	98±1	62±2	98±1	β-Myrcene 72	Geranial 45, neral 36
Origanum vulgare	100±0	33±11	97±3	γ-Terpinene 36, *p*-cymene 11	α-Terpineol 26, thymol 23, terpinen-4-ol 16, carvacrol 15, linalool 14
Satureja montana	98±2	52±6	97±2	γ-Terpinene 44, *p*-cymene 19	Carvacrol 96
Thymbra capitata	99±1	29±5	95±3	γ-Terpinene 36, *p*-cymene 23	Carvacrol 93
Thymus caespititius 4	95±2	43±2	92±3	*p*-Cymene 29, γ-terpinene 16, *trans*-dehydroagarofuran 12	Carvacrol 66, carvacryl acetate 14

[a] Fraction detailed composition in Faria et al. (2013), Annex 1. [b] EOs chosen for fractionation showed hatching inhibition ≥90% at 2 µL/mL

At the same concentration, the corresponding HM fractions showed activities ≤ 62% (Fig. 1, Table 3). The richness in oxygen compounds is reflected in cluster analysis (Fig. 1), as all OCM fractions grouped closely to their corresponding EOs ($S_{corr} \geq 0.8$). This was not observed for the HM fractions that grouped together ($S_{corr} \geq 0.7$), with the exception of *C. citratus*, showing similar chemical compositions (Fig. 1).

Given the results above, EC_{50} values were determined only for the most successful OCM fractions (Table 2), revealing additive/synergic interactions among the fractions. *S. montana* OCM fraction showed a higher EC_{50} value than that of the related EO, although the oxygen-containing compounds were present in higher proportions in the fraction. This suggests that in addition to the oxygen-containing compounds, the HM fraction also plays an important role in hatching toxicity of this EO. On the other hand, *Thymbra capitata* OCM fraction revealed a lower EC_{50} value than that of the related EO, suggesting that *M. chitwoodi* hatching toxicity may be EO specific.

4. Discussion

The present work is the first screening of essential oils and essential oil fractions with hatching inhibition activity on the CRKN. *D. ambrosioides*, *F. ulmaria*, *R. graveolens*, *S. montana* and *T. capitata* EOs were herewith shown to have the lowest EC_{50} values against CRKN hatching. The main compounds of these EOs, namely 2-undecanone, ascaridol, carvacrol, isoascaridol, methyl salicylate, *p*-cymene and/or γ-terpinene, were putatively considered responsible for CRKN hatching inhibition.

Ruta genus EOs nematotoxic activities have been previously described for other *Meloidogyne* species. *Ruta chalepensis* EO, also 2-undecanone-rich, displayed a high activity against *M. incognita* and *M. javanica* J2 motility (Ntalli et al. 2011b). Its mode of action is still unknown but aliphatic compounds are known to have high inhibition activities against acetylcholinesterase and glutathione S-transferase in the pinewood nematode (*B. xylophilus*) (Kang et al. 2013).

Thymus caespititius chemotypes, rich in carvacrol, thymol and α-terpineol, showed strong to very strong hatching inhibition, displaying CHI ≥ 78%, at 2 μL/mL. Nevertheless, as described by Faria et al. (2013) studying nematotoxic EOs against pinewood nematode *B. xylophilus* motility, the

occurrence of chemotypes must be taken into account when choosing a nematotoxic EO bearing-species, since EO particular chemotype may be determinant in this activity.

Methyl salicylate has shown strong nematicidal properties against *B. xylophilus*, both as a synthetic chemical (at 2 mg/mL) and as a major *Gaultheria fragrantissima* EO component (95%) (at 5 mg/mL) (Kim et al. 2011). This compound is known to be emitted by stressed plants as a signal involved in eliciting plant resistance (Loake and Grant 2007). Pest management using this EO may take advantage of these characteristics by inhibiting hatching and also stimulating the plant immune response.

D. ambrosioides EO and its components were assessed against *M. incognita* revealing low LC_{50} values (Bai et al. 2011). The EO showed LC_{50} values 20x lower than some of its main components, which indicates heavy additive or synergic compound relations.

Satureja montana 1, 2 and *T. capitata* EOs had similar volatile compositions, being carvacrol- (64%, 77% and 68%, respectively) and γ-terpinene-rich (18%, 5% and 11%, respectively). EOs rich in the oxygen-containing monoterpenoids carvacrol and thymol are known to have nematotoxic activity against plant parasitic nematodes (Oka et al. 2000, Kong et al. 2007, Barbosa et al. 2010, 2012, Faria et al. 2013). Anti-nematode activities of *S. montana* EOs have been demonstrated against hatching and J2 motility of *M. javanica* (Andrés et al. 2012), yielding similar results to the obtained in the present work.

Monoterpenoid activity against *M. incognita* hatching and J2 juvenile motility was tested, *in vitro*, by Echeverrigaray et al. (2010). Of the compounds tested, high nematotoxic activities were obtained for the oxygen-containing monoterpenes borneol, carveol, citral (mixture of geranial and neral), geraniol, and α-terpineol. Oka et al. (2000) showed that the monoterpenes carvacrol, thymol and *trans*-anethole also revealed high activities against *M. javanica* hatching and J2 juvenile motility. In the present study, high *M. chitwoodi* hatching inhibitions (≥ 90%), at 2 µL/mL, were observed for the monoterpene-rich EOs of *Thymus caespititius* 2 and *T. zygis* subsp. *silvestris* (α-terpineol, 62% and 60%, respectively), *C. citratus* (geranial - 34%, neral - 22% and geraniol - 18%) but also for *trans*-anethole-rich *Foeniculum vulgare* 1 (73%). The activity of geraniol-, citronellol- and linalool-rich *Pelargonium graveolens* EO was also tested against *M. incognita* J2 motility in direct contact assays, being highly nematotoxic. Commercial EO compounds were evaluated individually,

in the concentrations found in the EO, suggesting that the combined effect of the constituents also play a role in the EO nematicidal activity (Leela et al. 1992).

Synergistic action of basil (*Ocimum* spp.) EO components, methyl chavicol and linalool, was found against *Heterodera avenae*, *H. cajani*, *H. zeae* and *M. incognita* (Gokte et al. 1991), while individually each compound showed no appreciable nematicidal activity.

OCM fractions appear to contribute deeply to the EO hatching inhibition. The same has been suggested by Abd-Elgawad and Omer (1995), analysing EO effects on phytoparasitic nematodes hatching (*M. incognita*) and juvenile motility (*Criconemella* spp., *Hoplolaimus* spp., *Rotylenchulus reniformis*). In the present study, the activity of separate components was not assessed; nevertheless, evaluation of the isolated HM and OCM fractions against *M. chitwoodi* hatching showed that, for the activity of an EO, all its components play a distinct role, contributing in more than one way, either synergistically or antagonistically. Ntalli et al. (2011a) analyzed these types of interactions among the terpene components of EOs active against *M. incognita* showing that combinations of nematotoxic terpenes, such has carvacrol/thymol or carvacrol/geraniol, and/or phenylpropanoids, methyl chavicol/geraniol or *trans*-anethole, had a synergistic activity against J2 motility. This study did not include interactions among non-nematotoxic and nematotoxic EO components.

According to the present results, both the highly active oxygen-containing terpenes and the low hatching inhibition hydrocarbon terpenes cooperate against CRKN hatching. The higher activities of oxygen-containing monoterpenes against phytoparasitic nematodes has been described in several previous studies, but, to our knowledge, this is the first report on the hatching inhibition activity of EOs and their fractions against *M. chitwoodi*. Fractionation and evaluation of the fractions activities containing HM or OCM revealed that this approach, for some EOs, may improve hatching inhibition.

The high nematotoxic properties of some EOs encourage their use as environmentally safer nematicides for the management of the CRKN taken into account that the use of EOs is a highly complex method. Their use as pesticides must first be analyzed in a host/parasite environment, since allelopathic effects can be evident when applying EOs to plant tissues. Direct *in vitro* assays must be complemented by *in vivo*, soil-based experiments, in order to examine phytotoxicity or

plant biotransformation.

With a few exceptions, natural nematicides, like the 5 nematotoxic EOs presented herewith, are considered less hazardous than chemical synthetic nematicides (Figueiredo et al. 2008, Moharramipour and Negahban 2014); however, when considering an environmentally friendly pest management strategy for root-knot nematodes, some EOs volatility constraints, industrial process costs for large scale production, availability of primary biological material and needs for agricultural space should always be taken into consideration prior to any entrepreneurial endeavour.

To better assess the infection mechanism and the plant response to nematotoxics, laboratory assays using host / parasite *in vitro* cultures that mimic as closely as possible the field environment, are being conducted.

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Chapter 6

In vitro co-culture of *Solanum tuberosum* hairy roots with *Meloidogyne chitwoodi*: structure, growth and production of volatiles

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Abstract

Meloidogyne spp., commonly known as root-knot nematodes (RKNs), are economically important plant sedentary endoparasites that cause galls on susceptible hosts. The Columbia root-knot nematode (CRKN), *M. chitwoodi*, is a quarantine A2 type pest by the European and Mediterranean Plant Protection Organization (EPPO) since 1998. This nematode has been found associated with economically important crops such as potato and tomato, causing severe damage and making the agricultural products unacceptable for the fresh market and food processing. *In vitro* co-culture of host and parasite offers an advantageous experimental system for studying plant / RKN interactions. The structure, growth and production of volatiles of *Solanum tuberosum* hairy roots (HR) and of *S. tuberosum* HR with CRKN co-cultures were compared. HR were induced by inoculation of aseptic potato tuber segments with *Rhizobium rhizogenes*. Co-cultures were initiated by inoculating HR with sterilized CRKN eggs. Infection with CRKN induced the RKN symptomatology in the HR and several nematode life stages were observed by light and scanning electron microscopy. Potato HR and HR with CRKN co-cultures exhibited similar growth patterns, evaluated by measuring fresh and dry weight and by the dissimilation method. Volatiles, isolated by distillation-extraction and analyzed by GC and GC-MS, revealed that palmitic acid (37-52%), *n*-pentadecanal (10-16%) and linoleic acid (2-16%) were the main constitutive components of *S. tuberosum* HR, and of the HR with CRKN co-cultures (24-44, 8-22 and 4-18%, respectively). *S. tuberosum* HR with CRKN co-cultures can be considered a suitable biotechnological tool to study RKN infection mechanism by mimicking what occurs under field conditions.

Keywords: Plant biotechnology, Columbia root-knot nematode, potato, hairy root structure, root gall structure, volatiles

1. Introduction

The Columbia root-knot nematode (CRKN) *Meloidogyne chitwoodi* Golden, O'Bannon, Santo and Finley, 1980, is a sedentary and obligate plant endoparasite, which was described for the first time in the Pacific Northwest of the USA and has been reported in Africa (South Africa), Europe (Belgium, Germany, the Netherlands and Portugal), and North and South America (Mexico and Argentina, respectively) (Golden et al. 1980, da Conceição et al. 2009, OEPP/EPPO 2009). This species is a quarantine A2 type pest according to the European and Mediterranean Plant Protection Organization since 1998 (EPPO 2012) and has been found associated with economically important crops such as potato (*Solanum tuberosum* L.) and tomato (*S. lycopersicum* L.), causing severe damage responsible for heavy losses and/or unacceptable agricultural products for the fresh market and food processing (Santo et al. 1980, O'Bannon et al. 1982, Ferris et al. 1993, Perry et al. 2009).

These endoparasites mobilize the plant's photosynthates from shoots to roots and affect water and nutrient absorption and translocation by the roots to support their development and reproduction. Root-knot nematode (RKN) second-stage juveniles (J2), the infective stage, are attracted to a host plant, invade the root and move through the apoplast to the zone of differentiation. There, they induce the formation of giant cells (feeding site) and typical root galls (Perry et al. 2009). During this process, most of the J2 become sedentary and enlarge, assuming a pear shape and moult three times reaching the female adult stage and lay eggs into a gelatinous matrix produced by rectal glands. Males are vermiform and motile, leaving the roots, and are less frequent. However, the proportion increases under environmental stress conditions (Perry et al. 2009).

RKN pest management has been extensively documented (e.g. Griffin 1985, Chitwood 2002, 2003, Li et al. 2011, Andrés et al. 2012). Nevertheless, research on the effect of nematicidal compounds is commonly performed on the nematode species alone and rarely on the host / parasite system, not taking into account the cytotoxicity for the plant host or the plant's capability to metabolize or biotransform the nematicidal active substances. When studying complex *in vivo* organism-organism interactions, analyzing just one of the partners gives only a partial view of the existing relations. For this reason, the growth of more than one organism or cell type in a combined culture (*in vitro*

co-cultures) has the advantage of simulating the host / pathogen conditions and eliminating variables due to the environmental *in vivo* conditions.

In vitro axenic culture of hairy roots (HR), or transgenic roots, offers a suitable biotechnological model host system for analysis of RKN infection, due to their genetic and metabolomic stability as well as a rapid growth rate, compared to conventional root cultures, and to being free of plant growth regulators (Giri and Narasu 2000, Figueiredo et al. 2006). *In vitro* plant / nematode co-cultures have been commonly used since mid-1900's to increase and maintain nematodes and study plant / nematode interactions (Bonga and Durzan 1982, Maheshwari 1991). In monoxenic co-cultures of *Meloidogyne* spp., the host-pathogen system is free from contaminants such as soil flora and fauna which characterize the natural conditions (Bonga and Durzan 1982, Mitkowski and Abawi 2002). Moreover, in a controlled environment of an *in vitro* culture, single variables can be manipulated and plant / nematode responses can be observed directly, which is difficult under greenhouse or field conditions. Furthermore, *in vitro* cultures have the advantage of providing more biomass using fewer resources.

Although potato hairy roots have been previously established and used for metabolomic (Valancin et al. 2013) and genetic transformation evaluation (Nagy et al. 2005), and studies have addressed nematode effect on potato hairy root cultures (Hansen et al. 1996, Wiśniewska et al. 2013), no previous study established a *S. tuberosum* HR with *M. chitwoodi* co-culture system, which requires regular subculturing of the host / pathogen system.

Given that RKN quarantine species is a serious potato pest (Mojtahedi et al. 1988, Perry et al. 2009) and there is the need for a reliable host / pathogen system for phytopathological research, e.g. phytonematicidal research, the goals of this study were to: a) establish *S. tuberosum* HR, and *S. tuberosum* HR with *M. chitwoodi* co-cultures (*S. tuberosum* HR / CRKN co-cultures), b) evaluate HR and the co-cultures structure and growth, c) quantify the nematodes in *in vitro* co-cultures medium and d) characterize the constitutive and induced production of volatiles in the two independent *in vitro* culture systems. The present study proposes that the nematode life cycle in co-culture progresses in a similar way as under field conditions and that the co-cultures are an adequate biotechnological tool to study this pathology and to easily assess different approaches viewing to understand and combat the CRKN infection mechanism.

2. Materials and methods

2.1. Establishment of *Solanum tuberosum* hairy root cultures

Solanum tuberosum cv. Desiree tubers were obtained locally and prepared for inoculation using the methodology adapted from Kumar and Forrest (1990). The tubers were washed with commercial detergent (10 drops *per* 100 mL distilled water) and surface sterilized by immersion in ethanol 96% (Merck KGaA, Germany) for 10 min. Under asepsis, the potatoes were subsequently rinsed 3 times in 100 mL ultrapure sterile water, the peripheral portion removed, approximately 2 cm inwards from the surface, and the central piece divided into approx. 0.5 cm thick segments.

Hairy roots (HR) were induced by inoculation of the aseptic potato segments with *Rhizobium rhizogenes* A4 strain [according to new taxononomic revisions (Bull et al. 2010), *Rhizobium rhizogenes* (Riker et al. 1930) Young et al. 2001a is the most recent synonym of *Agrobacterium rhizogenes* (Riker et al. 1930) Conn 1942] carrying the *gus* reporter gene co-integrated in the Ri plasmid and driven by a double 35S promoter (A4 pRiA4::70GUS), using the methodology described by Santos et al. (1998). The potato segments were wounded with a scalpel previously dipped in an overnight grown *R. rhizogenes* suspension, to an A_{600} = 0.6, and diluted 1:10 (v/v) in liquid Schenk and Hildebrandt (SH) medium (Schenk and Hildebrandt 1972) supplemented with 30 g/L sucrose, pH = 5.6. After drying on sterile filter paper, for 1 min, the segments were placed on SH solid medium (8 g/L agar), and co-cultivated with the bacteria for 3 days after which they were transferred to SH solid medium supplemented with cefotaxime and carbenicillin (150 µg/mL each), with both medium and antibiotics renewed weekly, for over 3 months.

Solanum tuberosum HR were excised and used for propagation in antibiotic-free SH solid medium. After ±3 months free from contaminations, HR pieces were transferred to liquid SH medium without growth regulators and antibiotic and maintained on orbital shakers (80 rpm). Under a regular subculturing routine, a portion of the root clump was aseptically removed and transferred monthly to fresh culture medium. In every step the potato cultures were maintained in darkness at 24 ± 1 ºC. One-year-old *S. tuberosum* HR were characterized by evaluation of their structure, growth and production of volatiles compared with potato HR / CRKN co-cultures.

2.2. Establishment of *S. tuberosum* HR / *Meloidogyne chitwoodi* co-cultures

Meloidogyne chitwoodi (CRKN) egg masses were handpicked from infected tomato plants as described by Vieira dos Santos et al. (2013). The eggs were extracted with 0.52% (v/v) sodium hypochlorite (NaOCl) solution (Hussey and Barker 1973) and the debris removed with a 47% (w/v) sucrose solution according to McClure et al. (1973). Afterwards, the eggs were sterilized in a 0.05% (v/v) mercuric chloride ($HgCl_2$) solution for 3 min, centrifuged for 2 min at 500 G, rinsed in sterile water and centrifuged (4 times), as above, to remove $HgCl_2$ (adapted from Kumar and Forrest 1990).

Solanum tuberosum HR / CRKN co-cultures were established by adding 100-150 sterilized CRKN eggs to *S. tuberosum* HR cultures after 14 days of growth on solid SH medium. Co-cultures were maintained in darkness at 24±1 ºC for over 2 months, to ensure the completion of the nematode life cycle. Life cycle stages were identified using an inverted microscope Diaphot, Nikon, Japan. Subculturing of the co-cultures was performed every 4 weeks, by aseptically transferring a portion of the root clump to fresh solid SH medium, and maintained as described above. After approx. 6 months, root clumps were transferred to liquid SH medium and maintained in darkness at 24±1 ºC, on an orbital shaker (80 rpm), with monthly subculturing by refreshment of the culture medium over 3 months. Then, a portion of the root clump was removed and transferred to fresh liquid SH culture medium. The length of 30 ramdomly selected HR / CRKN galls, J2, and males as well as the length/width of 30 randomly selected HR / CRKN eggs and adult females were measured using a stage micrometer calibrated eyepiece reticle. Similar to *S. tuberosum* HR, one-year-old *S. tuberosum* HR / CRKN co-cultures were characterized by evaluation of their structure, growth and production of volatiles. Besides this initial infection stage, no further external nematodes were added to the co-culture, given that it would influence the natural co-culture equilibrium, which intends to mimic natural conditions.

2.3. Characterization of *S. tuberosum* HR and *S. tuberosum* HR / CRKN co-culture structure

Solanum tuberosum HR and galls, from selected co-cultures with at least one-year of *in vitro* culture, were collected from solid SH medium, for scanning electron microscopy (SEM), and from liquid SH medium for light microscopy (LM). *S. tuberosum* HR and galls were prepared for SEM following the methodology described by Figueiredo and Pais (1994). Samples were fixed with 1.5% (v/v) glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.0 for 45 min at room temperature. After 1-2 min under vacuum (26 mm Hg, 3.46 kPA), the fixative was substituted with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.0 for 2 h at room temperature. The material was rinsed thoroughly in the same buffer, post-fixed with a 2% osmium tetroxide aqueous solution (OsO_4) for 2 h at room temperature, dehydrated in a graded acetone series and critical point dried in a Polaron E 3500. Dried specimens were mounted on stubs, coated with gold in a Polaron E 5350. Observations were carried out on a JEOL T220 SEM (JEOL Ltd., Tokyo, Japan) at 15 kV.

For LM, galls excised from *S. tuberosum* HR / CRKN co-cultures grown in liquid SH medium were fixed with glutaraldehyde 2.5% (v/v) in 0.1 M sodium phosphate buffer, pH 7.2. Samples were kept in fixative under vacuum at room temperature for 20 min, followed by 24-48 h at 4 ºC. The material was then washed in the fixative buffer, dehydrated in a graded ethanol series, and embedded in Leica historesin® according to Ascensão et al. (2005). Longitudinal and cross sections (4 µm thick) were sequentially stained with periodic acid–Schiff's (PAS) reagent / Toluidine Blue O (Feder and O'Brien 1968) for polysaccharides and general histology. Observations were made under a Leica DM-2500 microscope (Leica Microsystems CMS GmbH, Wetzlar. Germany). The 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).

2.4. Time-course characterization of *S. tuberosum* HR and *S. tuberosum* HR / CRKN co-culture growth, nematode population density and production of volatiles

Erlenmeyer flasks with 100 mL SH medium were aseptically inoculated with 1 g fresh weight (FW) of *S. tuberosum* HR or *S. tuberosum* HR / CRKN kept under routine subculture, and maintained as above. Growth, nematode population density in the culture medium and production of volatiles were evaluated at inoculation time (t0) and weekly for 7 weeks. Two independent experiments were conducted separately, for each *in vitro* culture, and two replicates of each flask were used in each experiment. The data shown were calculated as mean values of all experiments.

2.4.1. Growth of *in vitro* cultures

Solanum tuberosum HR and *S. tuberosum* HR / CRKN co-cultures growth were evaluated by the dissimilation method, a non-invasive method that relates growth with energy-consumption of the cells (Schripsema et al. 1990), and by measuring the FW and DW. FW determination was performed after blotting the total HR cultures or HR / CRKN co-culture clumps on filter paper to remove excess culture medium. For dry weight (DW) calculation, samples from the clumps 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 ºC. The total DW was based on the relation between FW and DW determination after freeze-drying. Specific growth rate (μ) in wt/L/day and doubling time (dt) in days were determined for FW and DW growth curves, between the 4^{th} and 7^{th} day, using the formulae according to Kondo et al. (1989) and Mehrara et al. (2007): $\mu = (\log_e X - \log_e X_0) / t$; $dt = (\log_e 2)/\mu$, where X_0 is the initial weight, X is the final weight and t is the time between weighings. The remaining *S. tuberosum* HR or *S. tuberosum* HR / CRKN co-cultures were kept at -20 ºC until volatiles were extracted.

2.4.2. Nematode population density in co-cultures medium

Population of CRKN (J2 and males) in the liquid medium was evaluated by sampling 100 µL aliquots of each culture flask at each time-point. Three replicates of each flask were used for counts. Number of dead and live nematodes was recorded using an inverted microscope.

2.4.3. Isolation of volatiles from *in vitro* cultures

Constitutive volatiles as well as those induced by the phytoparasite were isolated from the *in vitro* cultures by distillation–extraction, for 3 h, using a Likens-Nickerson type apparatus (Likens and Nickerson 1964). Distillation was run at 3 mL/min rate, 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 (Yamato, Hitec RE-51). After collection in a vial, the volatiles were concentrated to a minimum volume under nitrogen flux, at room temperature. The volatile oils were stored at -20 ºC in the dark until analysis.

2.4.4. Analysis of volatiles from *in vitro* cultures

Volatiles were analyzed by gas chromatography (GC), for component quantification, and gas chromatography coupled to mass spectrometry (GC-MS) for component identification. Gas chromatographic analyses were performed using a Perkin Elmer Autosystem XL gas chromatograph (Perkin Elmer, Shelton, CT, USA) equipped with two flame ionization detectors (FIDs), a data handling system, and a vaporizing injector port into which two columns of different polarities were installed: a DB-1 fused-silica column (30 m × 0.25 mm i.d., film thickness 0.25 µm; J & W Scientific Inc., Rancho Cordova, CA, USA) and a DB-17HT fused-silica column (30 m × 0.25 mm i.d., film thickness 0.15 µm; J & W Scientific Inc., Rancho Cordova, CA, USA). Oven temperature was programmed to increase from 45 to 175°C, at 3°C/min increments, then up to 300°C at 15°C/min increments, and finally held isothermal for 10 min. Gas chromatographic settings were as follows: injector and detectors temperatures, 280°C and 300°C, respectively;

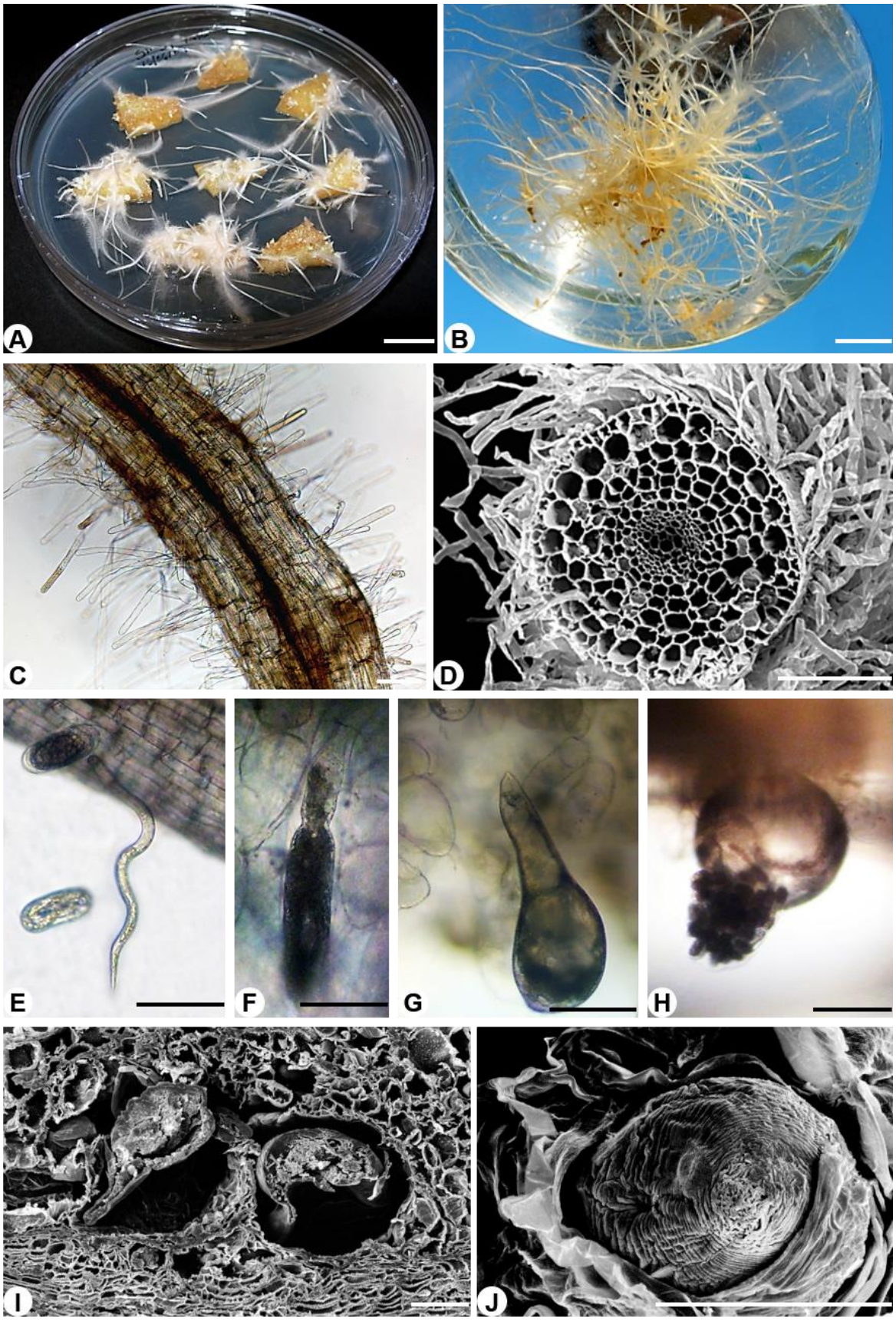
carrier gas, hydrogen, adjusted to a linear velocity of 30 cm/s. The samples were injected using a split sampling technique, ratio 1:50. The volume of injection was 0.1 µL of a pentane-oil solution (1:1). The percentage composition of the oils was computed by the normalization method from the GC peak areas, calculated as a mean value of two injections from each volatile oil, without response factors.

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

3. Results and discussion

3.1. Establishment of *Solanum tuberosum* hairy root cultures

Solanum tuberosum hairy roots (HR) were established on solid SH medium (Schenk and Hildebrandt 1972), after inoculation of aseptic potato segments with *Rhizobium rhizogenes* A4 strain. Approximately 2 weeks after infection, numerous HR were observed at the inoculation sites (Fig. 1a). Following establishment of the HR in liquid medium without antibiotics and growth regulators (Fig. 1b), potato HR were highly branched with the typical “rooty” phenotype (Fig. 1c and d). Structure, growth and volatile analyses were performed after approx. 12 months in culture with routine subculture.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3.2. Establishment of *S. tuberosum* HR / CRKN co-cultures

Solanum tuberosum HR / CRKN co-cultures were obtained by adding surface-sterilized nematode eggs to the HR cultures grown on solid SH medium. The first J2 appeared approximately 2 days after inoculation (DAI) and were seen on the vicinity of the transgenic roots (Fig. 1e). Within approximately 10 days, root tip enlargement was observed and galls and immature females were observed 25 DAI (Fig. 1f and g). Adult females in the galls were detected approximately 45 DAI followed by the production of the gelatinous matrix and eggs (Fig. 1h).

This species is known to cause numerous small, pimple-like, galls that are not always evident, without secondary roots emerging from them. Symptoms caused by *M. chitwoodi* vary according to host, nematode population density and environmental conditions (Perry et al. 2009).

3.3. Characterization of *S. tuberosum* HR and *S. tuberosum* HR / CRKN co-cultures

3.3.1. Structure of *in vitro* cultures

Hairy roots, grown on solid SH medium, showed the characteristic anatomy of primary root growth structures – a single-layered epidermis with a thin cuticle and numerous lateral hairs, a cortex with few cell layers, an endodermis and a pericycle surrounding the vascular cylinder (Fig. 1c and d). This typical primary root structure has been commonly reported for other HR, such as those of *Pimpinella anisum* (Santos 1997), *Levisticum officinale* (Costa 2005) and *Anethum graveolens* (Geraldes 2010). However, *S. tuberosum* HR, with an average diameter of 295±22 µm, differed from the previous examples by being slightly thinner. According to Verdejo et al. (1988),

Figure 1. *Solanum tuberosum* hairy (HR) roots and *S. tuberosum* HR with *Meloidogyne chitwoodi* (CRKN) co-cultures. a, b, *S. tuberosum* HR cultures grown on solid and liquid Schenk and Hildebrandt (1972) (SH) medium, respectively. c-j, Light and scanning electron microscopy micrographs of *S. tuberosum* HR (c, d) and *S. tuberosum* HR / CRKN co-cultures, grown on solid SH medium (e-h, i, j). Note the primary tissues of the root (c, d), the second-stage juvenile (e), females in different developmental stages (f, g) and an adult female with egg mass (h). Pear-shaped females with the head embedded in the periphery of the vascular tissue and the female perineal ridge pattern in i and j, respectively. Scale bars: 1 cm (a, b), 100 µm (c-j).

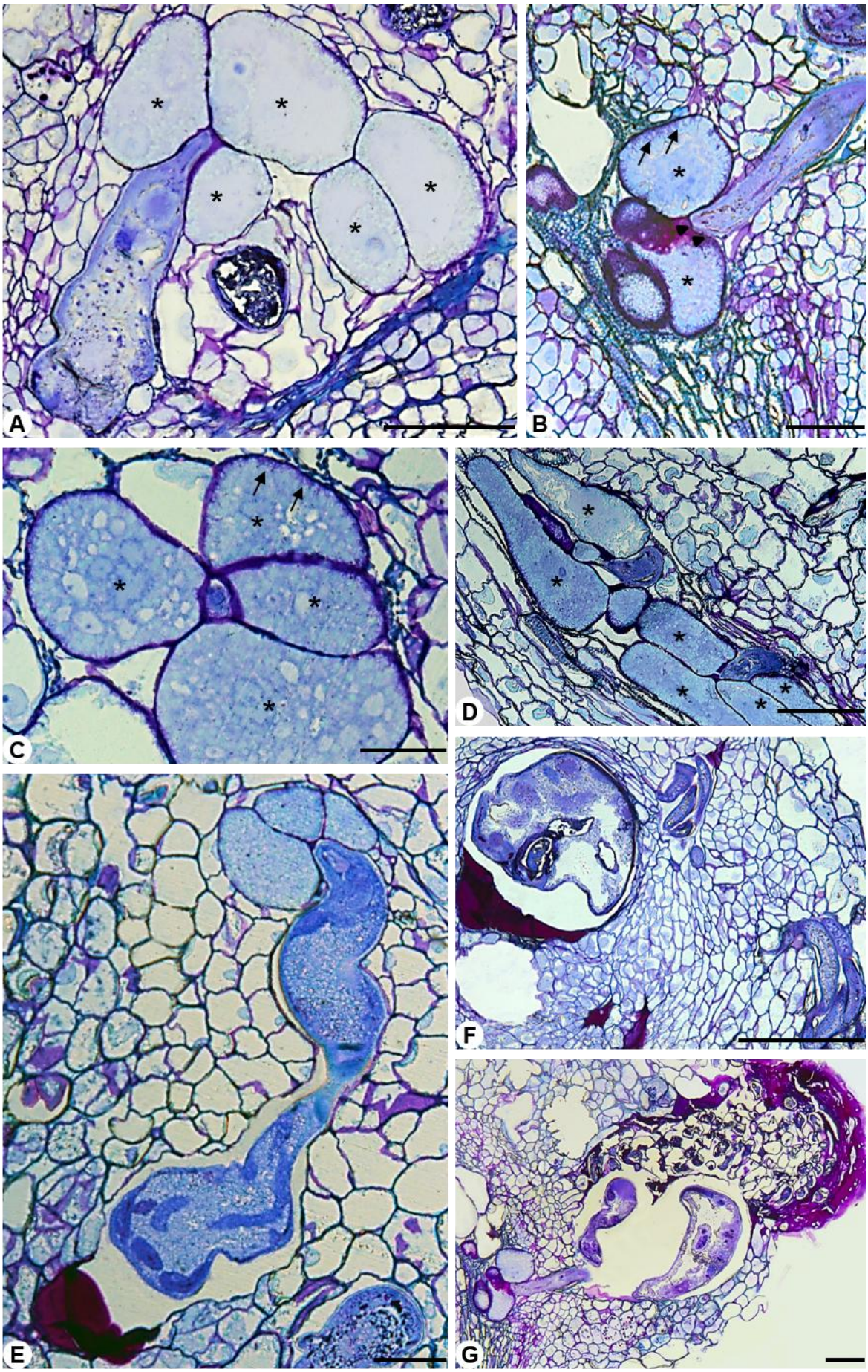
who studied the reproduction of *M. javanica* in several HR systems, thin roots that grow at moderate rates into the agar and produce many secondary roots could support high nematode reproduction. Thus, *S. tuberosum* HR seemed to have adequate characteristics for *M. chitwoodi* development and reproduction.

In *S. tuberosum* HR / CRKN co-cultures, the HR diameter did not show substantial differences (304±38 µm) compared with *S. tuberosum* HR (295±22 µm), except where root galls were formed (1375±260 µm). The formation of galls was more frequent in roots that grew inside the agar than on the agar surface, suggesting that the location of roots affected nematode penetration and reproduction. A similar observation was made by Verdejo et al. (1988).

Scanning electron micrographs (SEM) of co-culture root galls showed CRKN adult pear-shaped females (Fig. 1i and j). Morphometrics of CRKN adult females width/length (313±41 µm/488±33 µm), males length (950±91 µm), J2 length (336±12 µm) and eggs width/length (33±3 µm/88±7 µm) were consistent with the original description of Golden et al. (1980): adult female width/length (344-518 µm/430-740 µm); male length (887-1268 µm); J2 length (336-417 µm); egg width/length (40-46 µm/79-92 µm).

In response to signals from the infective stage, root cells from vascular cylinder, adjacent to the head of the nematode, redifferentiated into hypertrophied cells, giant cells (Fig. 2a-d, asterisks). These specialized cells were easily distinguished from their neighbouring cells by the increased volume, dense cytoplasm, numerous nuclei and small vacuoles. Another important characteristic feature of these metabolically active cells was the development of cell wall ingrowths (Fig. 2b and c, arrows).

Figure 2. Light micrographs of historesin galled root sections from *S. tuberosum* HR with CRKN co-cultures in liquid Schenk and Hildebrandt (1972) (SH) medium, stained with Periodic Acid–Schiff's (PAS) / Toluidine Blue O. a, b, Females feeding on a group of prominent giant cells (asterisks). Note in b, the nematode head embedded in the periphery of the vascular tissue (arrowheads) and the giant cell wall ingrowths (arrows). c, Detail of the giant cells with dense cytoplasm, small vacuoles, numerous nuclei and cell wall ingrowths (arrows). d, Giant cells and interspersed vascular elements are apparent. e-f, Longitudinal and cross sections of mature females revealing a pink PAS-positive exudate near their posterior ends. g, Eggs embedding in a polysaccharidic matrix are observed on the surface of galled roots. Scale bars: 100µm.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Giant cells are typical transfer cells that are involved in rapid solute transport from the contiguous xylem elements to the nematodes, supplying nutrients to nematode development and reproduction (Berg and Taylor 2009). Mature females secrete, at the posterior end, a material that stained dark pink with Periodic acid-Schiff stain (PAS), revealing a polysaccharide nature (Fig. 2e and f). This gelatinous matrix, embedding the eggs layed by females, was usually deposited on the surface of galled roots (Fig. 2g). The root-knot anatomy observed in this study was similar to that induced by RKN described by Berg and Taylor (2009).

Although previous studies adressed the morphology and anatomy of *Meloidogyne* genus infection and disease progression, the novelty of the present work is that it establishes and characterizes a plant / nematode co-culture as a biotechnological testing system that is reproducible and easy to use, as a tool to evaluate plant / nematode interactions in an *in vitro* culture system that mimics what occurs in natural conditions.

3.3.2. Growth of *in vitro* cultures

A 7 weeks' time-course study was performed to assess potato HR and *S. tuberosum* HR / CRKN co-culture growth, in liquid SH medium, by measuring FW and DW and using the dissimilation method. Compared to *S. tuberosum* HR cultures, *S. tuberosum* HR / CRKN co-cultures showed similar colour, as no browning was induced by the combined growth with *M. chitwoodi*. Aditionally, *S. tuberosum* HR growth was not influenced by the presence of the nematode (Fig. 3a and b).

This resemblance in growth profile was supported by the determination of specific growth rate and doubling time based on FW and DW growth curves. Specific growth rates (μ) for *S. tuberosum* HR cultures and *S. tuberosum* HR / CRKN co-cultures were 0.3 and 0.2 g FW/L/day, respectively, with doubling times (dt) of 2.6 and 3.0 days on FW basis and 0.3 and 0.2 DW/L/day with dt of 2.5 and 3.5 days, on a DW basis. These values were within the expected for HR cultures. Literature average growth rate for HR ranged from 0.1-2.0 g w/L/day (Oksman-Caldentey and Hiltunen 1996) and 1 to 11 days dt (Maldonado-Mendoza et al. 1993, Dhakulkar et al. 2005), being largely dependent on a high rate of linear extension, lateral branching and secondary increase in

root diameter (Figueiredo et al. 2006).

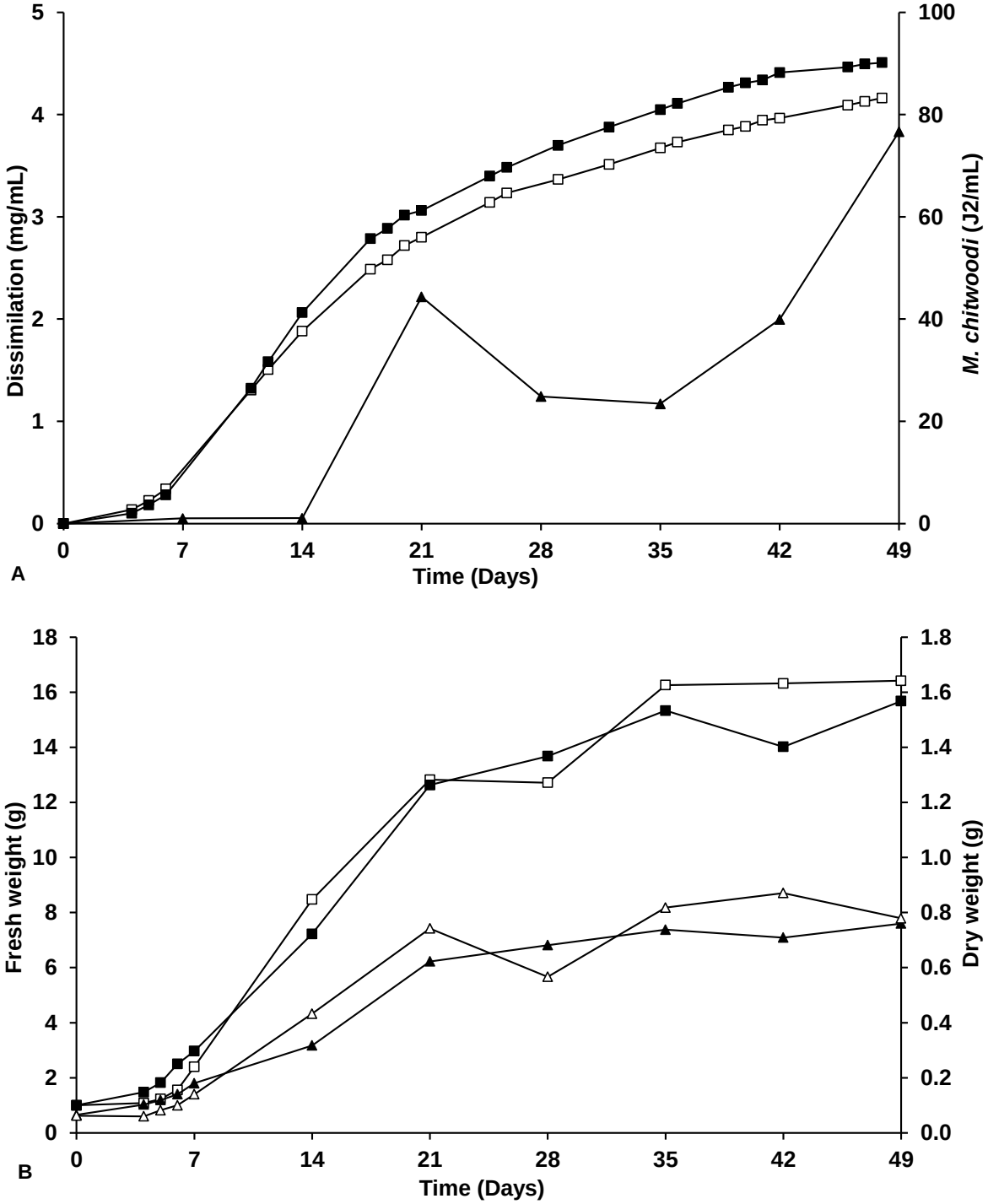

Figure 3. a, Dissimilation growth curves of *Solanum tuberosum* hairy roots (HR) (□) and *S. tuberosum* HR with *Meloidogyne chitwoodi* (CRKN) co-cultures (■), and number of nematodes in the culture medium (▲). b, Fresh (square symbols) and dry weight (triangle symbols) growth curves of *S. tuberosum* HR and *S. tuberosum* HR / CRKN co-cultures. Fresh weight growth curves: *S. tuberosum* HR (□) and co-cultures (■). Dry weight growth curves: *S. tuberosum* HR (∆) and co-cultures (▲).

Notwithstanding small variations in growth, probably due to variations in development stages of *S. tuberosum* HR starting inoculum, the outline of the growth curves was identical to those previously observed with *Anethum graveolens* (Faria et al. 2009) or *Levisticum officinale* HR (Nunes et al. 2009).

Potato HR growth seemed unaffected by the nematode which may be due to the constant optimal temperature and nutrient availability under *in vitro* culture conditions, and, as reported by Perry et al. (2009), a plant may be infected and still not show clear changes in growth and productivity. Santo and O'Bannon (1981) analysed differences in root weight and RKN reproduction, at different temperatures, in *S. tuberosum* roots inoculated with two starting inocula. At 25 ºC, a 10-fold increase in the starting inoculum induced an almost 3-fold decrease in root weight, and lead to the production of more CRKN eggs. In future experiments using this biotechnological model system, it would be advantageous to test different CRKN inoculum levels in order to determine the influence of this parameter on the co-cultures growth.

3.3.3. Nematode population density in co-culture medium

The number of *M. chitwoodi* motile forms (J2 and males) in the medium was quantified at different time-points, every week during 7 weeks, as an indicator of the nematode population density. Twenty one days after inoculation, the number of nematodes increased (±44 nematodes/mL), concomitant with the progressive growth deceleration of the co-culture, followed by a slight decrease and then another increase (±77 nematodes/mL) until the end of the sampling time-points, in the co-culture stationary growth phase (Fig. 3a).

The first peak may be due to J2 hatching from eggs produced by females present in the inoculated co-culture root clump (1st generation), and the second peak to the second generation of nematodes (Fig. 3a). Second generation of nematodes was originated from co-culture new infections and the production of new mature females, whose hatched juveniles contributed to the second peak in the nematode number.

Continuous subculturing of these co-cultures in SH medium reproduced the same growth pattern

leading to an equilibrium between nematode infection and development and HR growth, making possible the maintenance of this co-culture. Pak et al. (2009) analysed *M. incognita* infection and development in *Cucumis melo* HR grown in three culture media and found that the greatest number of egg masses were obtained on HR cultured in SH medium. Culture medium appears to contribute to the success in the establishment and maintenance of HR / nematode co-cultures.

3.3.4. Production of volatiles during *in vitro* culture

Thirty one compounds were identified in the constitutive volatiles isolated from *S. tuberosum* HR. The same compounds were identified in the volatiles isolated from *S. tuberosum* HR / CRKN co-cultures, maintained under the same growth conditions. The detailed relative amounts of the components identified in the volatiles, isolated from potato *in vitro* cultures, are listed according to their elution from a DB-1 column (Table 1). A limited number of components, with relative amounts of 0.5-3% each, could not yet be identified. Together they justify the lower identification attained at time 0 for HR / CRKN co-cultures volatiles. Nevertheless, their relative importance decreases during the time-course study of volatiles in these cultures.

Fatty acids (33-63%) and the fraction designated by others (23-39%), since components were neither terpenes nor C13 compounds, and which was mainly composed of non-aromatic alcohols, saturated and unsaturated non-aromatic aldehydes and hydrocarbons, dominated both the constitutive volatiles of the HR and those of the HR / CRKN co-cultures (Table 1). Palmitic acid (37-52% in the HR and 24-44% in the co-cultures), *n*-pentadecanal (6-16% in the HR and 8-22% in the co-cultures) and linoleic acid (2-16 % in the HR and 4-18% in the co-cultures) dominated *S. tuberosum* HR and *S. tuberosum* HR / CRKN volatiles. Fatty acid abundance, mostly resulting from cell membrane degradation, is common in constitutive volatile profiles of hairy roots (Faria et al. 2009, Nunes et al. 2009), as well as in plants producing small amount of volatiles.

Although Komaraiah et al. (2003) reported enhanced production of the stress related antimicrobial sesquiterpene phytoalexins rishitin, lubimin, phytuberin and phytuberol, extracted with organic solvents, from elicitor-treated HR cultures of *S. tuberosum*, these compounds were not detected in the present study.

Table 1. Percentage composition of the volatiles isolated from *Solanum tuberosum* HR and *S. tuberosum* HR with CRKN co-cultures, at the different time-points (t0, inoculation time).

		***S. tuberosum* HR**								***S. tuberosum* HR / CRKN**							
		Time (days)								**Time (days)**							
Component	**RI**	**0**	**7**	**14**	**21**	**28**	**35**	**42**	**49**	**0**	**7**	**14**	**21**	**28**	**35**	**42**	**49**
n-Hexanol	882	t	0.7	0.4	0.1	0.1	0.2	0.2	0.2	0.5	0.3	0.2	0.1	0.2	0.2	0.4	0.6
2-Pentyl furan	973	0.2	1.4	1.4	0.5	8.1	1.1	0.5	0.3	0.8	1.7	1.9	1.1	0.9	0.9	0.7	1.0
β-Myrcene	975	1.1	0.6	0.9	0.4	3.1	2.6	0.3	0.6	0.4	0.5	0.9	1.0	2.8	0.6	0.5	0.9
Benzyl alcohol	1000	4.2	4.4	4.9	2.8	6.9	4.3	5.4	5.4	3.3	6.0	4.7	5.7	4.6	5.6	7.4	3.0
Benzene acetaldehyde	1002	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t
n-Octanol	1045	0.3	0.1	0.4	0.1	0.1	0.2	0.2	0.4	0.3	0.1	0.1	0.2	0.1	0.6	0.3	0.2
o-Guaiacol*	1058	2.6	3.8	2.9	1.9	2.5	4.4	3.2	2.8	5.8	2.2	2.9	3.3	3.1	2.8	4.6	4.1
2-Methyl decane	1058	0.2	t	0.1	0.1	t	t	0.1	0.1	t	0.3	t	t	t	0.1	t	0.5
Phenyl ethyl alcohol	1064	0.5	1.1	0.4	0.3	0.5	0.5	1.7	1.6	0.3	0.3	0.4	0.5	0.4	0.3	0.6	0.2
trans-Pinocarveol	1106	t	0.3	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.3	0.2	0.1	0.3	0.1	0.3
3-*cis*-Nonen-1-ol	1124	t	0.7	0.7	1.0	0.6	1.0	0.8	0.8	2.2	0.7	1.5	0.8	1.1	1.4	1.4	2.1
trans-Mentha-1(7),8-dien-2-ol	1159	0.2	0.4	0.1	0.1	0.1	0.1	t	t	0.2	0.1	0.3	0.1	0.1	0.1	0.1	0.3
2-*trans*, 4-*trans*-Nonadienal	1184	0.4	0.7	0.2	0.3	0.1	0.2	0.1	0.2	0.4	0.2	0.3	0.3	0.2	0.1	0.1	0.3
Carvacrol	1286	1.7	1.3	1.1	0.9	0.6	1.6	1.6	1.6	2.2	2.2	0.9	1.6	1.7	1.8	2.2	2.7
n-Tetradecanal	1596	2.0	0.8	1.2	1.4	1.3	1.8	2.1	1.6	2.2	2.7	0.8	1.0	1.2	2.4	2.7	3.6
cis-Methyl dehydrojasmonate	1640	0.1	0.4	0.2	0.1	t	0.1	0.1	t	0.1	t	0.2	1.1	t	t	0.4	0.5
n-Pentadecanal	1688	11.0	5.6	9.7	12.0	9.8	15.2	16.2	13.7	11.4	8.5	7.6	8.2	9.6	17.0	15.6	21.7
Myristic acid[a]	1723	0.1	2.6	0.6	0.6	0.6	0.9	1.0	1.0	0.6	1.9	0.5	1.0	0.8	0.7	0.6	1.1
Isopropyl decanoate*	1753	0.8	1.5	1.0	0.8	0.5	0.2	0.4	0.4	1.4	2.0	0.9	0.6	0.5	0.4	0.3	0.1
Pentadecanoic acid*[b]	1776	2.2	2.3	2.5	3.1	3.0	4.2	4.1	4.2	1.5	3.4	2.2	3.9	4.4	3.5	3.7	1.6
n-Hexadecanol	1821	0.3	5.8	0.3	0.2	0.2	0.1	0.2	0.2	0.2	2.4	0.3	0.1	0.2	0.2	0.3	0.2
n-Heptadecanal	1894	0.5	1.1	0.8	0.8	0.6	0.5	0.6	0.4	0.9	2.5	0.8	0.4	0.4	0.4	0.4	0.4
Palmitic acid[c]	1908	51.6	35.5	36.6	44.2	37.6	36.7	37.1	39.5	23.8	39.5	37.8	43.7	42.7	38.9	38.6	32.4
Heptadecanol allyl ether	1987	0.4	0.9	0.6	1.0	1.1	1.2	0.2	0.2	0.5	0.7	1.0	1.1	0.6	0.9	1.0	0.9
Margaric acid*[d]	2032	0.5	0.5	0.2	0.2	0.2	0.3	1.8	1.9	0.1	0.4	0.1	0.2	0.3	0.2	0.2	t
n-Octadecanol	2071	0.1	0.2	0.1	0.1	t	t	0.1	0.1	t	0.3	0.1	t	0.1	0.7	t	t
Phytol acetate	2047	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t
Linoleic acid[e]	2125	1.7	9.9	16.1	12.4	6.9	6.5	7.4	8.4	6.3	11.5	18.1	10.1	8.0	6.5	3.7	5.7
Stearic acid[f]	2149	0.6	2.6	0.5	2.4	0.8	1.8	1.0	0.9	0.8	2.8	1.5	1.4	1.7	1.0	0.5	0.6
n-Eicosanal	2200	t	0.5	t	0.1	0.1	0.2	0.2	0.2	0.1	0.2	0.1	0.1	0.1	0.2	0.1	0.1
n-Docosanal	2426	t	0.6	t	0.1	0.2	t	t	t	t	0.3	t	t	t	0.3	t	0.1
% Identification		83.3	85.5	83.8	88.0	85.1	85.7	86.1	86.2	66.4	93.3	86.1	87.7	85.8	87.6	86.1	85.2
Grouped components																	
Monoterpene hydrocarbons		1.1	0.6	0.9	0.4	3.1	2.6	0.3	0.6	0.4	0.5	0.9	1.0	2.8	0.6	0.5	0.9

		S. tuberosum HR								*S. tuberosum* HR / CRKN							
		Time (days)								Time (days)							
Component	**RI**	**0**	**7**	**14**	**21**	**28**	**35**	**42**	**49**	**0**	**7**	**14**	**21**	**28**	**35**	**42**	**49**
Oxygen-containing monoterpenes		1.9	1.9	1.3	1.1	0.7	1.8	1.7	1.6	2.5	2.4	1.6	1.9	1.8	2.1	2.4	3.3
C13 compounds		0.1	0.1	0.2	0.1	t	0.1	0.1	t	0.1	t	0.2	1.1	t	t	0.4	0.5
Fatty acids		56.7	53.3	56.5	62.8	49.0	50.2	52.3	55.8	33.1	59.5	60.1	60.3	57.9	50.8	47.2	41.4
Others		23.5	29.4	24.9	23.6	32.3	31.0	31.8	28.2	30.3	31.0	23.3	23.4	23.2	34.1	35.7	39.1

RI, Lab calculated retention index relative to C_8-C_{25} n-alkanes on the DB-1 column; t, Trace (<0.05%); *Identification based on mass spectra only, [a] Tetradecanoic acid, [b] Pentadecylic acid, [c] Hexadecanoic acid, [d] Heptadecanoic acid, [e] *cis*,*cis*,-9,12-Octadecadienoic acid, [f] Octadecanoic acid.

These results can be explained by the different responses to elicitors or to CRKN, or that, if produced, the compounds can undergo glycosylation, as observed in *L. officinale* HR (Nunes et al. 2009), rendering them non-volatile and thus not directly extractable by hydrodistillation.

Other factors, such as increase in nematode load due to prolonged period of cultures might result in an altered stress-induced volatile response. Nevertheless, it was not the goal of this study to stress the HR / CRKN co-cultures but, instead, to obtain an equilibrated *in vitro* co-culture system where variations in volatiles can be easily detected, e.g, after the external application of nematicidals.

To our knowledge, this is the first report on the effect of *M. chitwoodi* infection on *S. tuberosum* HR production of volatiles. In other studies, Desjardins et al. (1997) identified altered ratios of solavetivone versus total sesquiterpenes in *Globodera rostochiensis* potato resistant genotypes. Veech (1978) and Khoshkhoo et al. (1994) related the resistance of cotton to *M. incognita* with increased production of terpene aldehydes and according to Edens et al. (1995), the resistance may be linked to the host's ability to perceive nematode infection, as resistant *Glycine max* plants showed greater expression of genes encoding enzymes from the phenylpropanoid pathway (defense response enzymes) in response to RKN infection while susceptible plants did not.

In conclusion, *S. tuberosum* HR cultures and *S. tuberosum* HR / CRKN co-cultures were successfully established and their structure, growth and volatiles evaluated. The presence of the phytoparasite was not detrimental as both HR cultures and co-cultures showed similar growth and volatile profiles. These results suggest that the established *in vitro* co-cultures may be used to

simulate the CRKN *in vivo* infection mechanism, making them a suitable biotechnological tool to research the effect of RKN nematotoxic compounds, while determining their effect on, or the biotransformation capacity of the host plant.

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Chapter 7

Ruta graveolens and *Satureja montana* essential oils as effective nematotoxics on *Solanum tuberosum* hairy roots with *Meloidogyne chitwoodi* co-cultures

Jorge MS Faria, Inês Sena, Richard N Bennett, Manuel Mota, A Cristina Figueiredo (2015) *Ruta graveolens* and *Satureja montana* essential oils as effective nematotoxics on *Solanum tuberosum* hairy roots with *Meloidogyne chitwoodi* co-cultures (*in preparation*)

Abstract

The root-knot nematodes, *Meloidogyne* spp., are among the most damaging species to agricultural productivity, causing decreased plant growth, crop quality and yield, reducing resistance to stress and ultimately leading to crop loss. Batch testing of pesticides would highly benefit from a biotechnological screening system for nematotoxics with activity directed at the nematode in a host-parasite system. *Solanum tuberosum* hairy roots (StHR) and *S. tuberosum* hairy roots with *M. chitwoodi* co-cultures (StHR / CRKN) were comparatively evaluated, with and without the addition of the nematotoxic essential oils (EOs) of *Satureja montana* (winter savory) and *Ruta graveolens* (rue). The effect of the addition of EOs, at 0.5 µL/mL, four weeks following subculture, was followed weekly over 7 weeks by evaluating nematode population density in the co-cultures, and the growth and volatile profiles of both *in vitro* cultures types. StHR and StHR / CRKN co-culture growth, measured by the dissimilation method and by fresh and dry weight determination, was inhibited after each EO addition. Nematode population increased in control cultures while in EO-added cultures CRKN numbers were kept stable. In addition to each of the EO compound volatile main components, and *in vitro* cultures constitutive volatiles, new volatiles were detected, by gas chromatography and gas chromatography coupled to mass spectrometry, in both culture types. StHR with CRKN co-cultures showed to be suitable for preliminary assessment of nematotoxic EOs.

Keywords: biotransformation, Columbia root-knot nematode, *in vitro* co-cultures, rue, winter savory

1. Introduction

Root-knot nematodes (RKNs), *Meloidogyne* spp., have been recently ranked first in the Top 10 list of plant-parasitic nematodes with scientific and economic importance (Jones et al. 2013). Root-knot is one of the five most damaging potato (*Solanum tuberosum* L.) pests in modern agriculture. Commonly used nematicides are broad-spectrum synthetic chemicals which have been shown to be linked to environment pollution and undesirable influences on non-target organisms and human health (Chitwood 2003, Palomares-Rius et al. 2014). This led to the search for environmentally-friendly natural nematicides that are, at the same time, cost-effective.

For displaying multiple biological activities, essential oils (EOs) are desirable biopesticides (Batish et al. 2008), able to control not only the targeted pest but also opportunistic species and resistant strains. EOs are complex mixtures of volatiles, mainly products from the plant's secondary metabolism, comprising terpenes (mostly mono-, sesqui- and a few diterpenes) and phenolic compounds (such as phenylpropanoids) although other groups of compounds can also occur in relevant amounts.

EO nematotoxic activity evaluation is commonly performed by direct contact bioassays and/or greenhouse and field assays (Oka et al. 2000, Pérez et al. 2003, Ibrahim et al. 2006, Meyer et al. 2008, Andrés et al. 2012). Despite the importance of these tests, the main problems associated with direct contact assays are the fact that they neither assess the phytotoxicity nor the biotransformation capacity of the host. On the other hand, greenhouse and field assays are very laborious and often environmentally dependent.

As a laboratory model, *in vitro* co-cultures, that is, the growth of more than one organism or cell type in a combined culture, provide a controlled environment and allow the analysis of metabolomic relationship between plant and nematode at various levels (Faria et al. 2014, 2015a). Particularly important is to follow, simultaneously, if the nematotoxic maintains its activity against the pathogen, while not showing phytotoxicity to the host.

Previous work has shown the anti-hatching potential of *Ruta graveolens* L. (rue) and *Satureja montana* L. (winter savory) EOs, and EO hydrocarbon and oxygen-containing molecules fractions, against the Columbia root-knot nematode (CRKN, *Meloidogyne chitwoodi*) hatching, in direct

contact bioassays (Faria et al. 2015b). These EOs have shown diverse behaviors when added to *Pinus pinaster* shoot cultures and *P. pinaster* with *Bursaphelenchus xylophilus in vitro* co-cultures (Faria et al. 2015c).

Using previously established *S. tuberosum* hairy roots (StHR) and *S. tuberosum* hairy roots with *M. chitwoodi* co-cultures (StHR / CRKN) (Faria et al. 2014), the present work aimed at assessing *R. graveolens* and *S. montana* EOs nematotoxicity and phytotoxicity by evaluating a) nematode population density in the co-cultures; b) growth; and c) volatile profiles of both *in vitro* culture types.

2. Material and methods

2.1. *Solanum tuberosum* HR and *S. tuberosum* HR with CRKN co-cultures

S. tuberosum HR (StHR) and *S. tuberosum* HR with CRKN co-cultures (StHR / CRKN) were previously established and routinely maintained in Schenk and Hildebrandt (SH) medium (Schenk and Hildebrandt 1972) with 30 g/L sucrose, in darkness at 24ºC on orbital shakers at 80 rpm, as detailed in Faria et al. (2014).

2.2. *Ruta graveolens* and *Satureja montana* essential oils bioactivity assays

Erlenmeyer flasks with 100 mL SH medium were aseptically inoculated with 1 g (fresh weight) of StHR or StHR / CRKN co-cultures and maintained as described above. Four weeks following subculture, a 1:1 solution (v/v) of *S. montana* or *R. graveolens* EO in methanol (Panreac Química S.A.U., Barcelona, Spain) was added to each culture flask, to obtain a final concentration of 0.5 µL EO / mL of culture medium. Methanol was chosen due to its high polarity and high solvent capacity. Two types of control cultures, StHR and StHR / CRKN co-cultures without EO, were maintained simultaneously, and were processed as the ones to which EOs were added. EO evaporation control experiments were performed by adding the same amount of EO to flasks containing only basal culture medium, and keeping them in the same conditions as the culture flasks throughout the experiment. Two independent experiments were separately run, for each EO,

and two replicates of each flask were used in each experiment. The data shown were calculated as mean values of all experiments. All statistical analyses were performed using Microsoft Excel 2013. *R. graveolens* and *S. montana* EOs addition phytotoxic effect on StHR and phyto- and nematotoxic effects on StHR / CRKN co-cultures were followed by measuring *in vitro* cultures growth, nematode population density and volatiles production, weekly during the 7 weeks.

2.2.1. StHR and StHR / CRKN growth

Growth was assessed in StHR and StHR / CRKN, with and without EO, by the dissimilation method, and by fresh and dry weight determination, as detailed in Faria et al. (2014).

2.2.2. CRKN population in co-culture medium

CRKN population density (J2 and males) in the liquid culture medium was evaluated by sampling 100 µL aliquots of each culture flask at each time-point. Three replicates of each flask were used for counts. Number of dead and live nematodes was recorded using an inverted microscope [Diaphot, Nikon, Japan (40x)].

2.2.3. *Ruta graveolens* and *Satureja montana* essential oils and volatiles from StHR and StHR / CRKN

Essential oils (EOs) from winter savory and rue were isolated from the dried aerial parts sold in local herbal shops and the chemical profiling was performed as detailed in Faria et al. (2013). Volatiles from StHR and StHR / CRKN were isolated and identified as detailed in Faria et al. (2014).

3. Results and discussion

3.1. StHR and StHR / CRKN growth and volatile profiles

S. tuberosum control hairy roots (StHR) showed the typical hairy root phenotype, highly branched roots with numerous root hairs (Fig 1a). Likewise, *S. tuberosum* hairy roots with *M. chitwoodi* control co-cultures (StHR / CRKN) were similar to StHR in morphology and growth (Figs. 1b, 2 and 3). CRKN in the culture medium showed the characteristic two-peak population curve (Fig. 2), as reported by Faria et al. (2014), with the first peak due to 1st generation hatching and the second peak to CRKN 2nd generation.

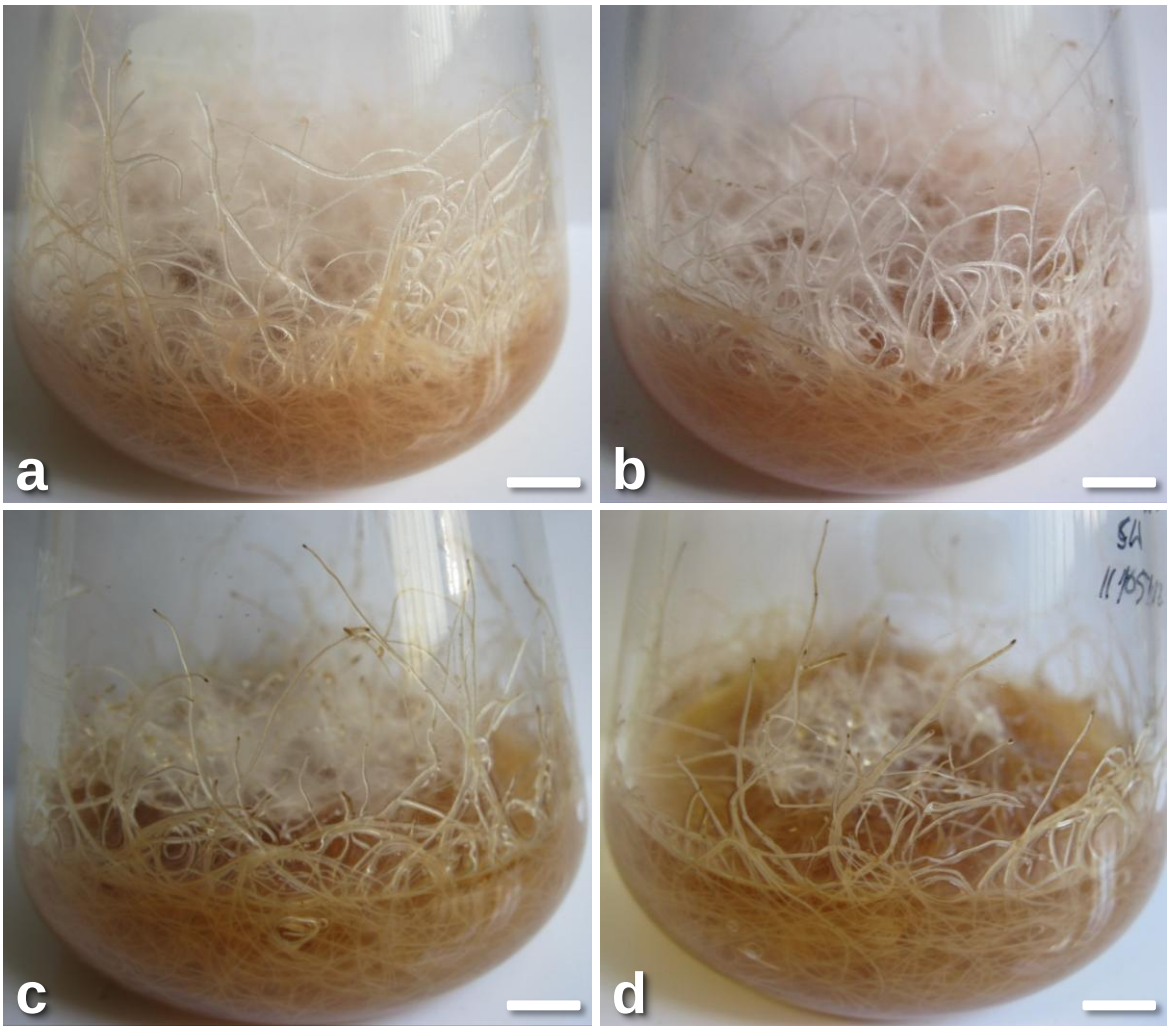

Figure 1. Aspect of a) *Solanum tuberosum* hairy roots (StHR), b) *S. tuberosum* HR with *Meloidogyne chitwoodi* co-cultures (StHR / CRKN) and StHR to which c) *Satureja montana* and d) *Ruta graveolens* EOs were added at 0.5 µL/mL, with 5 weeks in culture (1 week after EO addition). StHR / CRKN co-cultures grown in EOs-added culture media showed similar aspect to StHR. Scale bar 1 cm.

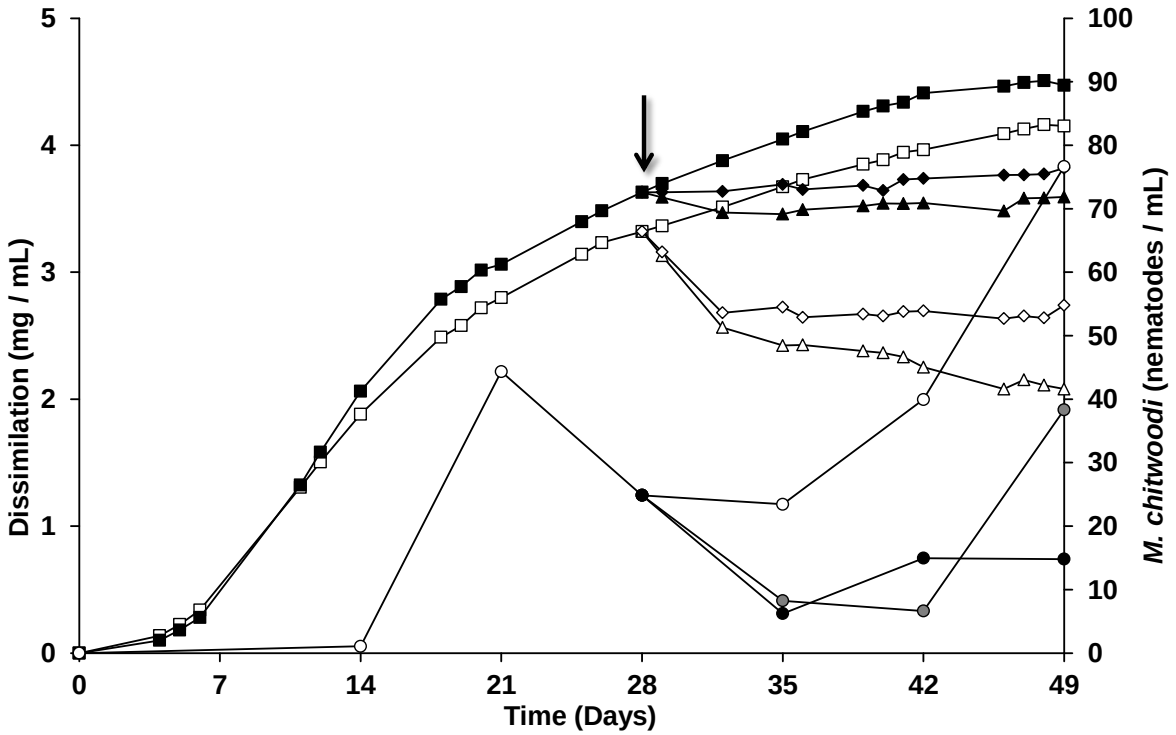

Figure 2. Dissimilation growth curves of *Solanum tuberosum* hairy roots (StHR, □) and *Solanum tuberosum* hairy roots with *Meloidogyne chitwoodi* co-cultures (StHR / CRKN, ■), without- (StHR and StHR / CRKN) and with the addition of *S. montana* (△ and ▲, respectively) or *R. graveolens* essential oils (◊ and ♦, respectively), at 0.5 µL/mL of in culture medium. Number of nematodes in StHR / CRKN culture medium without (○) and with the addition of *Satureja montana* (◍) or *Ruta graveolens* essential oils (●), at 0.5 µL/mL of in culture medium. Arrow: time point of EO addition to culture medium.

StHR and StHR / CRKN constitutive volatiles did not differ substantially, showing the characteristic volatile patterns reported in Faria et al. (2014). In total, 31 compounds were identified, of which palmitic acid (24–52%), *n*-pentadecanal (6-22%), linoleic acid (2-18%), 2-pentyl furan (0.2-8%), benzyl alcohol (3-7%), *o*-guaiacol (2-6%) and *n*-hexadecanol (0.1-6%) were the dominant ones (>5%).

3.2. *Satureja montana* essential oil nematotoxicity and phytotoxicity

Addition of winter savory EO, at 0.5 µL/mL, to StHR and StHR / CRKN co-cultures, four weeks following subculture (28 days, Fig. 2), revealed both nematotoxic and phytotoxic effects.

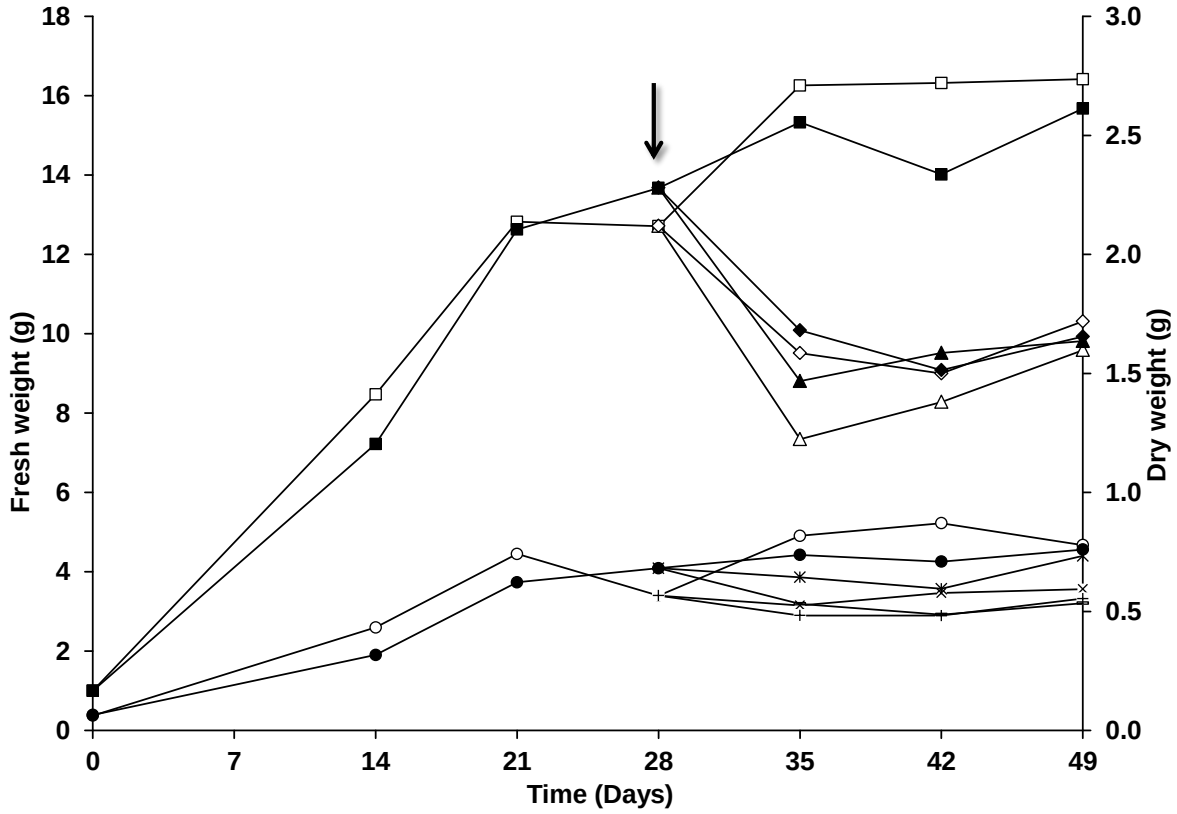

Figure 3. Fresh and dry weight growth curves of *Solanum tuberosum* hairy roots (StHR, □ and ○, respectively) and *S. tuberosum* hairy roots with *Meloidogyne chitwoodi* co-cultures (StHR / CRKN, ■ and ●, respectively), without- (StHR and StHR / CRKN) and with the addition of *Satureja montana* (fresh weight: △ and ▲, respectively; dry weight: x and ж, respectively) and *Ruta graveolens* essential oils (fresh weight: ◊ and ♦, respectively; dry weight: + and -, respectively), at 0.5 µL/mL. Arrow: time point of EO addition to culture medium.

CRKN population in co-cultures decreased in the 1st week after EO addition (35 days, Fig. 2) but EO diminished its effect at the 2nd week and population numbers increased to the end of culture time. Within the 1st week after EO addition, the culture medium became slightly brownish, due to phenolic exudates and oxidation, and the root tips dark brown, when compared to those from control StHR and control StHR / CRKN co-cultures (Fig. 1 c). One week after addition of *S. montana* EO, a sharp decrease in dissimilation and fresh and dry weight in both *in vitro* culture types was visible, comparatively to the corresponding control cultures (Figs. 2 and 3). Throughout culture time, growth was not recovered.

In the volatiles extracted from StHR and StHR / CRKN *S. montana*-EO added cultures, in addition to winter savory EO compounds, and *in vitro* cultures constitutive volatiles, new volatiles

were detected. From the winter savory EO, previously fully chemically characterized (Faria et al. 2013), main components, carvacrol (64%) and γ-terpinene (18%), only carvacrol was detected in high percentages, up to 84%, in both *in vitro* cultures volatiles, one week after EO addition. Although with a tendency to decrease, carvacrol percentage remained high even 3 weeks after EO addition (StHR 75%, StHR / CRKN 78%) (Table 1).

Table 1. Percentage composition (≥1%) of *Solanum tuberosum* hairy roots (StHR) and *S. tuberosum* HR with *Meloidogyne chitwoodi* co-cultures (StHR / CRKN) volatiles, 1, 2 and 3 weeks after the addition of *Satureja montana* EO to culture medium, at 0.5 µL/mL.

Components (≥1%)	RI	StHR			StHR / CRKN		
		Weeks after EO addition			Weeks after EO addition		
		1	2	3	1	2	3
Carvacrol	1286	84.3	85.0	75.1	84.1	83.8	78.3
β-Caryophyllene	1414	3.2	1.5	2.6	2.6	1.5	1.8
β-Bisabolene	1500	2.6	1.2	1.9	2.3	1.2	1.3
n-Pentadecanal	1688	1.6	1.9	3.2	1.5	1.8	2.5
Palmitic acid	1908	1.5	1.7	3.1	2.0	2.0	2.6
Linoleic acid	2101	1.1	2.6	5.1	1.3	3.1	5.5

RI: Lab calculated retention index relative to C_{12}-C_{22} *n*-alkanes on the DB-1 column. [Standard deviation <5%].

γ-Terpinene was only detected in trace amounts, which can be partly attributed to volatilization, as this was also detected in control experiments of EO evaporation and decomposition. However, substrate hydroxylation, glycosylation, oxidoreduction, hydrogenation, hydrolysis, methylation, acetylation, isomerization and esterification are some biotransformation reactions commonly found on plant *in vitro* cultures (Figueiredo et al. 1996, Giri et al. 2001, Faria et al. 2009, Nunes et al. 2009, Banerjee et al. 2012). For this reason, the biotransformation of γ-terpinene into non-volatile glycosylated compounds, can also partly explain the difference between γ-terpinene percentage in winter savory EO and winter savory EO added cultures. Seven new compounds were detected (all of which <0.3%) in the volatiles extracted from StHR and StHR / CRKN *S. montana*-EO added cultures, carvone, thymoquinone, tridecanal and 4, as yet unidentified compounds. Oxidation, isomerization and/or reduction (Fig. 4) are biotransformation reactions that can explain the conversion of *S. montana* EO dominant compounds, carvacrol and γ-terpinene, into

the new compounds, carvone and thymoquinone, present in the volatiles in trace amounts.

Winter savory EOs, commonly dominated by carvacrol, γ-terpinene and/or *p*-cymene, are known to possess high phytotoxic bioactivities. Angelini et al. (2003) reported complete *in vitro* germination inhibition of 3 weeds and 3 crops seeds subjected to winter savory EO at 0.5 mg/mL. In an attempt to pinpoint the main phytotoxic compound, further testing was performed with carvacrol, the main component (57%), and again similar inhibition activities were obtained.

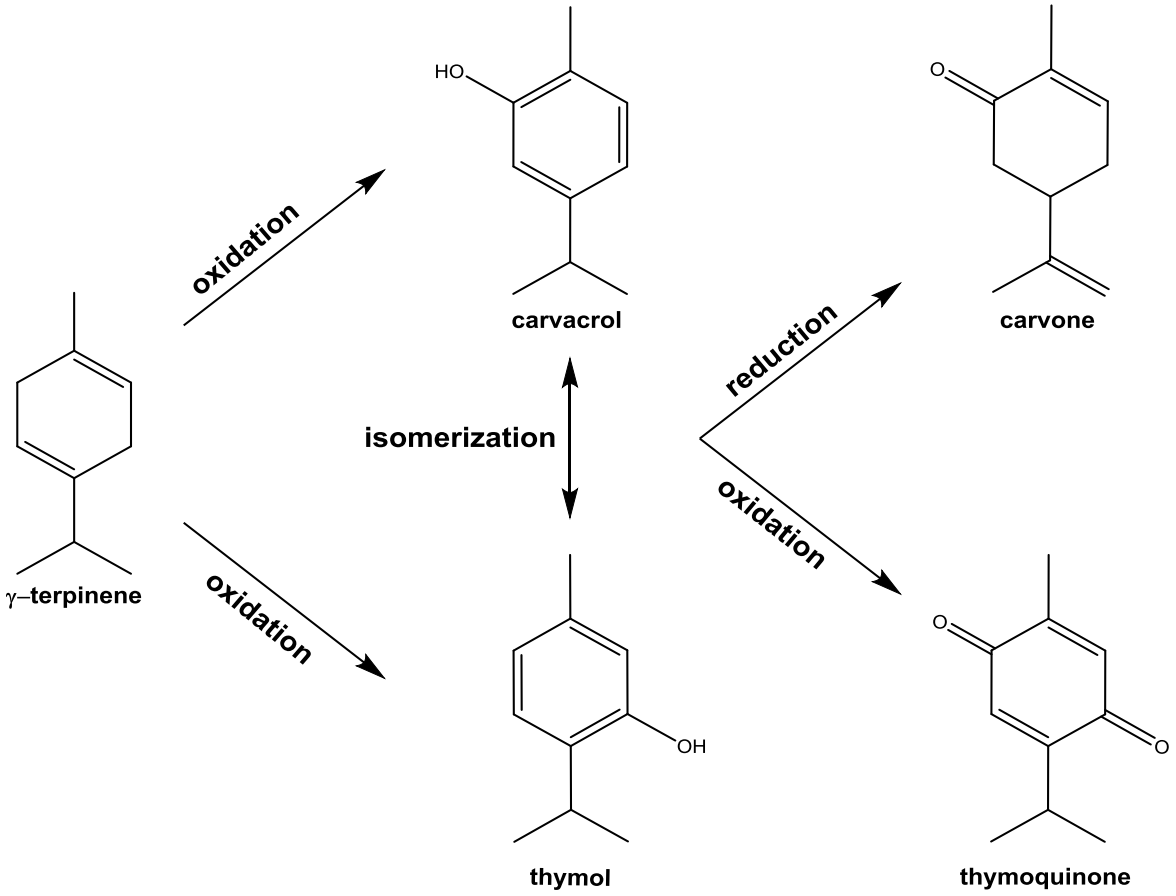

Figure 4. Putative biotransformation reactions of *Satureja montana* EO dominant compounds, carvacrol and γ-terpinene, by *Solanum tuberosum* hairy roots or *S. tuberosum* HR with *Meloidogyne chitwoodi* co-cultures.

Testing phytotoxic synergic activities of monoterpenes, carvacrol was assayed against *Lactuca sativa* (Vokou et al. 2003). Alone or in synergic combination with γ-terpinene or *p*-cymene, high activities were obtained in the inhibition of germination and seedling elongation. Carvacrol-rich EOs showed high sprouting inhibition on stored *S. tuberosum* tubers (Vokou et al. 1993, Baydar and Karadoğan 2003). Kordali et al. (2008) analyzed the phytotoxic potential of *Origanum acutidens* EO

and its three main components, carvacrol, thymol and *p*-cymene, on seeds of *Amaranthus retroflexus*, *Chenopodium album* and *Rumex crispus* weeds. This study showed that EO, carvacrol or thymol completely inhibited *in vitro* seed germination and seedling growth, and their activity was higher than that of commercial herbicide, 2,4-D isooctyl ester. Also Azirak and Karaman (2008) found high phytotoxic activities for carvacrol and thymol-rich EOs and respective synthetic chemicals. These EOs and compounds inhibited *in vitro* germination of six weeds found in field and horticultural crops. When added to *Pinus pinaster* shoot cultures and *P. pinaster* with *Bursaphelenchus xylophilus in vitro* co-cultures, winter savory EO also demonstrated a high phytotoxic activity, inducing shoot chlorosis and wilting while maintaining nematotoxic activity (Faria et al. 2015c).

3.3. *Ruta graveolens* essential oil nematotoxicity and phytotoxicity

Growth of StHR and StHR / CRKN co-cultures was completely inhibited after rue EO addition, the root tips turning dark brown (Fig. 1d). Similarly to what happened after the addition of winter savory EO, rue EO inhibited StHR / CRKN nematode population. One week after rue EO addition, CRKN population decreased <10 CRKN / mL culture medium and this inhibitory effect was maintained throughout (Fig. 2).

Rue EO compounds, *in vitro* cultures constitutive compounds and also new ones, were found in rue EO-added *in vitro* cultures isolated volatiles. Previously identified (Faria et al. 2013, 2015c) main rue EO compounds, 2-undecanone (91%), and 8-phenyl-2-octanone (7%), were detected on StHR and StHR / CRKN co-cultures EO-added volatiles. The ketone, 8-phenyl-2-octanone dominated StHR (54 to 67%) and StHR / CRKN co-cultures (52 to 68%) volatiles. Rue EO main compound, 2-undecanone, showed lower relative amounts than 8-phenyl-2-octanone, and decreased throughout experimental time in StHR (16 to 5%) and StHR / CRKN (17 to 5%) co-cultures (Table 2).

Twenty nine new compounds were detected after rue EO addition to StHR cultures and StHR / CRKN co-cultures: *n*-decanol, *n*-dodecanol, elemol, β-eudesmol, 2,4-heptadienal, *n*-hexadecanal, 5-methylene-2,3,4,4-tetramethylcyclopent-2-enone, 1-octen-3-ol, 6-phenyl-*n*-hexanol,

6-phenyl-2-hexanone, 2-*trans*-4-*cis*-decadienal, *trans*-nerolidol, *trans*-2-nonen-1-al, *n*-tetradecane, *n*-tetradecanol, tetradecanol allyl ether, 1-tetradecene, *n*-tridecanal, *n*-tridecane, *n*-tridecanol, *n*-undecanol and 8 unidentified compounds. Compounds present >1% are listed in Table 2.

Table 2. Percentage composition (≥1%) of *Solanum tuberosum* hairy roots (StHR) and *S. tuberosum* HR with *Meloidogyne chitwoodi* co-cultures (StHR / CRKN) volatiles, 1, 2 and 3 weeks after the addition of *Ruta graveolens* EO to culture medium, at 0.5 µL/mL.

Components (≥1%)	RI	StHR			StHR / CRKN		
		Weeks after EO addition			Weeks after EO addition		
		1	2	3	1	2	3
2-Undecanone	1275	15.6	5.1	4.7	16.5	5.9	4.6
2-Undecanol*	1288	6.1	3.7	1.3	5.7	1.9	1.5
2-Dodecanone	1389	2.8	0.5	0.3	3.6	0.6	0.3
UI F Rg[a]	1469	0.9	0.3	0.1	1.0	0.4	t
2-Tridecanone*	1479	5.5	2.6	0.6	5.8	3.8	0.5
n-Tridecanol	1565	0.3	0.9	1.2	0.3	0.9	1.2
n-Tetradecanal	1596	0.9	1.3	1.6	1.0	1.5	1.7
8-Phenyl-2-octanone	1626	54.3	66.1	66.9	52.4	65.7	68.1
trans-Amyl cinnamic alcohol*	1640	2.3	4.0	4.3	2.3	3.0	4.8
n-Tetradecanol	1659	1.1	3.1	4.3	1.0	3.0	4.7
n-Pentadecanal	1688	2.2	3.0	3.5	2.7	3.4	4.0
UI D Rg[a]	1775	0.6	0.7	1.0	0.5	0.6	0.9
UI E Rg[a]	1784	0.8	0.8	0.8	0.8	1.0	0.9
Palmitic acid	1908	0.8	1.1	2.0	0.5	0.9	1.3

RI: Lab calculated retention index relative to C_{12}-C_{20} *n*-alkanes on the DB-1 column. t: Trace (<0.05%). *Identification based on mass spectra only. [a] Unidentified compounds detected in trace amounts in *R. graveolens* essential oil. [Standard deviation <5%].

The phytotoxic properties of rue EO and some EO compounds were reported by de Feo et al. (2002) on *Raphanus sativus* seeds. *In vitro* germination and seedling radicle growth were inhibited by rue EO and some minor constituents, but not by the major components, 2-undecanone or 2-nonanone, when tested separately. The addition of rue EO to *Pinus pinaster* with *Bursaphelenchus xylophilus in vitro* co-cultures showed no visible phytotoxic effects in shoot aspect and volatiles composition (Faria et al. 2015c). Methyl ketones, particularly 2-undecanone, are currently used as insect and animal repellents, in households, paths, patios, solid waste

containers and on ornamental plants (EPPA 1995). Their activity against various *Solanum* spp. pests has been tested by Antonius et al. (2014). Isolated from crude extracts of resistant wild tomato plants, *Lycopersicon hirsutum*, it has shown to be promising as herbicidal for weed control (Bradow and Connick 1988).

Noma and Asakawa (1998) analyzed the biotransformation capacity of the alga *Euglena gracilis* Z strain by feeding a series of methyl nonyl ketones. The authors concluded that all compounds were reduced to the corresponding alcohols with a certain hierarchy of preference that was related do the length of the side chain. The longer side chain of aliphatic methyl ketones increased the reactivity for the reduction of the carbonyl group. In the present study, EO methyl nonyl ketones were also reduced to their corresponding alcohols, having been detected in the new induced compounds. Reduction reactions may constitute a detoxification response to the introduction of bioactive ketones.

4. Conclusion

Using *Solanum tuberosum* hairy roots with *Meloidogyne chitwoodi* co-cultures, the effect of adding nematotoxic winter savory and rue EOs was evaluated in a host-parasite system. Both EOs revealed phytotoxicity towards the StHR and StHR / CRKN co-cultures. In spite of this, rue EO was able to control parasite growth for a longer period, even though some major EO compounds may have been biotransformed. *In vitro* co-cultures used as biotechnological models can contribute to a more expeditious screening procedure and establishment of the effectiveness of nematotoxic EOs, by allowing a preview of how the plant host reacts to nematotoxics. Nevertheless, it must be noted that the observed nematoxicity in a co-culture system cannot be inputted exclusively to the EO, as the host, upon which the nematode feeds, can also biotransform the EO in such a way that it changes its composition and thus the EO bioactivity. Moreover, the pronounced phytotoxicity of these EOs on potato HR alerts to their cautionary use as nematicidals. This knowledge may help in designing further assays on *in vivo* root-knot diseased plants to determine its activity under field conditions.

5. Acknowledgments

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Chapter 8

Final considerations

1. Summary conclusions

Research on plant parasitic nematode (PPN) antagonist essential oils (EOs) is a fairly recent field in science. It arose from the need to develop environmental- and human health-friendly pesticides capable of reducing PPN impact on agriculture and forestry, which can sometimes be devastating. As discussed in **Chapter 1** commonly used nematicides are mostly broad-spectrum synthetic chemicals with a destructive influence on the surrounding ecosystem. They are lethal to many beneficial species and accumulate inside animals and plants, ultimately affecting human health.

EOs from many plant species have been tested against PPN, as potential environmentally-safe natural nematotoxics. As an example, plants tested against the pinewood nematode (PWN), *Bursaphelenchus xylophilus*, are distributed amongst 148 families (Barbosa et al. 2012). Nevertheless, the majority of studies employ direct contact bioassays that, although fundamental, focus solely on the parasite and do not take into account toxicity to the plant host.

The present work proposed the establishment and testing of plant with nematode co-cultures, as laboratory models to function as intermediate systems between direct contact bioassays and greenhouse or field assays, which give a notion of the host-parasite reaction to nematotoxic EOs addition. To accomplish this goal, and by using a) two types of nematodes that adversely affect the agro-food economy, *B. xylophilus*, responsible for pine wilt disease (PWD), by affecting the aerial parts of plants, and *Meloidogyne chitwoodi*, causative agent of root galls that infests the potato root system, and b) *in vitro* shoot cultures of *Pinus pinaster* and *Solanum tuberosum* hairy roots (HR), three main steps, detailed below, were followed, in order to:

1.1. Evaluate the nematotoxic potential of essential oils through direct contact assays

1.2. Establish *in vitro P. pinaster* with *B. xylophilus* co-cultures and also *S. tuberosum* HR with *M. chitwoodi* co-cultures

1.3. Determine the effect of the selected putative nematotoxic phytochemicals on the host with parasite co-cultures

1.1. Essential oil activity on plant parasitic nematodes

The first step was to identify highly nematotoxic EOs. In **Chapter 2** and **Chapter 5** research on potential effective nematotoxics through direct contact bioassays was reported. The main findings are highlighted and detailed below.

i. Of 84 EOs evaluated against PWN motility, *Ruta graveolens* (rue), *Satureja montana* (winter savory) and *Thymbra capitata* displayed the highest toxicity (**Chapter 2**).

ii. Of the 56 EOs evaluated against hatching of *Meloidogyne chitwoodi*, the Columbia root-knot nematode (CRKN), *Dysphania ambrosioides*, *Filipendula ulmaria*, *Ruta graveolens*, *Satureja montana* and *Thymbra capitata* displayed the highest toxicity (**Chapter 5**).

iii. EO oxygen-containing molecules fractions (OCM) were, generally, more effective.

iv. Hydrocarbon molecules fraction (HM) played an important role in PPN toxicity, contributing either positively or negatively with OCM.

As a first step to reach an effective PPN antagonist EO, direct contact bioassays are an exploratory method as its results do not encompass the plant's response to the nematotoxic phytochemicals applied. *R. graveolens*, *S. montana* and *Thymbra capitata* EOs were highly toxic against PWN motility, with $LC_{100/24h}$ <0.4 µL/mL. Also 9 decoction waters showed strong nematotoxic activities. The establishment of successful nematotoxic pesticides against this parasite may resort to formulations of EO / decoction water, which is a way to enhance EO activity while using hydrodistillation byproducts, reducing EO extraction costs. As hatching antagonists of CRKN, the previous EOs and those of *Dysphania ambrosioides* and *Filipendula ulmaria* were very successful with $EC_{50/72h}$ <0.15 µL/mL.

In the present work, EO activities on both PPN were not compared to commercial synthetic nematicides assayed in the same conditions, nevertheless, the results obtained can be placed into context when compared to the results obtained by Kong et al. (2006) for EOs and the commonly used trunk-injection nematicides fenitrothion, levamisol hydrochloride and morantel tartrate against the PWN, using direct contact bioassays. These authors obtained $EC_{50/24h}$ >10 mg/mL, a concentration well above those of the most successful EOs tested ($EC_{50/24h}$ <0.9 mg/mL).

Analysis and testing of HM and OCM fractions attempted to uncover interactions between EO compound groups within the whole EO, aiming at improving nematotoxic activity. The higher activity of the OCM when compared to the HM fraction was expected, as oxygen-rich compounds appear to be more active against PPN (Chitwood 2002, Ntalli and Menkissoglu-Spiroudi 2011, Andrés et al. 2012, Ntalli and Caboni 2012). Many of the dominant compounds in the most successful nematotoxic EOs in the present work were terpenic compounds with alcohol or phenol functional groups. These are known to induce cytotoxicity, damage to the cellular and organelle membranes, act as pro-oxidants on proteins and DNA, and produce reactive oxygen species (ROS) (Ntalli and Caboni 2012). Toxicity is exerted on PPN most likely through contact and not ingestion since these parasites show a very specific feeding mechanism, using a piercing stylet. The first barrier to nematotoxics in nematodes is, thus, the cuticle, the outer-most layer that provides protection against biotic and abiotic constraints. It is a highly structured extra-cellular matrix, composed predominantly of cross-linked collagens, additional insoluble proteins termed cuticlins, associated glycoproteins and lipids (Page and Johnstone 2007). Terpene compounds are known to be membrane penetration enhancers in the delivery of drugs through human skin. Williams and Barry (2012) found that terpene hydrocarbons are less potent enhancers than alcohol or ketone containing terpenes; the greatest enhancement activity was shown by the oxide terpenes and terpenoids. This permeability enhancement may lead to alterations in the intracellular ATP pool, membrane potential, pH gradient across the cytoplasmic membrane, and potassium gradient, which ultimately leads to impairment of essential processes in the organism and finally death. Some of these effects were detected by Ultee et al (1999) for carvacrol on the food-borne pathogen *Bacillus cereus*.

The main pioneering outcomes of this initial screening were that a) many EOs were tested for the first time against the PWN, b) a first report on EO direct contact assays against CRKN hatching was performed, c) fractions obtained from EOs were also tested for the first time against PPN, yielding promising results and d) a set of highly nematotoxic EOs has been reported.

1.2. Co-cultures as laboratory models

A second milestone was to establish biotechnological laboratory models that could supply a controlled environment likely to simulate the host with parasite condition. **Chapter 3** and **Chapter 6** addressed the establishment and analysis of *in vitro Pinus pinaster* shoots with PWN and *Solanum tuberosum* hairy roots (HR) with CRKN, respectively, which try to replicate PPN infection, for the evaluation of nematotoxics. The leading outcomes are discussed below.

i. *In vitro P. pinaster* shoots with PWN co-cultures (**Chapter 3**) were established and characterized, revealing shoot structure and volatiles pattern comparable to that of one-year old plantlets.

ii. *S. tuberosum* HR with CRKN co-cultures (**Chapter 6**) root gall structure and volatiles were characterized throughout culture time ascertaining many similarities with *in vivo* infection.

iii. The co-cultures established resembled the *in vivo* counterparts in a way that could be used to test for efficient putative nematotoxic EOs.

As a pioneering laboratory methodology, plant biotechnology can provide co-cultures as tools to effectively screen natural products. PPN feeding habits determine primarily the type of plant tissue required for their culture so co-cultures were established accordingly. The selected *in vitro* cultures tried to mimic, as closely as possible, the *in vivo* disease biology. The PWN is introduced in nature into healthy pines through young branches, in newly formed shoot tissue, where the adult vector beetles feed (“maturation feeding”). This is a very important step in PWN infection that ultimately leads to shoot wilting, with chlorosis and drooping. *In vitro P. pinaster* with PWN co-culture tried to emulate this infection step; *in vitro* shoots presented an immature young structure grown in a water saturated environment and revealed capable of supporting PWN feeding. PWNs grown in this co-culture successfully reproduced and presented morphometric parameters very close to the ones reported by Penas et al. (2008) and Fonseca et al. (2008), for *P. pinaster* infections in natural conditions.

The root-knot nematode CRKN is a soil dwelling obligate PPN that infects the root system of susceptible species and installs in the differentiation zone, reproducing and developing solely in the roots. These parasites are able to self-sustain inside root tissue without killing the host and persist

through various cultures. The HR model was the most advantageous biotechnological system to analyze these parasites since it provided a stable *in vitro* culture root system with primary growth characteristics. Potato HR with CRKN co-cultures were successfully established and maintained over time with successive subcultures. Potato HR growth characteristics and culture conditions allowed homeostasis between CRKN infection and root development, creating a true co-culture system able to sustain the nematode indefinitely. Root gall structure closely resembled the disease development described for this species in field infections.

The establishment and analysis of plant with PPN co-cultures have provided some innovative outcomes and may be regarded as an important step in phytopathology research. Besides being the first report on a co-culture system with the PWN, *in vitro P. pinaster* with PWN co-cultures present an unlimited source for microorganism-free PWNs for nematology research. Also CRKN was co-cultured for the first time with potato hairy roots, which may lead to novel approaches to deal with this crop parasite. Additionally, both co-cultures were analyzed for variations on volatiles which had not been performed before.

1.3. *Satureja montana* and *Ruta graveolens* EOs activity on co-cultures

The EOs reported as nematotoxic in **Chapter 2** and **Chapter 5**, were then tested with the established co-cultures. **Chapter 4** and **Chapter 7** report on the effects of the addition of the nematotoxic EOs of winter savory and rue to the host with parasite co-cultures. The main highlights of this work were the following, as detailed below.

i. Winter savory EO was phytotoxic to *in vitro Pinus pinaster* shoots and both phytotoxic and nematotoxic to *P. pinaster* with PWN co-culture, while rue EO revealed less phytotoxicity and maintained nematotoxic activity (**Chapter 4**).

ii. Winter savory and rue EOs revealed phytotoxic and nematotoxic activity to *S. tuberosum* HR and *S. tuberosum* HR with CRKN co-culture (**Chapter 7**).

iii. Testing in co-cultures contributes to the identification of effective anti-PPN natural products by allowing an indication of how the plant host reacts to nematotoxics.

Two milestone steps were important in reaching a laboratory model for effective nematotoxic

EO testing. On one hand, screening phytochemicals, using direct contact bioassays, allowed the establishment of a dose-response relation, indicating a concentration range to be used in the phytotoxic and nematotoxic assays in co-cultures. On the other hand, the establishment of an adequate biotechnological model system (*in vitro* co-cultures) able to withstand nematotoxic EOs addition. From these co-cultures information could be gathered on the phytotoxic EO effects that could impair EO activity, like phytotoxicity and the biotransformation of nematotoxic volatile compounds.

The EOs of winter savory and rue were added to the culture medium, at 0.5µL/mL, to the established co-cultures. Both EOs maintained their nematotoxic activity yet phytotoxic activity was also revealed. Winter savory EO showed to be toxic to *in vitro* co-cultures, inhibiting HR growth and promoting chlorosis and drooping to *in vitro* pine shoots. Rue EO was toxic against potato HRs with CRKN co-cultures but revealed no observable adverse activity to *in vitro* pine with PWN co-culture shoots. Volatile analysis revealed the fate of EO nematotoxic compounds. Although biotransformation reactions were observed, EO main compounds were retained in pine tissue maintaining nematotoxic activity and appeared to have low impact on constitutive *in vitro* pine volatiles. Although using different *in vitro* culture approaches, liquid or solid culture medium, rue EO revealed to affect these very sensitive plant tissues in different ways, at the concentration assayed. In both EOs assayed the volatile composition after addition varied considerably; this must be taken into account when choosing the nematotoxic EO, given that lower weight compounds appear to be volatilized more rapidly.

The assay of effective nematotoxic EOs on plant with nematode co-cultures is an approach to PPN disease research not assayed previously, as such some pioneering results were obtained. The analysis of volatiles induced in *in vitro* culture with the addition of complex mixtures of compounds was determined for the first time, tackling the difficulties of analysing multiple volatile patterns simultaneously, in the same sample. Also, the nematotoxic strength of EOs was determined for the first time on *in vitro* plant with nematode conditions, which is the first step towards standardizing testing conditions for other EOs.

Overall, *in vitro* co-cultures are a step forward in establishing faithful host-parasite laboratory models, retaining most of the fundamental biological processes that characterize the host with

parasite environment and serving as an intermediate measure for screening effective nematotoxic EOs. The advantages of this process are remarkable. As a lab model it allows molecular studies, analysis of biosynthetic pathways, precursor feeding, anatomy, morphology and ultrastructure determinations, among others; as an industrial tool it allows a rapid, high-throughput screening method obviating excessive greenhouse or field assays and a follow-up of the nematotoxic phytochemical fate in the host with parasite system while identifying its activity.

2. Future directions

Being mainly part of a more encompassing study, the present work intended to contribute to underpinning *in vitro* co-cultures as laboratory model tools for biopesticide research. As such, many aspects can continue to be improved and optimized in every section of the work.

2.1. Direct contact bioassays

Although a great number of EOs were evaluated in the present work, screening should continue using EOs from other species and also further analyzing the EOs tested. Many of the latter may possess chemical variability, chemotypes, as was detected in *Thymus caespititius* [see also Trindade et al. (2008) and Mendes et al. (2013)], and will undoubtedly yield different nematotoxic activities.

Testing should not be restricted to EOs but could also include other classes of phytochemicals. Potential nematotoxics can be identified from many other sources such as alkaloids, cyanogenic glycosides, diterpenoids, glucosinolates, isothiocyanates, lipids, polyacetylenes, polythienyls, quassinoids, simple and complex phenolics, steroids and triterpenoids [for example as detailed in the works of Zhao (1999), Caboni et al. (2013), Wen et al. (2013) and reviewed by Chitwood (2002)]. Also, highly active nematotoxics from fungi, algae or bacteria are already being analysed (El-Ansary and Hamouda 2014, Holajjer et al. 2014, Silva et al. 2014, Soares et al. 2014).

Towards improving EO nematotoxic activities, fractionation of EOs which showed low activities might uncover antagonistic interactions between compounds; particular interest should be given to

the oxygen-containing molecules fractions since these usually show the highest activities. Also, fractionated hydrodistillation, varying distillation time, could refine EO composition (Sintim et al. 2014) and nematotoxic EO compounds could be obtained in higher proportions.

Concerning direct contact bioassays, nematode counting, using a microscope or magnifier, is a commonly reliable methodology regularly used, but some limitations, like being operator dependent, can introduce heterogeneity in the results. For a more accurate determination of nematotoxic activity, nematode direct contact assays can use other standard scientific methodologies, such as colorimetric assays through the use of cell viability stains. Towards this objective some researchers have tested several stains; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide-formazan (MTT-formazan) is noteworthy given that it forms dark-blue water-insoluble formazan crystals in the presence of viable cells. The formation of formazan is directly proportional to cell number, so it is adequate to be used in PPN survival colorimetric assays (Robinson et al. 1992, James and Davey 2007, Smith et al. 2009). Implementation of this technique would allow screening a larger amount of phytochemicals in a shorter amount of time, speeding an otherwise lengthy process.

In the present work some preliminary assays were performed testing MTT in the PWN. After a 3 h exposure many PWNs showed a dark blue colour, intense in the initial part of the digestive tract (mouth and oesophagus) and losing intensity as it progressed to rest of the nematode body. PWNs were not all stained using this methodology so differential susceptibility to MTT in different nematode life stages may have been detected. Many optimizations must be performed before a reliable MTT methodology can be implemented. With assay optimization, other PPN life stages can be tested, as well as ascertain the effect of nematotoxic EOs on the ratio of particular developmental stages, using for e.g. flow cytometry. This would contribute in reaching an EO formulation capable of affecting PPN survival at its every developmental stage, while not affecting the plant host.

2.2. *In vitro* co-cultures

The culture conditions used in the present research revealed to be adequate for co-culture establishment and development. Nevertheless, optimization of culture conditions can be performed trying to simulate those of *in vivo* on-site environmental conditions (pH, temperature variations, nutrient content, etc.), enabling a more accurate co-culture response to external stimuli (e.g. the addition of phytochemicals). This would be advantageous in precision agriculture, targeting site-specific delivery of nematicides in individual fields, as described by Liu et al. (2014). Since culture medium affects root growth, ultimately affecting PPN development and reproduction, optimizations can be performed in culture medium composition. Changes in culture media composition should stimulate nematode population development in a way that the co-culture becomes sustainable between subculture periods, given that roots can overgrow nematode development or nematodes can kill the root if there is not enough root mass available. Also optimal temperatures for reproduction must be determined; generally PPN reproduce more rapidly when maintained at higher temperature but culture will decline more rapidly due to nutrient consumption (Verdejo-Lucas 1995). Subculture period is of the utmost importance and is highly dependent and specific to each host with parasite combination. According to Verdejo-Lucas (1995) the factors involved in such specificity are nematode multiplication rate, pathogenicity, nutrients and mass of tissue available, incubation temperature, initial inoculum density and culture age, and of course only experience and careful observation can determine the optimal time for subculture.

To further assess co-culture adequacy for nematotoxics testing and to explore issues that are difficult to assess *in vivo*, the use of other methodologies will be essential, using the co-cultures presently established as well as establishing new co-cultures. Firstly, further anatomical and ultrastructural studies, by light and scanning and transmission electron microscopy, should continue to be performed, in order to determine nematode routes within *in vitro* plant tissue and comparing to infected plant material grown in greenhouse or in the field. Changes in the cellular and metabolic pathways in response to infection, as reviewed in Kyndt et al. 2014, would be a good approach to be used on both co-cultures and compared to those of *in vivo* infections.

Co-cultures were established with highly damaging parasitic species and with the most affected

hosts, nevertheless, establishment of co-cultures as testing models requires that adequacy be ascertained through the establishment of new co-cultures with different hosts, when possible with resistant cultivars, as well as with different PPN and test co-culture conditions.

For the PWN new testing models should be established that co-culture *Pinus pinaster* with, for e.g. avirulent isolates of *B. xylophilus* [like the one isolated by Takeuchi and Futai (2007)] or its closest relative *B. mucronatus* Mamiya & Enda 1979 that shows little pathogenicity to conifers, according to that described in the EPPO bulletin (EPPO 2012a). This would contribute to uncover the in-depth cellular invasion mechanism of this parasite, trying to discriminate the trigger for its aggressive pathogenicity. Useful *in vitro* co-culture systems can also be established using virulent *B. xylophilus* to infect pine species with intermediate levels of susceptibility as well as species considered resistant, according to the classification of Evans et al. (1996). Co-cultures established with the intermediate susceptible *Pinus pinea* or *P. halepensis* or the resistant *P. elliottii*, would contribute to a more expeditious way for analyzing susceptibility and resistance and understand the specific resistance mechanisms developed. One application would be the analysis of lignification of cell walls thought responsible for effective inhibition of migration and reproduction in PWN on resistant varieties of *P. thunbergii* as detailed by Kusumoto et al. (2014).

Concerning the CRKN, new co-cultures should be established not only with other races but also with different plant host species, such as *Solanum lycopersicum* or *Medicago sativa* (for CRKN race 2) (EPPO 2012b). Root-knot is also caused by many other *Meloidogyne* species, sometimes by more than one, so it would be important do establish *S. tuberosum* co-cultures with other economically important RKNs such as *M. arenaria*, *M. hapla*, *M. incognita* and *M. javanica*, which are major pests worldwide, and show characteristic environmental adaptations. Additionally, ascertaining RKN interspecies behaviour, collaboration or competition, towards the disease development could be tested in different culture conditions, emulating different local seasonal environmental variations.

The co-cultures established are a very good tool to study key steps in root-knot nematode infection, such as the triggers for the induction and formation of the giant feeding cells formed at nematode moulting to adulthood; the molecular and cellular transport processes are still poorly understood as reviewed by Rodiuc et al. (2014). Given the transgenic nature of HR, directed mutagenesis may be

easily employed, in the *R. rhizogenes* infection step, facilitating obtaining and analyzing mutant lines for genes encoding important proteins in the infection mechanism, like those controlling cell cycle and formation of giant cells (as seen in Vieira 2012).

The metabolomics interplay between host and parasite can be studied in more detail, analyzing specific parasitism proteins that can be pinpointed in the nematode secretome (reviewed by Davis et al. 2008), as well as resistance or induced compounds from the host. These compounds can be, in this way, obtained in quantity and free from contamination due the stability in production and sterilized environment of an *in vitro* culture. Furthermore, co-cultures allow the analysis of the host genetic response to infection and testing genetic engineering approaches to inhibit infection in key stages [particularly the introduction of interference RNA, an approach described in Park et al. (2008) and Wang et al. (2012)].

2.3. Nematotoxics addition to co-cultures

EO addition to *in vitro* co-cultures is a good basis for experimental research given that, to date, bibliographic survey has yielded no experimental data on the subject.

Concerning the parasite, anatomical and ultrastructural studies, by light and electron microscopy (scanning and transmission) can be used to determine the exact reaction of the nematode to the external supply of nematotoxic pesticides. Also, a molecular and metabolic follow up of this supply can be performed to analyze the way in which the nematode reacts to the newly introduced phytochemical nematotoxics and how this affects the host with parasite system.

Regarding the plant host, much information can be gathered, particularly in phytochemical uptake rates and times. Following phytochemicals effect at the gene level would provide a notion of how the plant biosynthetic apparatus responds to the introduction of nematotoxic pesticides. As before, anatomical and ultrastructural studies would allow determining the effects of the phytochemical at the cellular level and if long-term damaging effect could be induced that would impair production. Preliminary results not discussed in the present work revealed that winter savory EO may have exerted its phytotoxicity by affecting plasma membrane structure.

Overall, effective nematotoxic pesticide screening for industrial purposes would require several

process optimizations to *in vitro* co-cultures to diminish methodology costs. Optimization of culture media and culture conditions, optimizations in temperature and pH, phytochemical delivery (solvents, surfactants, microencapsulation, etc.) are examples of pertinent subjects of study before *in vitro* co-cultures are to be established as successful pesticide industry screening tools.

Although results obtained from tissue culture may differ from those obtained *in vivo*, as biotic and abiotic factors can sometimes determine the fate of the nematode population, the use of co-cultures may contribute as an intermediate method for analyzing nematotoxics, between the conventional direct contact assays and the laborious and expensive greenhouse or environment-dependent *in vivo* assays.

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Annex 1

Supplementary data to Chapter 1

Tables ST1 to 3. Percentage composition of the 84 essential oils isolated from samples of 13 families, and of the corresponding oxygen-containing molecules fractions, assayed against PWN. For abbreviations and cluster analysis see Table 1 and Fig. 1, respectively.

Table ST1. Percentage composition of the essential oils isolated from Apiaceae / Umbelliferae, Asteraceae / Compositae, Cupressaceae, Fabaceae / Leguminosae and Geraniaceae assayed against PWN. For abbreviations and cluster analysis see Table 1 and Fig. 1, respectively.

		Apiaceae / Umbelliferae					Asteraceae/ Compositae		Cupressaceae			Fabaceae / Leguminosae	Geraniaceae
Components	**RI**	**Al[i]**	**Ag**	**Ca[i]**	**Fv[i]**	**Pc**	**Am**	**Iv**	**Cj[i]**	**Jb1[i]**	**Jb2[i]**	**Gt**	**Pg[i]**
n-Octane	800											t	
trans-2-Hexenal	866									t	t	t	
cis-3-Hexen-1-ol	868									t	t	t	
n-Hexanol	882											t	
2-Heptanone	886											t	
2-Methyl octane	887												
cis-Salvene*	887												
trans-Salvene*	893												
n-Heptanal	897	t										t	
n-Nonene	900						0.1						
Santolina triene	911						0.4						
3-Methyl cyclohexanone	914												
Tricyclene	921								t	t	t		
Terbutyl isovalerate	924												
α-Thujene	924			t	t	t	t	1.0	1.7	t	t		
Benzaldehyde	927			t			0.2					t	
α-Pinene	930	0.3	0.1	0.3	13.0	3.9	3.9	2.4	22.9	17.8	10.8		0.9
α-Fenchene	938												
Butyl isobutyrate	938												
Camphene	938				t		0.2		0.9	0.2	0.1		
Thuja-2,4(10)-diene	940												
n-Heptanol	952									t	t	t	
Sabinene	958	0.2		2.7	t	t	3.7	1.2	17.4	t	t		
6-Methyl-5-hepten-2-one	960												
1-Octen-3-ol	961			t					t	1.7	1.0	8.6	
3-Octanone	961											t	
β-Pinene	963	0.2	t	t	1.1	1.5	5.9	1.8	0.1	0.4	t		t
Hexanoic acid	968											t	
Dehydro 1,8-cineole	973				t	t		4.8					
2-Pentyl furan	973					t	0.1					1.8	
n-Octanal	973	3.9											

		Apiaceae / Umbelliferae					Asteraceae/ Compositae		Cupressaceae			Fabaceae / Leguminosae	Geraniaceae
Components	**RI**	**Al**[i]	**Ag**	**Ca**[i]	**Fv**[i]	**Pc**	**Am**	**Iv**	**Cj**[i]	**Jb1**[i]	**Jb2**[i]	**Gt**	**Pg**[i]
trans-Dehydroxy linalool oxide*	973												
3-Octanol	974									t	t	0.2	
1,2,4-Trimethyl benzene	975						0.1						
β-Myrcene	975	11.7	0.2	0.1	0.9	12.6	0.1	t	2.8	2.0	1.9	0.2	t
δ-2-Carene	983									t	t		
Isobutyl isovalerate	986												
α-Phellandrene	995	0.1		0.4	8.0	0.9	0.1	0.1	0.4	t	t		
Isopentyl isobutyrate	995												
δ-3-Carene	1000								2.2	t	t		
o-Cymene	1000												
Benzene acetaldehyde	1002	t			t							t	t
α-Terpinene	1002			t			0.5	2.6	5.5	0.2	t		
p-Cymene	1003	t		0.3	0.2	t	1.4	0.3	t	t	t	0.7	t
1,8-Cineole	1005						0.9	30.0				1.1	0.1
β-Phellandrene	1005	12.9		t	0.6	2.8	t	t	0.8	t	t		t
Limonene	1009	64.5	91.6	1.9	1.0	4.5	0.6	t	1.7	63.3	82.4	0.2	t
cis-β-Ocimene	1017		2.4	t	t		0.1			t	t		t
Bergamal*	1024												
trans-β-Ocimene	1027		t	t		t	0.2			t	1.4		t
γ-Terpinene	1035		0.6	2.5	0.1	t	1.0	4.7	8.4	0.4	0.2	0.3	
trans-Sabinene hydrate	1037						0.1		t				
cis-Linalool oxide	1045												t
p-Cresol	1045	0.1											
n-Octanol	1045											t	
p-Mentha-3,8-diene	1049												
Fenchone	1050				0.6					t	t	0.3	
2-Nonanone	1058												
trans-Linalool oxide	1059											t	
2,5-Dimethyl styrene	1059			t		1.9		t		t	t		
Myrcene epoxide	1064												
2-Phenylethyl alchool	1064											t	
Terpinolene	1064	t		29.8	t	7.3	0.2	0.7	2.4	0.9	0.6		
6-Methyl-3,5-heptadien-2-one	1064											t	t
cis-Sabinene hydrate	1066												
n-Nonanal	1073	t						t				t	
α-Thujone	1073						0.7						t
Linalool	1074	t		t		t		t	t	0.2	t	7.2	2.5
2-Methyl butyric acid,	1074												

		Apiaceae / Umbelliferae					Asteraceae/ Compositae		Cupressaceae			Fabaceae / Leguminosae	Geraniaceae
Components	**RI**	**Al**[i]	**Ag**	**Ca**[i]	**Fv**[i]	**Pc**	**Am**	**Iv**	**Cj**[i]	**Jb1**[i]	**Jb2**[i]	**Gt**	**Pg**[i]
isoamyl ester													
1,3,8-*p*-Menthatriene	1074					49.5							
Perillene*	1076												
Propionic acid hexyl ester	1079												
β-Thujone	1081						33.0					0.4	t
Chrysanthenone	1081						3.8						
cis-Rose oxide	1083											t	2.3
Isopentyl isovalerate	1084												
endo-Fenchol	1085									0.1	t		
Albene	1085								t				
Oct-1-en-3-yl acetate	1086	t		t									
α-Campholenal	1088									t	t		
cis-Limonene oxide	1095												
trans-*p*-2-Menthen-1-ol	1099							t	t	t	t		
cis-Chrysanthenol*	1099						1.1						
trans-Rose oxide	1100												0.3
trans-Sabinol	1101												
Camphor	1102						0.4			0.1	t	0.4	0.2
trans-Pinocarveol	1106						0.2						
cis-Pinocarveol*	1106												
cis-Verbenol	1110												
allo-Ocimene	1110												
cis-*p*-2-Menthen-1-ol	1110							t	0.2	t	t		
trans-Limonene oxide	1112												
trans-Verbenol	1114												
2-*trans*-6-*cis*-Nonadienal	1114											t	
Isopulegol	1116												
Geigerene isomer	1116												
trans-Pinocamphone	1116									t	t		
3-*cis*-Hexenyl butanoate	1118												
Menthone	1120											2.7	t
Citronellal	1121												
Geigerene	1121												
Pinocarvone	1121						0.2						
neo-Isopulegol	1121												t
Benzyl acetate	1123												
2-*trans*-Nonen-1-al	1124											t	t
iso-Menthone	1126											t	6.0
Nerol oxide	1127												
Hexyl isobutanoate	1127												

		Apiaceae / Umbelliferae					Asteraceae/ Compositae		Cupressaceae			Fabaceae / Leguminosae	Geraniaceae
Components	**RI**	**Al**[i]	**Ag**	**Ca**[i]	**Fv**[i]	**Pc**	**Am**	**Iv**	**Cj**[i]	**Jb1**[i]	**Jb2**[i]	**Gt**	**Pg**[i]
iso-Borneol	1132												
Menthofuran	1134												
δ-Terpineol	1134												
p-Mentha-1,5-dien-8-ol*	1134							2.2					
Borneol	1134						0.1			0.5	t	t	
cis-Isopulegone	1134												
Neomenthol	1139											t	
Lavandulol	1142											t	
Cryptone*	1143												
p-Methyl acetophenone	1143					t							
Rose furan epoxide*	1143												
neo-iso-Isopulegol	1148												
Menthol	1148											3.1	
Terpinen-4-ol	1148				t		1.1	7.2	24.3	2.0	0.1	0.7	
p-Cymen-8-ol	1148			0.2		t							
neo-iso-Menthol	1151												
Octanoic acid	1152											t	
Myrtenal	1153						0.1						
iso-Menthol	1154												t
Dill ether	1155									t	t		
cis-Dihydrocarvone	1159												
trans-Mentha-1,(7),8-dien-2-ol*	1159												
Methyl salicylate	1159											t	
α-Terpineol	1159				t	t	0.4	0.5	t	1.1	0.1	0.8	t
Methyl chavicol (= Estragole)	1163				0.8				t			t	
trans-Dihydrocarvone	1164												
Verbenone	1164									0.1	t		
2-Decanone	1166												
Dihydrocarveol	1167												
Myrtenol	1168						0.2	t					
Hexyl butanoate	1173												
n-Decanal	1180											0.2	
trans-Carveol	1189						0.2			t	t		
Bornyl formate	1199												
Cuminaldehyde	1200												
cis-p-Mentha-1,(7),8-dien-2-ol*	1200												
α-Fenchyl acetate	1201												
cis-Carveol	1202						t			t	t		

		Apiaceae / Umbelliferae					Asteraceae/ Compositae		Cupressaceae			Fabaceae / Leguminosae	Geraniaceae
Components	**RI**	**Al**[i]	**Ag**	**Ca**[i]	**Fv**[i]	**Pc**	**Am**	**Iv**	**Cj**[i]	**Jb1**[i]	**Jb2**[i]	**Gt**	**Pg**[i]
Nerol	1206												
Citronellol	1207												34.3
Thymol methyl ether	1210												
cis-Ocimenone	1210												
Pulegone	1210						0.2					8.4	
Carvone	1210						0.2			t	t	1.0	
Neral	1210											0.2	
cis-Piperitone epoxide	1211												
trans-Ocimenone	1211												
Piperitone	1211									t	t	t	
trans-Chrysanthenyl acetate*	1213						19.1						
2-Methyl butyric acid hexyl ester	1220												
cis-Anethole	1220				t								
2-*trans*-Decenal	1224											t	
Carvacrol methyl ether	1224									0.2	t		
2-Phenyl ethyl acetate	1228												
Geraniol	1236											2.7	4.9
Perilla aldehyde	1237												
Geranial	1240											1.1	t
cis-Chrysanthenyl acetate	1241						0.1						
Linalyl acetate	1245												
Methyl citronellate	1245												
Dehydrocarvacrol	1252												
trans-Anethole	1254			0.1	73.1		0.1		0.3	t	t	1.1	
n-Decanol	1259												
Thymyl formate	1262												
Citronellyl formate*	1262												14.4
Neryl formate	1263												t
Bornyl acetate	1265								t				
Nonanoic acid	1273												
Perilla alcohol	1274												
Thymol	1275						t					0.1	
2-Undecanone	1275											t	
Neryl acetate	1275												
trans-Sabinyl acetate	1277								t				
trans-Pinocarvyl acetate	1278												
Menthyl acetate	1278												
Lavandulyl acetate	1278												
Geranyl formate	1285												2.3

		Apiaceae / Umbelliferae					Asteraceae/ Compositae		Cupressaceae			Fabaceae / Leguminosae	Geraniaceae
Components	**RI**	**Al**[i]	**Ag**	**Ca**[i]	**Fv**[i]	**Pc**	**Am**	**Iv**	**Cj**[i]	**Jb1**[i]	**Jb2**[i]	**Gt**	**Pg**[i]
Carvacrol	1286						0.5			t	t	0.2	
cis-Theaspirane	1288											26.9	
n-Undecanal	1288												
Dihydrocarvyl acetate	1288												
Methyl geranate*	1288												
Piperitenone	1289												
Myrtenyl acetate	1290						0.1						
Terpinen-4-yl acetate*	1297												
Methyl anthranilate	1300												
trans-Theaspirane	1300											22.2	
iso-Dihydrocarvyl acetate	1310												
4A-α, 7-α, 7Aα-Nepetalactone	1319												
Geranyl 2-propyl ether	1322												
Eugenol	1327						0.6					0.1	
2-Phenyl ethyl propanoate	1328												t
Thymyl acetate	1330												
Piperitenone oxide	1330												
δ-Elemene	1332												t
α-Terpinyl acetate	1334								t			0.1	
UI A	1334	t											
Citronellyl acetate	1343												t
α-Cubebene	1345						0.1						t
cis-Carvyl acetate*	1346												
Hydrocinnamyl acetate*	1346												
Carvacryl acetate	1348												
Nepetalactone (2 unidentified isomers)	1348												
Neryl acetate	1353												
trans-β-Damascenone	1356											t	
Geranyl acetate	1370											t	t
α-Ylangene	1371							0.2					
cis-Jasmone	1372						0.7						
α-Copaene	1375	t			0.1		0.1	0.2					0.6
Methyl eugenol	1377											0.2	
β-Bourbonene	1379												0.9
α-Bourbonene*	1379												
β-Cubebene	1385												
β-Elemene	1388					0.6			t				0.2
2-Dodecanone*	1389												

		Apiaceae / Umbelliferae					Asteraceae/ Compositae		Cupressaceae			Fabaceae / Leguminosae	Geraniaceae
Components	**RI**	**Al**[i]	**Ag**	**Ca**[i]	**Fv**[i]	**Pc**	**Am**	**Iv**	**Cj**[i]	**Jb1**[i]	**Jb2**[i]	**Gt**	**Pg**[i]
α-Gurjunene	1400			0.3				0.3					t
α-Cedrene	1400											t	
Decyl acetate	1400												
Acora-3,5-diene	1414			0.8									
β-Cedrene	1414			t									
trans-β-Caryophyllene	1414		1.2	9.2		0.6	1.2	6.8		t	0.1		1.5
trans-Cinnamyl acetate	1414												
trans-Isoeugenol	1422												
β-Gurjunene	1426												
β-Copaene*	1426	t							t				t
Aromadendrene	1428												
UI B	1430	0.1											
trans-Ethyl cinnamate	1431												
Selina-1,5-diene	1432												
Geranyl acetone	1434											0.2	
trans-α-Bergamotene	1434			t									
cis-trans-α-Farnesene*	1436												
Sesquisabinene B	1438												
cis-Muurola-3,5-diene*	1445												
Citronellyl propanoate*	1446												t
Guaia-6,9-diene *	1447												14.6
α-Humulene	1447		t	0.4			0.1	0.3	t	0.1	t		0.7
γ-Thujaplicin*	1447												
trans-β-Farnesene	1455			2.4									
Cabreuva oxide A*	1455						0.1						
allo-Aromadendrene	1456							0.1					0.2
trans-β-Ionone	1456											t	
Geranyl propionate	1461												t
Cabreuva oxide B*	1463						0.1						
Geraniol butyl ether	1466												
γ-Gurjunene	1467												
Phenyl ethyl 2-methyl butanoate*	1467												
γ-Muurolene	1469	t					0.4	0.2					t
α-Amorphene	1469							0.5					
Dehydroaromadendrene*	1469												
trans-Methyl isoeugenol	1471												
Germacrene-D	1474	4.3			t	0.6	0.1		0.6	0.1	0.1		2.7
α-Curcumene	1475			0.6								0.2	
γ-Curcumene	1475			0.6									

		Apiaceae / Umbelliferae					Asteraceae/ Compositae		Cupressaceae			Fabaceae / Leguminosae	Geraniaceae
Components	**RI**	**Al**[i]	**Ag**	**Ca**[i]	**Fv**[i]	**Pc**	**Am**	**Iv**	**Cj**[i]	**Jb1**[i]	**Jb2**[i]	**Gt**	**Pg**[i]
β-Selinene	1476		1.9										
γ-Humulene	1477												
cis-β-Guaiene*	1478							1.5					
2-Tridecanone	1479												
Eremophilene*	1480												
Valencene	1484												
Dodecanol allyl ether	1488												
trans-Dehydroagarofuran	1489												
Viridiflorene	1487												
Bicyclogermacrene	1487	0.3											
3,3,5,5,8,8-Hexamethyl-7-oxabicyclo[4.3.0]non-1(6)-ene-2,4-dione*	1488												
α-Zingiberene	1492						0.1						
Eugenol acetate	1493												
Myristicin	1493			25.9		0.3							
α-Muurolene	1494	t					0.1	0.9	t				0.3
α-*trans*, *trans*-Farnesene	1500			1.0									
β-Bisabolene	1500			t			0.5			t	t	t	
γ-Cadinene	1500	0.1						1.0	t				0.3
7-epi-α-Selinene	1500												
trans-Calamene	1505											t	0.1
δ-Cadinene	1505	0.3					0.5	4.3	0.2	t	t		1.3
β-Sesquiphellandrene	1508			1.5									
Kessane*	1517					0.2							
Elemicin	1525					t							
α-Calacorene	1525											t	0.2
α-Cadinene	1529							0.1					
Elemol	1530								1.0				
Germacrene B*	1533	0.6											
trans-α-Bisabolene	1536												
Geranyl butyrate	1544												t
γ-Maaliene*	1544												
trans-Nerolidol	1549			t			0.8						
β-Caryophyllene alcohol	1550												
ar-Turmerol	1551						0.1						
Spathulenol	1551						0.1						0.4
Dodecanoic acid	1551											0.6	
Phenyl ethyl tiglate	1553												t
Furopelargone A *	1558												2.3

		Apiaceae / Umbelliferae					Asteraceae/ Compositae		Cupressaceae			Fabaceae / Leguminosae	Geraniaceae
Components	**RI**	**Al**[i]	**Ag**	**Ca**[i]	**Fv**[i]	**Pc**	**Am**	**Iv**	**Cj**[i]	**Jb1**[i]	**Jb2**[i]	**Gt**	**Pg**[i]
β-Caryophyllene oxide	1561						0.8	2.9				0.3	
Globulol	1566												
Viridiflorol	1569						0.2						
trans-Nuciferyl acetate	1571												
Guaiol	1575												
Anhydro-oplopanone	1576								t				
Cedrol	1579			t									
Ledol	1580												
Humulene epoxide	1580												
Geranyl 2-methyl butyrate	1586												t
Geranyl isovalerate	1590												t
10-epi-γ-Eudesmol	1593								t				
UI C	1597												
epi-Cubenol	1600												
UI D	1609												
γ-Eudesmol	1609								1.1				
epi-α-Cadinol	1616	0.1					0.5		0.2				
δ-Cadinol	1618												
α-Muurolol	1618							1.7					0.2
β-Eudesmol	1620								0.5				
β-Sinensal*	1622												
Valerianol	1623												
Cubenol	1624												
Intermedeol	1626												
α-Cadinol	1626	0.2					0.2			t	t		0.2
UI E	1626												
Geranyl valerate	1633												t
α-Eudesmol	1634								1.0				
α-Muurolol	1634									t	t		
Anastreptene*	1634												
Cadalene	1640												
Apiole	1640					11.3							
UI F	1641			t									
Citronellyl tiglate	1643												t
Acorenone B*	1645			16.8									
UI G	1648												
α-Bisabolol	1656						0.2						
epi-α-Bisabolol	1658	t					0.1						
UI H	1662												
α-Sinensal*	1667												

		Apiaceae / Umbelliferae					Asteraceae/ Compositae		Cupressaceae			Fabaceae / Leguminosae	Geraniaceae
Components	**RI**	**Al**[i]	**Ag**	**Ca**[i]	**Fv**[i]	**Pc**	**Am**	**Iv**	**Cj**[i]	**Jb1**[i]	**Jb2**[i]	**Gt**	**Pg**[i]
Geranyl tiglate	1671												2.0
trans,*cis*-Farnesol	1693												
n-Nonadecane	1900												
Palmitic acid	1908												
epi-13-Manool	1946									6.9	1.0		
Falcarinol	2000			t									
Phyllocladene	2006								1.4				
Abietatriene	2027									0.4	0.1		
Phytyl acetate	2047			0.4									
Abietadiene	2060									t	t		
cis-Totarol methyl ether	2175									0.1	t		
trans-Totarol	2234									0.4	0.1		
Phytyl acetate 2	2243												
n-Tricosane	2300						0.1						
n-Pentacosane	2500						0.1						
% Identification		99.8	98.0	98.2	99.5	98.5	89.5	80.5	98.0	99.2	100.0	94.5	96.9
Monoterpene hydrocarbons		89.9	94.9	38.0	24.9	83.0	18.3	14.8	67.2	85.2	97.4	1.4	0.9
Oxygen-containing monoterpenes		t		0.2	0.6	t	62.7	44.7	24.5	4.3	0.2	30.5	69.3
Sesquiterpene hydrocarbons		5.6	3.1	16.8	0.1	1.8	3.2	16.4	0.8	0.2	0.2	0.2	23.6
Oxygen-containing sesquiterpenes		0.3		16.8		0.2	3.2	4.6	3.8	t	t	0.3	3.1
Diterpene hydrocarbons									1.4	0.4	0.1		
Oxygen-containing diterpenes										7.4	1.1		
Phenylpropanoids				26.0	73.9	11.6	0.7		0.3	t	t	1.4	
Polyacetylenes				t									
C11 molecules							0.7						
C12 molecules													
C13 molecules												49.3	
N-containing molecules													
Fatty acids												0.6	
Others		4.0		0.4	t	1.9	0.7	t	t	1.7	1.0	10.8	t

RI, Calculated retention index relative to C_8-C_{25} *n*-alkanes on the DB-1 column; t, trace (<0.05%); UI = unidentified compounds. *identification based on mass spectra only. [i] - EOs previously tested (Barbosa et al., 2010; Barbosa et al., 2012).

Table ST2. Percentage composition of the 84 essential oils isolated from Lamiaceae samples and of the corresponding hydrocarbon molecules and oxygen-containing molecules fractions, assayed against PWN. For abbreviations and cluster analysis see Table 1 and Fig. 1, respectively.

		Lamiaceae / Labiatae																																									
Components	**RI**	**Cn**	**Mo[i]**	**Ma**	**Mc1**	**Mc2**	**Mp1**	**Mp2**	**Mpu**	**Ms1**	**Ms2[i]**	**Nc[i]**	**Om**	**Ov1**	**Ov2**	**Ov2H**	**Ov2O**	**Ovi**	**Ro[i]**	**So[i]**	**Sm1[i]**	**Sm2**	**Sm2H**	**Sm2O**	**Tc**	**TcH**	**TcO**	**Thc1[i]**	**Thc2[i]**	**Thc3[i]**	**Thc4[i]**	**Thc5**	**Thc6**	**Thc6H**	**Thc6O**	**Thc7**	**Thca[i]**	**Thm**	**Thvl[i]**	**Thv**	**Thz**	**Thzs1[i]**	**Thzs2**
n-Octane	800																																										
trans-2-Hexenal	866																																										
cis-3-Hexen-1-ol	868																																										
n-Hexanol	882																																										
2-Heptanone	886																																										
2-Methyl octane	887																																										
cis-Salvene*	887																			0.1																							
trans-Salvene*	893																			t																							
n-Heptanal	897																																										
n-Nonene	900																																										
Santolina triene	911																																										
3-Methyl cyclohexanone	914				t	t			0.5																																		
Tricyclene	921			0.1															0.2	0.1	t	t	t			t						t									0.1		
Terbutyl isovalerate	924																																										
α-Thujene	924			t	t	t		t		t	0.1		0.3	0.3	1.3	3.7		0.4	t	0.4	0.3	1.4	3.5		2.3	6.8	0.1	1.5	0.5	1.7	0.4	1.9	2.7	5.9		1.9		0.1	0.2	1.2	0.7	0.4	2.0
Benzaldehyde	927																																										
α-Pinene	930	0.8		1.1	0.7	0.7	0.5	0.7	0.4	0.6	0.6	t	0.5	0.4	0.5	1.5		0.3	14.6	10.4	1.6	1.2	2.1		0.9	2.6	0.2	0.7	0.4	1.1	0.4	0.6	1.5	2.2		1.2	0.7	3.3	1.2	0.8	0.8	0.2	0.7
α-Fenchene	938																																										
Butyl isobutyrate	938																																										
Camphene	938			0.6	t	t		0.1			0.2		0.1	0.2	0.1	0.3		0.1	3.4	2.6	1.6	0.2	0.3		0.1	0.4		0.1	t	1.1	t	0.1	0.1	t		0.1		0.2	0.8	0.4	1.1	0.1	0.1

Components	RI	Lamiaceae / Labiatae																																							
		Cn	Mo[i]	Ma	Mc1	Mc2	Mp1	Mp2	Mpu	Ms1	Ms2[i]	Nc[i]	Om	Ov1	Ov2	Ov2 H	Ov2 O	Ovi	Ro[i]	So[i]	Sm1[i]	Sm2	Sm2 H	Sm2 O	Tc	TcH	TcO	Thc1[i]	Thc2[i]	Thc3[i]	Thc4[i]	Thc5	Thc6	Thc6 H	Thc6 O	Thc7	Thca[i]	Thm	Thvl[i]	Thv	Thz	Thzs 1[i]	Thzs 2
Thuja-2,4(10)-diene	940																		0.6													0.1					t						
n-Heptanol	952			t																																							
Sabinene	958	0.4		t	t	t	t	0.2		0.1	0.2	t	3.3	1.2	0.3	1.7		t		0.2			0.1		0.1	0.3		0.4	t	1.7	0.5	0.2	0.1	t		t		2.5	0.2	t	t	0.2	0.5
6-Methyl-5-hepten-2-one	960		0.1																																		t						
1-Octen-3-ol	961			1.7			t	t		0.1	t	t		0.8	0.3		0.5	0.1	0.7	t	t	t		1.0			t	0.1	t	t	t	t	t		0.1	t			0.2	0.4	0.7	0.2	0.5
3-Octanone	961				t	t								0.5			0.1																								0.4		
β-Pinene	963	1.9		1.1	t	t	0.8	1.1	0.3	0.8	0.7	t	0.3		0.4	0.7		0.2	0.7	4.4	0.2	0.2	0.5		0.3	0.7		0.2	0.2	1.9	t	0.2	0.3	0.3		0.2		4.7	1.0	0.4		0.2	0.3
Hexanoic acid	968																																										
Dehydro 1,8-cineole	973												t		0.1		t	0.1										t	t	0.1	t	t	t			t		0.2	0.8			0.1	0.2
2-Pentyl furan	973																											t	t	0.1	t	t	t			t							
n-Octanal	973																																										
trans-Dehydroxy linalool oxide*	973												0.1																								t						
3-Octanol	974			0.1	0.4	0.4		t	0.4								t							t								t							t				
1,2,4-Trimethyl benzene	975																																										
β-Myrcene	975	0.1		0.3	0.3	0.3	2.3	t		0.5	2.7		1.2	1.1	1.2	3.4	t	0.2	29.0	2.0	t	1.4	5.2		2.4	6.9	0.2	0.5	t	2.1	0.1	t	t	0.4		t	0.3	1.2	t	1.5	1.1	1.3	0.3
δ-2-Carene	983																																										
Isobutyl isovalerate	986																																										
α-Phellandrene	995							t		0.1	0.1			t	0.3	0.7	t	t	2.0	t	t	0.1	0.7		0.4	1.0		0.3	t	0.4	t	0.2	t	0.3		t				0.1	0.1	0.2	t
Isopentyl isobutyrate	995																																										
δ-3-Carene	1000															0.1					t	t	0.2		0.1	0.3		0.1	t	0.1	t	0.1	0.1	0.4		t				0.1	0.1		
o-Cymene	1000																																										
Benzene acetaldehyde	1002		0.1	0.7													t																				0.8						

Components	RI	Cn	Mo[i]	Ma	Mc1	Mc2	Mp1	Mp2	Mpu	Ms1	Ms2[i]	Nc[i]	Om	Ov1	Ov2	Ov2H	Ov2O	Ovi	Ro[i]	So[i]	Sm1[i]	Sm2	Sm2H	Sm2O	Tc	TcH	TcO	Thc1[i]	Thc2[i]	Thc3[i]	Thc4[i]	Thc5	Thc6	Thc6H	Thc6O	Thc7	Thca[i]	Thm	Thvl[i]	Thv	Thz	Thzs1[i]	Thzs2
		Lamiaceae / Labiatae																																									
α-Terpinene	1002						t	t		0.8	t		2.6	2.6	3.2	8.3	t	0.7	0.9	t	0.3	1.9	5.7	t	1.8	6.3	0.1	1.1	0.1	1.8	0.2	1.3	0.5	3.5		t		0.3	t	1.0	0.8	1.2	1.3
p-Cymene	1003				0.2	0.2	t	t	0.2	t	t		1.4	4.3	4.4	10.6	t	3.8	2.7	0.4	20.3	8.1	19.0	t	7.2	23.2	0.7	19.1	5.8	13.4	6.2	13.5	8.4	28.7		11.2	t	0.3	t	19.5	15.1	1.3	0.4
1,8-Cineole	1005	11.0		t	0.1	t	2.0	3.1	0.4	2.5	t						t		3.7	25.5	t	t		0.2														45.9	5.0	0.4			
β-Phellandrene	1005	t								t	t		0.9	0.4	0.4	1.0		0.1	3.7	t	t	0.2	0.5		0.3	t	t	0.3	t	0.4	t	0.2	0.2	0.9		t	t				0.1	0.2	t
Limonene	1009	4.0		1.2	9.5	10.1	3.2	1.5	0.2	7.5	9.9		1.5	0.5	0.5	1.1	t	0.2	6.3	0.8	0.6	0.3	0.8		0.4	0.9	t	3.2	1.4	2.3	2.8	0.4	0.5	1.4		t	0.5	22.9	2.0	0.4	0.4	1.0	1.2
cis-β-Ocimene	1017			6.5	0.1	t	t	0.1		0.5	1.1	t	0.7	2.4	1.7	4.7	t	1.3	0.8		t	t	1.6			t		t	t	t	t	t	t			t	t	0.1	t			5.9	3.0
Bergamal*	1024																																										
trans-β-Ocimene	1027		t	0.3	t	t		t		t	0.3	t	0.3	0.4	0.7	2.0	t	0.8	t	t			0.3		t	0.2				0.1	t	t					1.6	0.8	0.6	t		1.6	2.2
γ-Terpinene	1035			t	t	t	0.2	0.1		1.4	t		4.9	9.5	14.6	35.7	0.3	7.5	1.9	0.6	4.3	18.1	44.4	t	10.9	35.6	0.9	7.6	2.1	12.6	8.0	6.0	3.8	16.1		4.1	0.2	0.3	0.2	12.2	9.1	15.6	5.6
trans-Sabinene hydrate	1037						t	0.3					4.9	1.3	t		0.5	t		t	t	t		0.2	0.2		0.2					0.1			0.2		t	0.4	0.6	0.6	0.4	0.2	0.6
cis-Linalool oxide	1045				t	t									0.3		t	t																			3.4		0.4				
p-Cresol	1045																																										
n-Octanol	1045																																										
p-Mentha-3,8-diene	1049								0.6																																		
Fenchone	1050																											0.1	t	t	t		0.1			t							
2-Nonanone	1058																																										
trans-Linalool oxide	1059														t			t																			1.4		0.2				
2,5-Dimethyl styrene	1059															0.1			t		t	t				t		0.2	t	t	t	t	t	0.1		t				t			
Myrcene epoxide	1064																																										
2-Phenylethyl alchool	1064																																										
Terpinolene	1064						t	t	t	0.3	t		1.1	0.9	0.7	2.0		t	0.3	t	0.4	t	0.2		t	0.3		0.6	0.1	0.8	0.5	0.1	t	t		t	t	0.1		t	0.1	0.2	2.0
6-Methyl-3,5-heptadien-2-one	1064																																										

		Lamiaceae / Labiatae																																									
Components	RI	Cn	Mo[i]	Ma	Mc1	Mc2	Mp1	Mp2	Mpu	Ms1	Ms2[i]	Nc[i]	Om	Ov1	Ov2	Ov2 H	Ov2 O	Ovi	Ro[i]	So[i]	Sm1[i]	Sm2	Sm2 H	Sm2 O	Tc	TcH	TcO	Thc1[i]	Thc2[i]	Thc3[i]	Thc4[i]	Thc5	Thc6	Thc6 H	Thc6 O	Thc7	Thca[i]	Thm	Thvl[i]	Thv	Thz	Thzs 1[i]	Thzs 2
cis-Sabinene hydrate	1066							t		0.7	t		32.5	14.0	t		0.6	t			t	0.1		t								t					t	0.1	0.5	0.2	0.2		
n-Nonanal	1073																															t											
α-Thujone	1073																			28.5																							
Linalool	1074		0.8	t				t		t	t	t	2.9	4.9	7.4		13.6	15.6	1.0	t	t	t		1.1	0.9		1.5	t	t	t	t	t	t			t	25.5	3.7	68.5	1.9	1.5	11.2	9.5
2-Methyl butyric acid, isoamyl ester	1074			0.2				t																																			
1,3,8-*p*-Menthatriene	1074																																										
Perillene*	1076																																										
Propionic acid hexyl ester	1079																																										
β-Thujone	1081																			10.0																							
Chrysanthenone	1081																																										
cis-Rose oxide	1083		t																																								
Isopentyl isovalerate	1084																																										
endo-Fenchol	1085																		t																								
Albene	1085																											0.1	t				t			t							
Oct-1-en-3-yl acetate	1086			0.9						0.1	t			0.3																0.3	t	0.6			0.2								
α-Campholenal	1088																		t																		t						
cis-Limonene oxide	1095																																										
trans-*p*-2-Menthen-1-ol	1099									0.1	t		0.9	0.6	0.3		0.6	t		t												t											
cis-Chrysanthenol*	1099																																										
trans-Rose oxide	1100																																										
trans-Sabinol	1101																																										

		Lamiaceae / Labiatae																																									
Components	RI	Cn	Mo[i]	Ma	Mc1	Mc2	Mp1	Mp2	Mpu	Ms1	Ms2[i]	Nc[i]	Om	Ov1	Ov2	Ov2H	Ov2O	Ovi	Ro[i]	So[i]	Sm1[i]	Sm2	Sm2H	Sm2O	Tc	TcH	TcO	Thc1[i]	Thc2[i]	Thc3[i]	Thc4[i]	Thc5	Thc6	Thc6H	Thc6O	Thc7	Thca[i]	Thm	Thvl[i]	Thv	Thz	Thzs1[i]	Thzs2
Camphor	1102																		7.4	6.0	0.1	t																0.2	0.5	0.3	1.1		
trans-Pinocarveol	1106																																					0.2					
cis-Pinocarveol*	1106																																										
cis-Verbenol	1110																			t												t					0.3						
allo-Ocimene	1110			0.1											0.1	t		t	t																							0.1	t
cis-*p*-2-Menthen-1-ol	1110												0.4	0.2			0.3															t											
trans-Limonene oxide	1112												0.2																														
trans-Verbenol	1114																				t	t																					
2-*trans*-6-*cis*-Nonadienal	1114																																										
Isopulegol	1116																		0.4																								
Geigerene isomer	1116																																										
trans-Pinocamphone	1116																		0.1																								
3-*cis*-Hexenyl butanoate	1118																																										
Menthone	1120	0.9			0.2	t	19.3	56.0																																			
Citronellal	1121		0.3																																								
Geigerene	1121																																										
Pinocarvone	1121						0.1																															0.1					
neo-Isopulegol	1121																		t																								
Benzyl acetate	1123																																										
2-*trans*-Nonen-1-al	1124																																										
iso-Menthone	1126	51.5			3.6	1.5	0.6	3.7																																			
Nerol oxide	1127		t										0.2																								3.6	0.1					

		Lamiaceae / Labiatae																																										
																	Ov2	Ov2					Sm2	Sm2				Thc1	Thc2	Thc3	Thc4		Thc6	Thc6	Thca					Thzs	Thzs
Components	**RI**	**Cn**	**Mo**[i]	**Ma**	**Mc1**	**Mc2**	**Mp1**	**Mp2**	**Mpu**	**Ms1**	**Ms2**[i]	**Nc**[i]	**Om**	**Ov1**	**Ov2**	**H**	**O**	**Ovi**	**Ro**[i]	**So**[i]	**Sm1**[i]	**Sm2**	**H**	**O**	**Tc**	**TcH**	**TcO**	[i]	[i]	[i]	[i]	**Thc5**	**Thc6**	**H**	**O**	**Thc7**	[i]	**Thm**	**Thvl**[i]	**Thv**	**Thz**	**1**[i]	**2**
Hexyl isobutanoate	1127																																						v				
iso-Borneol	1132																																										
Menthofuran	1134				t	t	5.9	8.5	0.6																																			
δ-Terpineol	1134	t								0.1	t									0.1																		1.7	0.4				
p-Mentha-1,5-dien-8-ol*	1134																																										
Borneol	1134			1.5							0.5		0.4	0.5	0.2		0.5	0.2	1.1	0.1	3.9	0.1		0.3	0.2	0.3			1.3	t	0.1						0.4	0.3	2.3	0.3	t
cis-Isopulegone	1134				0.5	0.1			1.2																																	
Neomenthol	1139	0.8					2.8	2.4																																		
Lavandulol	1142						t																																			
Cryptone*	1143																																									
p-Methyl acetophenone	1143																																									
Rose furan epoxide*	1143																																									
neo-iso-Isopulegol	1148																																									
Menthol	1148						31.1	5.4																																		
Terpinen-4-ol	1148				t	t	1.0	0.4		4.3	0.1		12.9	8.1	8.5		15.9	1.8	1.2	0.7	2.3	0.1		0.7	0.8	1.2	1.7	0.4	2.5	0.6	0.7	0.6		0.8	t	1.3	0.8	0.3	0.5	1.9	0.3	3.0
p-Cymen-8-ol	1148			t																										t												
neo-iso-Menthol	1151	0.8																																								
Octanoic acid	1152																																									
Myrtenal	1153																																					t					
iso-Menthol	1154	19.0					t																																				
Dill ether	1155																																									
cis-Dihydrocarvone	1159										1.8																																

		Lamiaceae / Labiatae																																									
Components	RI	Cn	Mo[i]	Ma	Mc1	Mc2	Mp1	Mp2	Mpu	Ms1	Ms2[i]	Nc[i]	Om	Ov1	Ov2	Ov2 H	Ov2 O	Ovi	Ro[i]	So[i]	Sm1[i]	Sm2	Sm2 H	Sm2 O	Tc	TcH	TcO	Thc1[i]	Thc2[i]	Thc3[i]	Thc4[i]	Thc5	Thc6	Thc6 H	Thc6 O	Thc7	Thca[i]	Thm	Thvl[i]	Thv	Thz	Thzs 1[i]	Thzs 2
trans-Mentha-1,(7),8-dien-2-ol*	1159																																										
Methyl salicylate	1159																																										
α-Terpineol	1159		t	0.2				t	0.1	0.1	t		2.9	1.1	15.8		26.1	39.7	3.1		t	t		0.1				3.5	2.4	36.1	62.1	2.5	2.4		2.4	t	3.2	4.0	9.1	0.1	t	31.5	60.0
Methyl chavicol (= Estragole)	1163																											0.1	t				t			t							
trans-Dihydrocarvone	1164													0.1																													
Verbenone	1164				t	t			1.2										9.8																		t						
2-Decanone	1166																																										
Dihydrocarveol	1167									2.9	1.6																																
Myrtenol	1168				t	t																																0.1					
Hexyl butanoate	1173																																										
n-Decanal	1180																																				t						
trans-Carveol	1189								0.3	3.1	0.5																			t	t	0.1											
Bornyl formate	1199																															t									t		
Cuminaldehyde	1200																																										
cis-*p*-Mentha-1,(7),8-dien-2-ol*	1200																																										
α-Fenchyl acetate	1201																																										
cis-Carveol	1202									t	t																																
Nerol	1206													0.1																							t	0.1					
Citronellol	1207		31.9										0.2						t																			0.2					
Thymol methyl ether	1210														0.1	0.4		t																						0.1		0.4	t
cis-Ocimenone	1210																		t																								
Pulegone	1210	5.4			79.6	86.1	0.2	13.2	49.1										t																							0.1	t

		Lamiaceae / Labiatae																																									
Components	RI	Cn	Mo[i]	Ma	Mc1	Mc2	Mp1	Mp2	Mpu	Ms1	Ms2[i]	Nc[i]	Om	Ov1	Ov2	Ov2 H	Ov2 O	Ovi	Ro[i]	So[i]	Sm1[i]	Sm2	Sm2 H	Sm2 O	Tc	TcH	TcO	Thc1[i]	Thc2[i]	Thc3[i]	Thc4[i]	Thc5	Thc6	Thc6 H	Thc6 O	Thc7	Thca[i]	Thm	Thvl[i]	Thv	Thz	Thzs 1[i]	Thzs 2
Carvone	1210									53.5	69.5				t			t						0.1				0.2	t			t	t			t							
Neral	1210																																										
cis-Piperitone epoxide	1211								0.9																																		
trans-Ocimenone	1211																																										
Piperitone	1211	t			t	t	5.0	0.5											t																								
trans-Chrysanthenyl acetate*	1213																																										
2-Methyl butyric acid hexyl ester	1220																																										
cis-Anethole	1220																																										
2-*trans*-Decenal	1224																																										
Carvacrol methyl ether	1224													8.2	0.5	1.3	t	0.1			3.7	t						t	t	0.5	t	t	0.1	2.1		t				t		0.7	t
2-Phenyl ethyl acetate	1228																																										
Geraniol	1236		18.0										0.5	0.3	0.1			t																			t						
Perilla aldehyde	1237																																										
Geranial	1240		38.0																																								
cis-Chrysanthenyl acetate	1241																																										
Linalyl acetate	1245												8.8	2.0			0.3																				18.2	0.4					
Methyl citronellate	1245																																										
Dehydrocarvacrol	1252																											t	t				t			t							
trans-Anethole	1254																																				t						
n-Decanol	1259																																										
Thymyl formate	1262																		t																					0.7	0.6		
Citronellyl formate*	1262																																										

Components	RI	Cn	Mo^{i}	Ma	Mc1	Mc2	Mp1	Mp2	Mpu	Ms1	Ms2^{i}	Nc^{i}	Om	Ov1	Ov2	Ov2 H	Ov2 O	Ovi	Ro^{i}	So^{i}	Sm1^{i}	Sm2	Sm2 H	Sm2 O	Tc	TcH	TcO	Thc1^{i}	Thc2^{i}	Thc3^{i}	Thc4^{i}	Thc5	Thc6	Thc6 H	Thc6 O	Thc7	Thca^{i}	Thm	Thvl^{i}	Thv	Thz	Thzs 1^{i}	Thzs 2
		Lamiaceae / Labiatae																																									
Neryl formate	1263																																										
Bornyl acetate	1265			0.5									1.0	0.9					t									0.1	t	0.5	t	t	t			t							
Nonanoic acid	1273								0.1																																		
Perilla alcohol	1274																																				t						
Thymol	1275						0.5		0.7				0.5	0.5	14.7		22.5	11.8	t		15.2	t		0.2	0.1		0.2			t	0.4	42.2								47.8	50.0	3.8	0.9
2-Undecanone	1275																		t																								
Neryl acetate	1275		t																																		t						
trans-Sabinyl acetate	1277																																										
trans-Pinocarvyl acetate	1278																																										
Menthyl acetate	1278						8.2	0.1																																			
Lavandulyl acetate	1278									0.1	t		0.1																														
Geranyl formate	1285																																										
Carvacrol	1286								0.8					14.3	10.3		15.1	5.9	t	t	40.0	63.9	0.2	95.5	68.2	3.3	92.9	34.6	47.1	0.1	2.8	2.8	53.7	0.5	66.2	58.7				3.2	3.2	1.3	t
cis-Theaspirane	1288																																										
n-Undecanal	1288																																										
Dihydrocarvyl acetate	1288	1.5																																									
Methyl geranate*	1288																																										
Piperitenone	1289			0.2	0.5	0.1	0.3	0.1	10.4										t																								
Myrtenyl acetate	1290																																										
Terpinen-4-yl acetate*	1297																																										
Methyl anthranilate	1300																																										
trans-Theaspirane	1300																																										

Components	RI	Lamiaceae / Labiatae																																									
		Cn	Mo[i]	Ma	Mc1	Mc2	Mp1	Mp2	Mpu	Ms1	Ms2[i]	Nc[i]	Om	Ov1	Ov2	Ov2 H	Ov2 O	Ovi	Ro[i]	So[i]	Sm1[i]	Sm2	Sm2 H	Sm2 O	Tc	TcH	TcO	Thc1[i]	Thc2[i]	Thc3[i]	Thc4[i]	Thc5	Thc6	Thc6 H	Thc6 O	Thc7	Thca[i]	Thm	Thvl[i]	Thv	Thz	Thzs 1[i]	Thzs 2
iso-Dihydrocarvyl acetate	1310									1.9	0.2																																
4A-α, 7-α, 7Aα-Nepetalactone	1319											88.8																															
Geranyl 2-propyl ether	1322																																										
Eugenol	1327																t										0.1																
2-Phenyl ethyl propanoate	1328																																										
Thymyl acetate	1330																													t	t	15.2								0.2			
Piperitenone oxide	1330			56.3																																							
δ-Elemene	1332						t						0.1	0.2		0.1																0.4							t			0.1	t
α-Terpinyl acetate	1334																																										
UI A	1334																																										
Citronellyl acetate	1343																																										
α-Cubebene	1345																																										
cis-Carvyl acetate*	1346									3.2	t																																
Hydrocinnamyl acetate*	1346																																										
Carvacryl acetate	1348																							0.1	0.1		0.3	6.1	11.5	t	0.4	0.7	10.4		13.7	1.2							
Nepetalactone (2 unidentified isomers)	1348											2.5																															
Neryl acetate	1353																																										
trans-β-Damascenone	1356																																										
Geranyl acetate	1370		1.3											0.4			t																				0.6	0.1					
α-Ylangene	1371																		t																								
cis-Jasmone	1372						0.1			0.3	t								t																								

		Lamiaceae / Labiatae																																									
Components	RI	Cn	Mo[i]	Ma	Mc1	Mc2	Mp1	Mp2	Mpu	Ms1	Ms2[i]	Nc[i]	Om	Ov1	Ov2	Ov2H	Ov2O	Ovi	Ro[i]	So[i]	Sm1[i]	Sm2	Sm2H	Sm2O	Tc	TcH	TcO	Thc1[i]	Thc2[i]	Thc3[i]	Thc4[i]	Thc5	Thc6	Thc6H	Thc6O	Thc7	Thca[i]	Thm	Thvl[i]	Thv	Thz	Thzs1[i]	Thzs2
α-Copaene	1375									0.3	0.3												0.1							t	t	t											
Methyl eugenol	1377																		0.1																								
β-Bourbonene	1379			0.3			0.1	t	t	1.9	0.2		0.4			t				t	t	t	0.1							0.3	t	t						0.1	0.1	t		0.1	t
α-Bourbonene*	1379									0.2	1.1																																
β-Cubebene	1385																																										
β-Elemene	1388			t			0.2	0.3		0.3	0.2																			0.1	t	0.1		0.6			0.3		0.7				
2-Dodecanone*	1389																																										
α-Gurjunene	1400						0.5																									t											
α-Cedrene	1400																																										
Decyl acetate	1400																																				t						
Acora-3,5-diene	1414																																										
β-Cedrene	1414																																										
trans-β-Caryophyllene	1414	1.1	8.6	2.7	0.1	0.2	4.5	1.0		3.6	1.9	7.8	3.0	3.7	4.3	9.5	t	4.0	1.5	2.5	2.6	2.4	8.5		2.0	7.6	0.3			3.0		t		2.0			0.9	0.5	0.9	0.7	2.7	9.2	3.0
trans-Cinnamyl acetate	1414																																										
trans-Isoeugenol	1422																																										
β-Gurjunene	1426																																										
β-Copaene*	1426			0.1						0.2	0.1					t					t	t	0.2									t											
Aromadendrene	1428														0.1	0.3		t		0.1	0.3	t				t																	
UI B	1430																																										
trans-Ethyl cinnamate	1431																																										
Selina-1,5-diene	1432																																										
Geranyl acetone	1434																		t											t	0.1						t						

		Lamiaceae / Labiatae																																									
Components	**RI**	**Cn**	**Mo[i]**	**Ma**	**Mc1**	**Mc2**	**Mp1**	**Mp2**	**Mpu**	**Ms1**	**Ms2[i]**	**Nc[i]**	**Om**	**Ov1**	**Ov2**	**Ov2 H**	**Ov2 O**	**Ovi**	**Ro[i]**	**So[i]**	**Sm1[i]**	**Sm2**	**Sm2 H**	**Sm2 O**	**Tc**	**TcH**	**TcO**	**Thc1[i]**	**Thc2[i]**	**Thc3[i]**	**Thc4[i]**	**Thc5**	**Thc6**	**Thc6 H**	**Thc6 O**	**Thc7**	**Thca[i]**	**Thm**	**Thvl[i]**	**Thv**	**Thz**	**Thzs 1[i]**	**Thzs 2**
trans-α-Bergamotene	1434																																									t	t
cis-trans-α-Farnesene*	1436																																										
Sesquisabinene B	1438																																										
cis-Muurola-3,5-diene*	1445																															0.1											
Citronellyl propanoate*	1446																																										
Guaia-6,9-diene *	1447																																										
α-Humulene	1447		0.4	0.3			0.2		t	0.5	0.3	0.5	0.1	0.4	0.6	1.3		0.5	0.7	1.3	t	t	0.2		0.1	0.3				0.2	t	t		0.7								0.9	0.4
γ-Thujaplicin*	1447								t																																		
trans-β-Farnesene	1455			1.6			0.6	t		0.5	0.6	t																												0.2			
Cabreuva oxide A*	1455																																										
allo-Aromadendrene	1456		t																	t						t				0.4	0.4	0.4		2.0			1.3	0.2					
trans-β-Ionone	1456																																										
Geranyl propionate	1461																																										
Cabreuva oxide B*	1463																																										
Geraniol butyl ether	1466																																					t					
γ-Gurjunene	1467																																										
Phenyl ethyl 2-methyl butanoate*	1467																																										
γ-Muurolene	1469									3.8	0.2					t			0.2				0.5									t											
α-Amorphene	1469																																										
Dehydroaromadendrene*	1469																																										
trans-Methyl isoeugenol	1471																																										
Germacrene-D	1474	0.4	0.1	6.9			4.4	1.0		0.1	2.5		0.7		0.4	1.1		0.5					0.4							0.6	0.3	0.1						0.1	0.3	0.2	0.4	2.4	0.8

Components	RI	Cn	Mo[i]	Ma	Mc1	Mc2	Mp1	Mp2	Mpu	Ms1	Ms2[i]	Nc[i]	Om	Ov1	Ov2	Ov2H	Ov2O	Ovi	Ro[i]	So[i]	Sm1[i]	Sm2	Sm2H	Sm2O	Tc	TcH	TcO	Thc1[i]	Thc2[i]	Thc3[i]	Thc4[i]	Thc5	Thc6	Thc6H	Thc6O	Thc7	Thca[i]	Thm	Thvl[i]	Thv	Thz	Thzs1[i]	Thzs2
		Lamiaceae / Labiatae																																									
α-Curcumene	1475																		t																								
γ-Curcumene	1475																																										
β-Selinene	1476																																										
γ-Humulene	1477																																										
cis-β-Guaiene*	1478																																					0.2					
2-Tridecanone	1479																																										
Eremophilene*	1480			0.1																												0.1											
Valencene	1484																																										
Dodecanol allyl ether	1488																																										
trans-Dehydroagarofuran	1489																											3.3	1.7			0.8	2.1	11.5	1.5	3.8							
Viridiflorene	1487																			0.2			0.3											1.3					0.5				
Bicyclogermacrene	1487	0.4		0.3			1.2	0.2		0.2	0.3		1.5	5.6	0.8	1.8		0.9								t					1.3						1.5		1.2		0.6	2.5	1.1
3,3,5,5,8,8-Hexamethyl-7-oxabicyclo[4.3.0]non-1(6)-ene-2,4-dione*	1488																																										
α-Zingiberene	1492																		t																								
Eugenol acetate	1493																																										
Myristicin	1493																																										
α-Muurolene	1494			0.2												t			t	t			0.1							0.2	0.6	0.4					0.5						
α-*trans*, *trans*-Farnesene	1500		t																																							t	t
β-Bisabolene	1500												1.6	3.1	2.1	4.6		1.7	0.1		0.1	t	3.1			t															1.0	3.7	0.3
γ-Cadinene	1500			0.3											0.2			t										3.0	3.3	1.7	1.2	1.2	0.8	8.5		1.4	6.2			0.7		0.1	t

		Lamiaceae / Labiatae																				
Components	RI	Cn	Mo[i]	Ma	Mc1	Mc2	Mp1	Mp2	Mpu	Ms1	Ms2[i]	Nc[i]	Om	Ov1	Ov2	Ov2 H	Ov2 O	Ovi	Ro[i]	So[i]	Sm1[i]	Sm2
7-epi-α-Selinene	1500																					
trans-Calamene	1505			0.2															t		0.1	0.1
δ-Cadinene	1505			0.7			0.5	t		0.5	0.2					0.3			0.4	t	0.1	0.1
β-Sesquiphellandrene	1508																					
Kessane*	1517																					
Elemicin	1525																					
α-Calacorene	1525																		0.1			
α-Cadinene	1529			0.2																		
Elemol	1530																					
Germacrene B*	1533																					
trans-α-Bisabolene	1536															0.1						
Geranyl butyrate	1544																					
γ-Maaliene*	1544																					
trans-Nerolidol	1549																					
β-Caryophyllene alcohol	1550																					
ar-Turmerol	1551																					
Spathulenol	1551												0.4	0.7	0.3		0.6	0.7				
Dodecanoic acid	1551																					
Phenyl ethyl tiglate	1553																					
Furopelargone A *	1558																					
β-Caryophyllene oxide	1561		t		0.6	0.1			1.0			0.4	0.6	0.8	0.4		0.7	0.5	0.1	0.5	0.6	t
Globulol	1566						1.3							0.2								

Components	RI	Sm2 H	Sm2 O	Tc	TcH	TcO	Thc1[i]	Thc2[i]	Thc3[i]	Thc4[i]	Thc5	Thc6	Thc6 H	Thc6 O	Thc7	Thca[i]	Thm	Thvl[i]	Thv	Thz	Thzs 1[i]	Thzs 2
7-epi-α-Selinene	1500																					
trans-Calamene	1505						0.6	0.9	0.2	t	0.3	0.1	2.0		t	1.2			0.2			
δ-Cadinene	1505	0.5			t				0.6	1.9	0.2		1.8			0.8			0.2	0.3		
β-Sesquiphellandrene	1508																					
Kessane*	1517						1.3	0.8	t	0.3	0.3	1.1		1.3	0.9							
Elemicin	1525																					
α-Calacorene	1525										t					0.4						
α-Cadinene	1529										t		0.4									
Elemol	1530								0.4	0.1	t						0.9					
Germacrene B*	1533																					
trans-α-Bisabolene	1536	0.1			t											3.3						
Geranyl butyrate	1544																					
γ-Maaliene*	1544																					
trans-Nerolidol	1549																					
β-Caryophyllene alcohol	1550																					
ar-Turmerol	1551																					
Spathulenol	1551					t					t					1.5		0.1				
Dodecanoic acid	1551																					
Phenyl ethyl tiglate	1553																					
Furopelargone A *	1558																					
β-Caryophyllene oxide	1561			0.2		0.3					t					1.1			0.2	0.5		
Globulol	1566										t					0.5	0.4					

		Lamiaceae / Labiatae																							
Components	RI	Cn	Mo[i]	Ma	Mc1	Mc2	Mp1	Mp2	Mpu	Ms1	Ms2[i]	Nc[i]	Om	Ov1	Ov2	Ov2 H	Ov2 O	Ovi	Ro[i]	So[i]	Sm1[i]	Sm2	Sm2 H	Sm2 O	Tc
Viridiflorol	1569			0.3				t												1.9					
trans-Nuciferyl acetate	1571																								
Guaiol	1575																								
Anhydro-oplopanone	1576																								
Cedrol	1579																								
Ledol	1580																								
Humulene epoxide	1580				0.2	0.1			2.5								t								
Geranyl 2-methyl butyrate	1586																								
Geranyl isovalerate	1590																								
10-epi-γ-Eudesmol	1593																								
UI C	1597																								
epi-Cubenol	1600																								
UI D	1609																								
γ-Eudesmol	1609																								
epi-α-Cadinol	1616			0.4																					
δ-Cadinol	1618																								
α-Muurolol	1618			0.1			0.2			0.2	0.1														
β-Eudesmol	1620																								
β-Sinensal*	1622																								
Valerianol	1623																								
Cubenol	1624										0.3														
Intermedeol	1626														0.1		0.3	0.2							

		Lamiaceae / Labiatae																	
Components	RI	TcH	TcO	Thc1[i]	Thc2[i]	Thc3[i]	Thc4[i]	Thc5	Thc6	Thc6 H	Thc6 O	Thc7	Thca[i]	Thm	Thvl[i]	Thv	Thz	Thzs 1[i]	Thzs 2
Viridiflorol	1569					0.4	t												
trans-Nuciferyl acetate	1571																		
Guaiol	1575																		
Anhydro-oplopanone	1576																		
Cedrol	1579																		
Ledol	1580												0.3						
Humulene epoxide	1580																		
Geranyl 2-methyl butyrate	1586																		
Geranyl isovalerate	1590																		
10-epi-γ-Eudesmol	1593																		
UI C	1597			0.8	0.8	0.3	0.7	0.3	1.0		1.3	1.7							
epi-Cubenol	1600				1.6			0.6					1.9						
UI D	1609			1.8	1.8	0.9	1.7	0.6	3.3		4.0	5.1							
γ-Eudesmol	1609					0.9	0.1	t						0.3					
epi-α-Cadinol	1616			3.3	11.8	3.2	2.5	2.5	2.6		3.0	4.3	8.4			1.2			
δ-Cadinol	1618																		
α-Muurolol	1618					t	0.4	t											
β-Eudesmol	1620					0.8	t	t						0.3					
β-Sinensal*	1622																		
Valerianol	1623																		
Cubenol	1624															0.2			
Intermedeol	1626				1.3						1.0		t		2.5				

		Lamiaceae / Labiatae																																							
Components	RI	Cn	Mo[i]	Ma	Mc1	Mc2	Mp1	Mp2	Mpu	Ms1	Ms2[i]	Nc[i]	Om	Ov1	Ov2	Ov2H	Ov2O	Ovi	Ro[i]	So[i]	Sm1[i]	Sm2	Sm2H	Sm2O	Tc	TcH	TcO	Thc1[i]	Thc2[i]	Thc3[i]	Thc4[i]	Thc5	Thc6	Thc6H	Thc6O	Thc7	Thca[i]	Thm	Thvl[i]	Thv	Thz	Thzs1[i]	Thzs2
α-Cadinol	1626			0.8			0.5			0.3	0.3																			t	1.4						3.1			t			
UI E	1626																																										
Geranyl valerate	1633																																										
α-Eudesmol	1634																													1.1	t	1.3						0.3					
α-Muurolol	1634																																										
Anastreptene*	1634																																										
Cadalene	1640																																				0.2						
Apiole	1640																																										
UI F	1641																																										
Citronellyl tiglate	1643																																										
Acorenone B*	1645																																										
UI G	1648																											0.6	0.6	0.3	0.5	0.3	1.1		1.3	2.0							
α-Bisabolol	1656																		0.2										0.1			t											
epi-α-Bisabolol	1658																																										
UI H	1662																													t	0.6	0.1											
α-Sinensal*	1667																																										
Geranyl tiglate	1671																																										
trans,cis-Farnesol	1693																																										
n-Nonadecane	1900																																										
Palmitic acid	1908								2.8																																		
epi-13-Manool	1946																																										
Falcarinol	2000																																										

		Lamiaceae / Labiatae																																									
Components	RI	Cn	Mo[i]	Ma	Mc1	Mc2	Mp1	Mp2	Mpu	Ms1	Ms2[i]	Nc[i]	Om	Ov1	Ov2	Ov2H	Ov2O	Ovi	Ro[i]	So[i]	Sm1[i]	Sm2	Sm2H	Sm2O	Tc	TcH	TcO	Thc1[i]	Thc2[i]	Thc3[i]	Thc4[i]	Thc5	Thc6	Thc6H	Thc6O	Thc7	Thca[i]	Thm	Thvl[i]	Thv	Thz	Thzs1[i]	Thzs2
Phyllocladene	2006																																										
Abietatriene	2027																																										
Phytyl acetate	2047																																										
Abietadiene	2060																																										
cis-Totarol methyl ether	2175																																										
trans-Totarol	2234																																										
Phytyl acetate 2	2243			0.3									0.2	0.2																													
n-Tricosane	2300																																										
n-Pentacosane	2500																																										
% Identification		100.0	99.6	89.4	96.6	99.9	98.3	100.0	74.7	98.2	98.7	100.0	97.1	98.2	98.3	98.4	98.5	99.9	99.0	99.4	98.6	99.9	99.4	99.5	100.0	96.7	99.5	94.0	93.5	97.1	96.0	98.7	92.2	93.6	90.4	89.0	95.0	98.4	99.4	98.1	97.4	98.8	99.9
Monoterpene hydrocarbons		7.2	t	11.3	10.8	11.3	7.0	3.8	1.7	12.6	15.9	t	19.1	24.2	30.4	77.5	0.3	15.6	67.1	21.9	29.6	33.1	85.1	t	27.2	85.5	2.2	35.7	10.6	41.5	19.1	24.8	18.2	60.1		18.7	3.3	36.8	6.2	37.6	29.6	29.7	19.6
Oxygen-containing monoterpenes		90.9	90.3	58.7	84.5	87.8	77.0	93.7	65.7	72.5	74.2	91.3	69.4	57.5	58.3	1.7	96.0	75.2	27.8	70.9	65.2	64.2	0.2	98.5	70.5	3.3	96.6	46.3	61.4	41.1	66.3	64.4	67.3	2.6	83.3	59.9	57.5	58.3	86.7	56.3	61.2	49.9	74.2
Sesquiterpene hydrocarbons		1.9	9.1	13.9	0.1	0.2	12.2	2.5	t	12.1	7.9	8.3	7.4	13.0	8.5	19.1	t	7.6	3.0	4.1	3.2	2.6	14.1	t	2.1	7.9	0.3	3.7	4.2	7.3	5.7	3.4	0.9	19.3	t	1.4	16.6	1.1	3.7	2.2	5.0	19.0	5.6
Oxygen-containing sesquiterpenes			t	1.6	0.8	0.2	2.0	t	3.5	0.5	0.7	0.4	1.0	1.7	0.8		1.6	1.4	0.3	2.4	0.6	t			0.2		0.3	7.9	17.3	6.8	4.8	5.5	5.8	11.5	6.8	9.0	16.8	2.2	2.6	1.6	0.5		
Diterpene hydrocarbons																																											
Oxygen-containing diterpenes																																											
Phenylpropanoids																			0.1								0.1	0.1	t				t			t	t						
Polyacetylenes																																											
C11 molecules							0.1			0.3	t								t																								
C12 molecules																																											
C13 molecules																			t											t	0.1												

Components	RI	Lamiaceae / Labiatae																																								
		Cn	Mo[i]	Ma	Mc1	Mc2	Mp1	Mp2	Mpu	Ms1	Ms2[i]	Nc[i]	Om	Ov1	Ov2	Ov2 H	Ov2 O	Ovi	Ro[i]	So[i]	Sm1[i]	Sm2	Sm2 H	Sm2 O	Tc	TcH	TcO	Thc1[i]	Thc2[i]	Thc3[i]	Thc4[i]	Thc5	Thc6	Thc6 H	Thc6 O	Thc7	Thca[i]	Thm	Thvl[i]	Thv	Thz	Thzs 1[i]	Thzs 2
N-containing molecules																																											
Fatty acids									2.9																																		
Others			0.2	3.9	0.4	0.4	t	t	0.9	0.2	t	t	0.2	1.8	0.3	0.1	0.6	0.1	0.7	0.1	t	t		1.0				0.3	t	0.4	t	0.6	t	0.1	0.3	t	0.8		0.2	0.4	1.1	0.2	0.5

RI, Calculated retention index relative to C_8-C_{25} *n*-alkanes on the DB-1 column; t, trace (<0.05%); UI = unidentified compounds. *identification based on mass spectra only.

[i] - EOs previously tested (Barbosa et al., 2010; Barbosa et al., 2012).

Table ST3. Percentage composition of the 84 essential oils isolated from Lauraceae, Myrtaceae, Pittosporaceae, Poaceae / Gramineae, Rutaceae, Verbenaceae and Zingiberaceae samples, and of the corresponding hydrocarbon molecules and oxygen-containing molecules fractions, assayed against PWN. For abbreviations and cluster analysis see Table 1 and Fig. 1, respectively.

		Lauraceae			Myrtaceae																		Pittosporaceae		Poaceae / Gramineae					Rutaceae										Verbenaceae	Zingiberaceae
Components	**RI**	**Cc**	**La**[i]	**Ln**	**Eb**[ii]	**Ebo**[ii]	**Ec**[ii]	**Eci**[ii]	**Ect**[ii]	**Eco**[ii]	**Ed**[ii]	**Ef**[ii]	**Eg**[ii]	**Ep**[ii]	**Epo**[ii]	**Er**[ii]	**Es**[ii]	**Esm**[ii]	**Eu**[ii]	**Ev**[ii]	**Mco**	**Sa**	**Pu1**[i]	**Pu2**[i]	**Cci1**[i]	**Cci2**	**Cci3**	**H**	**O**	**Cl1**	**Cl2**	**Cl3**	**Cs1**	**Cs2**	**Cs3**	**Cs4**	**Rg1**[i]	**Rg2**[i]	**Rg3**	**Ac**[i]	**Zo**
n-Octane	800																																							
trans-2-Hexenal	866																																							
cis-3-Hexen-1-ol	868																																							
n-Hexanol	882																																							
2-Heptanone	886																					t																		0.1
2-Methyl octane	887																																			t	t	t		
cis-Salvene*	887																																							
trans-Salvene*	893																																							
n-Heptanal	897																																							
n-Nonene	900																																							
Santolina triene	911																																							
3-Methyl cyclohexanone	914																																							
Tricyclene	921	0.1	t							t				t																										0.2
Terbutyl isovalerate	924				t								t				t	t		t																				
α-Thujene	924	0.5	0.1	0.6	t	0.5	t				5.8	t		t	2.2	3.0			1.2	t	0.2		1.1	1.3						t	0.1	t	0.4	0.5					t	t
Benzaldehyde	927																																							
α-Pinene	930	10.1	34.8	5.7	14.2	43.2	31.6	7.7	1.2	6.3	1.0	43.5	15.5	82.2	4.2	2.6	40.2	6.7	2.0	12.5	24.4		2.2	5.5	t	t	t			2.9	2.1	1.8	2.3	2.9	0.4	0.3				0.6	4.5
α-Fenchene	938				t												0.2																							
Butyl isobutyrate	938																						t	t																

		Lauraceae			Myrtaceae																		Pittosporaceae		Poaceae / Gramineae					Rutaceae										Verbenaceae	Zingiberaceae
Components	**RI**	**Cc**	**La[i]**	**Ln**	**Eb[ii]**	**Ebo[ii]**	**Ec[ii]**	**Eci[ii]**	**Ect[ii]**	**Eco[ii]**	**Ed[ii]**	**Ef[ii]**	**Eg[ii]**	**Ep[ii]**	**Epo[ii]**	**Er[ii]**	**Es[ii]**	**Esm[ii]**	**Eu[ii]**	**Ev[ii]**	**Mco**	**Sa**	**Pu1[i]**	**Pu2[i]**	**Cci1[i]**	**Cci2**	**Cci3**	**H**	**O**	**Cl1**	**Cl2**	**Cl3**	**Cs1**	**Cs2**	**Cs3**	**Cs4**	**Rg1[i]**	**Rg2[i]**	**Rg3**	**Ac[i]**	**Zo**
Camphene	938	4.6	4.4	0.1	0.1	0.2	0.1	0.1	t	0.1		0.2	0.1	0.8		t	0.2	0.1	t	0.1	t		t	t	t	t	t			0.2	0.1	0.1	0.1	0.1						13.9
Thuja-2,4(10)-diene	940				t	t							t			t	t			t																			
n-Heptanol	952																																							
Sabinene	958	1.6	0.2	6.9		t	t		t		t	t		t	t	t			t		t		30.5	17.8						3.4	t	t	47.2	63.7	0.3	0.2				t	
6-Methyl-5-hepten-2-one	960																								t	t	t		1.1										6.0	0.8
1-Octen-3-ol	961																																							
3-Octanone	961																																							
β-Pinene	963	4.5	16.0	4.2	0.4	2.8	0.4	t	1.6	0.1	t	8.8	0.2	0.2	0.2	0.7	0.2	0.1	2.6	0.2	0.2		t	1.0						33.8	13.9	13.9	t	2.0	0.3	0.3					t
Hexanoic acid	968																																							
Dehydro 1,8-cineole	973		t	t	t								t			t				t			0.1	0.3	t	0.5	0.4	0.5	t				t						0.1	t
2-Pentyl furan	973																																						t	
n-Octanal	973																			t									t	0.4	t			0.8	1.3	t	t	t		t
trans-Dehydroxy linalool oxide*	973												t																											
3-Octanol	974																																						0.3	
1,2,4-Trimethyl benzene	975																																							
β-Myrcene	975	2.0	0.7	0.5	0.3	0.2	t	t	t	t	2.0	t	t	t	1.0	1.1	t		0.4	t	0.3		5.5	5.9	24.7	38.2	19.5	71.6	0.1	0.7	0.4	0.4	5.6	3.6	0.8	1.5				0.3	2.6
δ-2-Carene	983				0.1																																			
Isobutyl isovalerate	986			t																	0.2																			
α-Phellandrene	995	3.6	t	0.1		5.7	0.8	t		0.1	19.3	t		t	7.8	4.2			45.3	0.1	t		0.6	0.6							t	t	0.3	0.3	0.1	0.1					0.4
Isopentyl isobutyrate	995				t	t											t		t	t	t																			
δ-3-Carene	1000			0.3										0.3							0.1											1.4	2.5	0.1	t					0.1

		Lauraceae			Myrtaceae																		Pittosporaceae		Poaceae / Gramineae					Rutaceae										Verbenaceae	Zingiberaceae
																										Cci3	Cci3														
Components	**RI**	**Cc**	**La[i]**	**Ln**	**Eb[ii]**	**Ebo[ii]**	**Ec[ii]**	**Eci[ii]**	**Ect[ii]**	**Eco[ii]**	**Ed[ii]**	**Ef[ii]**	**Eg[ii]**	**Ep[ii]**	**Epo[ii]**	**Er[ii]**	**Es[ii]**	**Esm[ii]**	**Eu[ii]**	**Ev[ii]**	**Mco**	**Sa**	**Pu1[i]**	**Pu2[i]**	**Cci1[i]**	**Cci2**	**Cci3**	**H**	**O**	**Cl1**	**Cl2**	**Cl3**	**Cs1**	**Cs2**	**Cs3**	**Cs4**	**Rg1[i]**	**Rg2[i]**	**Rg3**	**Ac[i]**	**Zo**
o-Cymene	1000						0.5																																	
Benzene acetaldehyde	1002																														t								
α-Terpinene	1002		t	0.5		0.1	t				0.7	t	t	t	0.3	0.7			0.5	t	t		7.1	7.3						0.1	0.2	0.2	1.8	1.0	0.1	t					
p-Cymene	1003	1.2	t	t	0.1	0.6	0.5	0.1	t	1.1	18.5	t	0.7	0.3	6.4	12.8	0.1	0.8	4.0	6.0	0.2		t	0.8	t	t	t				t	0.5	t	t					0.1	t
1,8-Cineole	1005	4.3	7.4	34.8	59.3	35.0	50.8	67.4	11.4	71.9	t	2.0	70.3	1.4	27.2	47.9	47.9	83.3	22.6	46.4	37.4				t	t	t			0.1	17.2	14.9							4.6	t
β-Phellandrene	1005	t				t					3.5	t			9.1	t			t				0.7	0.7						0.1			0.6	0.5	13.6	13.0					16.9
Limonene	1009	6.9	1.1	1.8	4.5	4.3	3.3	5.3	0.2	6.3	0.7	41.1	2.5	3.0	1.3	4.8	3.1	4.0	3.0	5.1	13.1		14.4	21.8	t	t	t			31.6	51.7	44.6	9.9	3.4	81.4	77.8				11.4	2.7
cis-β-Ocimene	1017	t	0.3	t		t	t			t	t	t		t		t	t		t	t	t		0.3	0.8	0.4	t	t	1.1		t	t	t	t	t					0.2	
Bergamal*	1024								0.1																																t
trans-β-Ocimene	1027	t	1.2			t					t	t				t					0.4		0.3	1.1	0.1	t	t	0.5		2.7	t	t	5.7	5.6	t	t					
γ-Terpinene	1035	t	0.2	1.1	0.1	0.2	t	t	0.1	0.4	0.6	1.7	t		0.8	1.9	t	t	7.9	11.5	0.3		9.9	11.5						1.0	9.3	7.4	3.2	2.0	t	0.3			0.1	t
trans-Sabinene hydrate	1037			0.1												t							0.2	0.5						t			0.2	0.1					0.5	
cis-Linalool oxide	1045				t	t							t	t					t	t																				
p-Cresol	1045																																							
n-Octanol	1045																														t	t			t	0.3				
p-Mentha-3,8-diene	1049								0.2																																
Fenchone	1050																																							
2-Nonanone	1058		t																			t			t	t	t										t	t	t		t
trans-Linalool oxide	1059				0.1	t																																	t	
2,5-Dimethyl styrene	1059				t		t				t		t							t															t	t					
Myrcene epoxide	1064																										1.3	0.3													
2-Phenylethyl alchool	1064																																							
Terpinolene	1064	0.3	t	0.3	0.1	0.2	0.1	t		0.1	1.4	0.1	t	0.1	0.2	0.4	0.1		0.6	0.6	0.4		1.8	2.5						0.1	0.5	0.2	0.9	0.7		t				0.3

		Lauraceae			Myrtaceae																			Pittosporaceae		Poaceae / Gramineae					Rutaceae										Verbenaceae	Zingiberaceae
Components	**RI**	**Cc**	**La[i]**	**Ln**	**Eb[ii]**	**Ebo[ii]**	**Ec[ii]**	**Eci[ii]**	**Ect[ii]**	**Eco[ii]**	**Ed[ii]**	**Ef[ii]**	**Eg[ii]**	**Ep[ii]**	**Epo[ii]**	**Er[ii]**	**Es[ii]**	**Esm[ii]**	**Eu[ii]**	**Ev[ii]**	**Mco**	**Sa**	**Pu1[i]**	**Pu2[i]**	**Cci1[i]**	**Cci2**	**Cci3**	**H**	**Cci3 O**	**Cl1**	**Cl2**	**Cl3**	**Cs1**	**Cs2**	**Cs3**	**Cs4**	**Rg1[i]**	**Rg2[i]**	**Rg3**	**Ac[i]**	**Zo**
6-Methyl-3,5-heptadien-2-one	1064																																					0.3	
cis-Sabinene hydrate	1066			0.1																												0.2	t							
n-Nonanal	1073												t																0.1	t	t			t	t	t	t	t		
α-Thujone	1073																																							
Linalool	1074	t	0.4	9.1	t	t	t		0.2	t	t	t		1.3	0.1	t	t		t	t	0.8		0.2	0.2	0.7	2.6	0.2		1.3	0.3	0.1	0.5	8.4	4.0	1.3	2.4				1.1	1.0
2-Methyl butyric acid, isoamyl ester	1074									t									t		0.8																			
1,3,8-*p*-Menthatriene	1074				t																																			
Perillene*	1076																							t	t	t	0.7													
Propionic acid hexyl ester	1079																					t	t																	
β-Thujone	1081																																							
Chrysanthenone	1081																																							
cis-Rose oxide	1083																																							
Isopentyl isovalerate	1084				0.1			t	0.1	t			t				t	t	t	t	0.2																			
endo-Fenchol	1085				0.1	0.1	0.1	t		t		t	t	0.5			0.3	t			t																			t
Albene	1085																																							
Oct-1-en-3-yl acetate	1086																																							
α-Campholenal	1088				t	t							t	0.5			0.1			t	t																			
cis-Limonene oxide	1095																																					0.4	
trans-p-2-Menthen-1-ol	1099			t		t					0.1				0.4	1.6			0.1			0.4	0.3									0.2	0.1							0.2
cis-Chrysanthenol*	1099																																							
trans-Rose oxide	1100																																							

		Lauraceae			Myrtaceae																	Pittosporaceae		Poaceae / Gramineae					Rutaceae										Verbenaceae	Zingiberaceae
Components	**RI**	**Cc**	**La[i]**	**Ln**	**Eb[ii]**	**Ebo[ii]**	**Ec[ii]**	**Eci[ii]**	**Ect[ii]**	**Eco[ii]**	**Ed[ii]**	**Ef[ii]**	**Eg[ii]**	**Ep[ii]**	**Epo[ii]**	**Er[ii]**	**Es[ii]**	**Esm[ii]**	**Eu[ii]**	**Ev[ii]**	**Mco**	**Sa**	**Pu1[i]**	**Pu2[i]**	**Cci1[i]**	**Cci2**	**Cci3**	**H**	**O**	**Cl1**	**Cl2**	**Cl3**	**Cs1**	**Cs2**	**Cs3**	**Cs4**	**Rg1[i]**	**Rg2[i]**	**Rg3**	**Ac[i]**	**Zo**
trans-Sabinol	1101										0.1																													
Camphor	1102	48.6																																					0.1
trans-Pinocarveol	1106		0.1		1.0	0.3	1.8	0.6	0.9	0.4			2.8	0.2			3.8	0.7		0.2	t																		
cis-Pinocarveol*	1106																			t																			
cis-Verbenol	1110		t																					0.5	t	t														
allo-Ocimene	1110																					0.1	0.1	t	t	t														
cis-p-2-Menthen-1-ol	1110										t				0.2	1.0			0.1													0.2	t						0.1
trans-Limonene oxide	1112				t																																	0.2	
trans-Verbenol	1114																							1.0	0.4	0.4														
2-*trans*-6-*cis*-Nonadienal	1114																																							
Isopulegol	1116								13.4																															0.2
Geigerene isomer	1116																																			t	t	t		
trans-Pinocamphone	1116													0.1																										
3-*cis*-Hexenyl butanoate	1118																					t	0.1																	
Menthone	1120																																							
Citronellal	1121								35.8															t	t	t	0.3	0.1	0.4	t	t	t	0.2					0.3	1.9
Geigerene	1121																																			0.5	0.1	0.5		
Pinocarvone	1121		0.1		0.3	0.1	2.1	t		0.1			2.2				1.6	0.3		t																			
neo-Isopulegol	1121								5.8																															t
Benzyl acetate	1123																				t																		
2-*trans*-Nonen-1-al	1124																																						
iso-Menthone	1126																																						

		Lauraceae			Myrtaceae																		Pittosporaceae	Poaceae / Gramineae				Rutaceae										Verbenaceae	Zingiberaceae
																									Cci3	Cci3													
Components	**RI**	**Cc**	**La[i]**	**Ln**	**Eb[ii]**	**Ebo[ii]**	**Ec[ii]**	**Eci[ii]**	**Ect[ii]**	**Eco[ii]**	**Ed[ii]**	**Ef[ii]**	**Eg[ii]**	**Ep[ii]**	**Epo[ii]**	**Er[ii]**	**Es[ii]**	**Esm[ii]**	**Eu[ii]**	**Ev[ii]**	**Mco**	**Sa**	**Pu1[i]**	**Pu2[i]**	**Cci1[i]**	**Cci2**	**Cci3**	**H**	**O**	**Cl1**	**Cl2**	**Cl3**	**Cs1**	**Cs2**	**Cs3**	**Cs4**	**Rg1[i]**	**Rg2[i]**	**Rg3**	**Ac[i]**	**Zo**
Nerol oxide	1127																																					t	
Hexyl isobutanoate	1127																						t	t															
iso-Borneol	1132																																						t
Menthofuran	1134																																						
δ-Terpineol	1134			t	0.4			0.1		0.1			0.1				t	0.1		t																		0.1	
p-Mentha-1,5-dien-8-ol*	1134																																						
Borneol	1134	0.2				0.2	0.1	0.1		0.1		t	0.1	1.1			0.5	0.1		t	t																		t
cis-Isopulegone	1134								t																														
Neomenthol	1139																																						
Lavandulol	1142									t							t			t																			
Cryptone*	1143																																						t
p-Methyl acetophenone	1143					t													t																			t	
Rose furan epoxide*	1143																																						0.2
neo-iso-Isopulegol	1148								1.0																														
Menthol	1148																																						
Terpinen-4-ol	1148	0.8	t	2.3	0.3	0.4	t	0.1	0.1	0.5	4.3	t	t	0.1	2.0	9.1	t	0.1	1.3	1.2	0.3		20.8	14.8					0.2	0.9	1.2	5.2	3.1	0.3	0.3				0.4	0.2
p-Cymen-8-ol	1148																																						
neo-iso-Menthol	1151																																						
Octanoic acid	1152																																						
Myrtenal	1153		0.1										0.1																										
iso-Menthol	1154																																						
Dill ether	1155																																						

		Lauraceae			Myrtaceae																			Pittosporaceae		Poaceae / Gramineae					Rutaceae										Verbenaceae	Zingiberaceae
Components	**RI**	**Cc**	**La[i]**	**Ln**	**Eb[ii]**	**Ebo[ii]**	**Ec[ii]**	**Eci[ii]**	**Ect[ii]**	**Eco[ii]**	**Ed[ii]**	**Ef[ii]**	**Eg[ii]**	**Ep[ii]**	**Epo[ii]**	**Er[ii]**	**Es[ii]**	**Esm[ii]**	**Eu[ii]**	**Ev[ii]**	**Mco**	**Sa**	**Pu1[i]**	**Pu2[i]**	**Cci1[i]**	**Cci2**	**Cci3**	**Cci3 H**	**Cci3 O**	**Cl1**	**Cl2**	**Cl3**	**Cs1**	**Cs2**	**Cs3**	**Cs4**	**Rg1[i]**	**Rg2[i]**	**Rg3**	**Ac[i]**	**Zo**
cis-Dihydrocarvone	1159																																						
trans-Mentha-1,(7),8-dien-2-ol*	1159							0.4		0.1			0.1				t	0.3																					
Methyl salicylate	1159																				0.1																		
α-Terpineol	1159	0.2	0.9	2.4	6.9	1.6	1.2	3.4	0.4	4.4	0.6	0.2	0.6	2.7	0.4	6.3	1.4	1.5	0.3	2.5	3.5		0.8	0.6						0.2	1.4	2.1	0.8	0.3	0.3	0.5				2.0	1.9
Methyl chavicol (= Estragole)	1163																				0.3																		0.2	
trans-Dihydrocarvone	1164																																							
Verbenone	1164						0.1																																	
2-Decanone	1166																																			t	t	0.5		
Dihydrocarveol	1167																																							
Myrtenol	1168		0.1		t								t	t			0.1			t																			t
Hexyl butanoate	1173																						t	t																
n-Decanal	1180																												t	t	t		t	t	0.5					
trans-Carveol	1189				t	t	t	t		t			t	0.1			t	t		t	t																		0.5	
Bornyl formate	1199																																							
Cuminaldehyde	1200					t																																		
cis-*p*-Mentha-1,(7),8-dien-2-ol*	1200							1.6		0.5								0.4	t	t																				
α-Fenchyl acetate	1201					t																																		
cis-Carveol	1202				t		t	t					t				t																						0.2	
Nerol	1206																																t							
Citronellol	1207			0.1					12.4					t									t	t						t	t	t		t	t	0.2				14.4
Thymol methyl ether	1210																																							

		Lauraceae			Myrtaceae																		Pittosporaceae		Poaceae / Gramineae					Rutaceae									Verbenaceae	Zingiberaceae
Components	**RI**	**Cc**	**La[i]**	**Ln**	**Eb[ii]**	**Ebo[ii]**	**Ec[ii]**	**Eci[ii]**	**Ect[ii]**	**Eco[ii]**	**Ed[ii]**	**Ef[ii]**	**Eg[ii]**	**Ep[ii]**	**Epo[ii]**	**Er[ii]**	**Es[ii]**	**Esm[ii]**	**Eu[ii]**	**Ev[ii]**	**Mco**	**Sa**	**Pu1[i]**	**Pu2[i]**	**Cci1[i]**	**Cci2**	**Cci3**	**H**	**O**	**Cl1**	**Cl2**	**Cl3**	**Cs1**	**Cs2**	**Cs3**	**Cs4**	**Rg1[i]**	**Rg2[i]**	**Rg3**	**Ac[i]**	**Zo**
cis-Ocimenone	1210																																						
Pulegone	1210																																					0.3	t
Carvone	1210	t			t		t	t		t			t				t																					0.3	
Neral	1210																							28.6	19.6	21.5	9.0	35.7	6.4	0.3	5.5		t	t	0.2				10.0	
cis-Piperitone epoxide	1211																																						
trans-Ocimenone	1211																																t						
Piperitone	1211										40.2		t	t		0.5								t	t	t													0.3	
trans-Chrysanthenyl acetate*	1213																																						
2-Methyl butyric acid hexyl ester	1220																						t	0.1															
cis-Anethole	1220																																						
2-*trans*-Decenal	1224																																						
Carvacrol methyl ether	1224																																						
2-Phenyl ethyl acetate	1228												t	t																									
Geraniol	1236	t			0.1	t								t		t	t			t	0.5			0.5	13.8	17.7		5.2	t	t	t		t	t	t				0.7	t
Perilla aldehyde	1237																																	t	t				
Geranial	1240																							42.5	22.6	33.7	1.6	45.4	5.6	1.0	6.5		t	0.1	0.4				12.3	29.2
cis-Chrysanthenyl acetate	1241																																						
Linalyl acetate	1245			t																																			
Methyl citronellate	1245																						t	t															
Dehydrocarvacrol	1252																																						
trans-Anethole	1254																																					0.2	

		Lauraceae			Myrtaceae																		Pittosporaceae	Poaceae / Gramineae					Rutaceae										Verbenaceae	Zingiberaceae
Components	**RI**	**Cc**	**La[i]**	**Ln**	**Eb[ii]**	**Ebo[ii]**	**Ec[ii]**	**Eci[ii]**	**Ect[ii]**	**Eco[ii]**	**Ed[ii]**	**Ef[ii]**	**Eg[ii]**	**Ep[ii]**	**Epo[ii]**	**Er[ii]**	**Es[ii]**	**Esm[ii]**	**Eu[ii]**	**Ev[ii]**	**Mco**	**Sa**	**Pu1[i]**	**Pu2[i]**	**Cci1[i]**	**Cci2**	**Cci3**	**H**	**O**	**Cl1**	**Cl2**	**Cl3**	**Cs1**	**Cs2**	**Cs3**	**Cs4**	**Rg1[i]**	**Rg2[i]**	**Rg3**	**Ac[i]**	**Zo**
n-Decanol	1259											t																												
Thymyl formate	1262																			t																				
Citronellyl formate*	1262																																							
Neryl formate	1263																																							
Bornyl acetate	1265	6.5	2.6			t						0.2		0.5																										t
Nonanoic acid	1273																																							
Perilla alcohol	1274																																							
Thymol	1275												t						t	0.1																				
2-Undecanone	1275		t		t																			0.4	t	t	0.3	0.5								94.4	92.8	90.8		t
Neryl acetate	1275																																							
trans-Sabinyl acetate	1277																																							
trans-Pinocarvyl acetate	1278		0.1																																					
Menthyl acetate	1278																																							
Lavandulyl acetate	1278																																							
Geranyl formate	1285																																							
Carvacrol	1286				t	t			t		t		t			t		t	t	0.1																			t	
cis-Theaspirane	1288																																							
n-Undecanal	1288																																							
Dihydrocarvyl acetate	1288																																							
Methyl geranate*	1288					t														t																				
Piperitenone	1289																																							
Myrtenyl acetate	1290												t						t		5.0																			
Terpinen-4-yl acetate*	1297																																							

		Lauraceae			Myrtaceae																		Pittosporaceae	Poaceae / Gramineae				Rutaceae										Verbenaceae	Zingiberaceae
Components	**RI**	**Cc**	**La[i]**	**Ln**	**Eb[ii]**	**Ebo[ii]**	**Ec[ii]**	**Eci[ii]**	**Ect[ii]**	**Eco[ii]**	**Ed[ii]**	**Ef[ii]**	**Eg[ii]**	**Ep[ii]**	**Epo[ii]**	**Er[ii]**	**Es[ii]**	**Esm[ii]**	**Eu[ii]**	**Ev[ii]**	**Mco**	**Sa**	**Pu1[i]**	**Pu2[i]**	**Cci1[i]**	**Cci2**	**Cci3**	**Cci3 H**	**Cci3 O**	**Cl1**	**Cl2**	**Cl3**	**Cs1**	**Cs2**	**Cs3**	**Cs4**	**Rg1[i]**	**Rg2[i]**	**Rg3**	**Ac[i]**	**Zo**
Methyl anthranilate	1300																															0.1							
trans-Theaspirane	1300																																						
iso-Dihydrocarvyl acetate	1310																																						
4A-α, 7-α, 7Aα-Nepetalactone	1319																																						
Geranyl 2-propyl ether	1322																							t	t	t													
Eugenol	1327			0.5																	92.3																		
2-Phenyl ethyl propanoate	1328																																						
Thymyl acetate	1330																																						
Piperitenone oxide	1330																																						
δ-Elemene	1332				t	t									0.1	t							0.1	t						t										
α-Terpinyl acetate	1334	0.2	0.5	12.8				10.0		4.8			0.7						1.5		1.9																			
UI A	1334																																						
Citronellyl acetate	1343								2.8																				t	t	t		t						t
α-Cubebene	1345																						t	t																
cis-Carvyl acetate*	1346																																						
Hydrocinnamyl acetate*	1346		0.1	t																																			
Carvacryl acetate	1348																																						
Nepetalactone (2 unidentified isomers)	1348																																						
Neryl acetate	1353			t																									5.6	t	0.1		t						

		Lauraceae			Myrtaceae																		Pittosporaceae		Poaceae / Gramineae					Rutaceae										Verbenaceae	Zingiberaceae
Components	**RI**	**Cc**	**La[i]**	**Ln**	**Eb[ii]**	**Ebo[ii]**	**Ec[ii]**	**Eci[ii]**	**Ect[ii]**	**Eco[ii]**	**Ed[ii]**	**Ef[ii]**	**Eg[ii]**	**Ep[ii]**	**Epo[ii]**	**Er[ii]**	**Es[ii]**	**Esm[ii]**	**Eu[ii]**	**Ev[ii]**	**Mco**	**Sa**	**Pu1[i]**	**Pu2[i]**	**Cci1[i]**	**Cci2**	**Cci3**	**H**	**O**	**Cl1**	**Cl2**	**Cl3**	**Cs1**	**Cs2**	**Cs3**	**Cs4**	**Rg1[i]**	**Rg2[i]**	**Rg3**	**Ac[i]**	**Zo**
trans-β-Damascenone	1356																																						
Geranyl acetate	1370		0.2	t		t							t	t			t		t	t	3.5				t	0.5	0.3	1.3	0.3	1.2	t	t		t					0.1	t
α-Ylangene	1371			t																																			
cis-Jasmone	1372																																						
α-Copaene	1375				t									t									t	0.1														1.6	t
Methyl eugenol	1377			6.1																	2.1																		
β-Bourbonene	1379																																					0.7	
α-Bourbonene*	1379																																						
β-Cubebene	1385																						t	t															
β-Elemene	1388	0.1	2.0	1.4		t									0.1	t					t		t	0.2							0.8	1.5							t
2-Dodecanone*	1389																																			t	t	0.8		
α-Gurjunene	1400			t	0.3	t	0.1	t			t									0.4																			
α-Cedrene	1400																																					0.6	
Decyl acetate	1400																																						
Acora-3,5-diene	1414																																						
β-Cedrene	1414																																						
trans-β-Caryophyllene	1414	1.1	1.1	0.9	0.1		t	0.6	6.1			0.1	0.2	t	4.8				1.9		1.4	1.9	t	0.1	t	0.1	1.2	0.8		1.6	0.1	t	0.5	0.6	t	t			1.7	t
trans-Cinnamyl acetate	1414		4.8	0.9																																			
trans-Isoeugenol	1422		t																																				
β-Gurjunene	1426				0.2		t													0.3																			
β-Copaene*	1426																		t																			0.1	
Aromadendrene	1428				3.0	0.2	0.2	0.2	0.3	0.3	t	0.1	0.4	0.3	0.1	t	t		0.2	2.8																			
UI B	1430																																						

		Lauraceae			Myrtaceae																		Pittosporaceae		Poaceae / Gramineae					Rutaceae										Verbenaceae	Zingiberaceae
Components	**RI**	**Cc**	**La[i]**	**Ln**	**Eb[ii]**	**Ebo[ii]**	**Ec[ii]**	**Eci[ii]**	**Ect[ii]**	**Eco[ii]**	**Ed[ii]**	**Ef[ii]**	**Eg[ii]**	**Ep[ii]**	**Epo[ii]**	**Er[ii]**	**Es[ii]**	**Esm[ii]**	**Eu[ii]**	**Ev[ii]**	**Mco**	**Sa**	**Pu1[i]**	**Pu2[i]**	**Cci1[i]**	**Cci2**	**Cci3**	**H**	**O**	**Cl1**	**Cl2**	**Cl3**	**Cs1**	**Cs2**	**Cs3**	**Cs4**	**Rg1[i]**	**Rg2[i]**	**Rg3**	**Ac[i]**	**Zo**
trans-Ethyl cinnamate	1431			0.1																																			
Selina-1,5-diene	1432				0.1																																		
Geranyl acetone	1434																																					0.1	
trans-α-Bergamotene	1434		0.1																				t	0.1	t	0.4	1.0	0.9		t	0.1	t								
cis-trans-α-Farnesene*	1436																								t	t	0.3													
Sesquisabinene B	1438																								t	t	0.2													
cis-Muurola-3,5-diene*	1445																																							
Citronellyl propanoate*	1446																																							
Guaia-6,9-diene *	1447																																							
α-Humulene	1447	0.2	0.5	0.3	t			t	0.4			t	0.1		0.5				0.2		0.5	0.2			t	t	t			0.2			0.1	t						0.1	
γ-Thujaplicin*	1447																																							
trans-β-Farnesene	1455		0.2																						t	t	t	0.5				0.2	0.2							
Cabreuva oxide A*	1455																																							
allo-Aromadendrene	1456				1.0	0.3	0.1	0.1	0.1		0.3	t	0.1	0.2	0.2	0.1	t		0.2	0.8																			0.7	0.2
trans-β-Ionone	1456																																							
Geranyl propionate	1461																																							
Cabreuva oxide B*	1463																																							
Geraniol butyl ether	1466																																							
γ-Gurjunene	1467				t																																			
Phenyl ethyl 2-methyl butanoate*	1467												t							t																					
γ-Muurolene	1469				t																		t	t																t
α-Amorphene	1469																																							
Dehydroaromadendre	1469					t				0.1																															

		Lauraceae			Myrtaceae																		Pittosporaceae		Poaceae / Gramineae					Rutaceae										Verbenaceae	Zingiberaceae
Components	**RI**	**Cc**	**La[i]**	**Ln**	**Eb[ii]**	**Ebo[ii]**	**Ec[ii]**	**Eci[ii]**	**Ect[ii]**	**Eco[ii]**	**Ed[ii]**	**Ef[ii]**	**Eg[ii]**	**Ep[ii]**	**Epo[ii]**	**Er[ii]**	**Es[ii]**	**Esm[ii]**	**Eu[ii]**	**Ev[ii]**	**Mco**	**Sa**	**Pu1[i]**	**Pu2[i]**	**Cci1[i]**	**Cci2**	**Cci3**	**H**	**O**	**Cl1**	**Cl2**	**Cl3**	**Cs1**	**Cs2**	**Cs3**	**Cs4**	**Rg1[i]**	**Rg2[i]**	**Rg3**	**Ac[i]**	**Zo**
ne*																										Cci3 H	Cci3 O												
trans-Methyl isoeugenol	1471																				0.1																		1.2
Germacrene-D	1474	0.2	0.2	0.1											0.1					t			t	t														
α-Curcumene	1475																																					6.9	0.3
γ-Curcumene	1475																																						
β-Selinene	1476			0.3	0.1																																		
γ-Humulene	1477	0.9																																					
cis-β-Guaiene*	1478																																						
2-Tridecanone	1479																																			t	t	0.1		
Eremophilene*	1480																																						
Valencene	1484			0.1																									t	t		0.1	0.2					0.4
Dodecanol allyl ether	1488																							0.2	t	0.4													
trans-Dehydroagarofuran	1489																																						
Viridiflorene	1487		0.1		0.4	0.6	0.1	0.5		0.4			t	0.1						0.5																			
Bicyclogermacrene	1487	0.9	1.5	0.5							0.4	0.1			2.2	0.2			1.1				1.6	1.5					0.5	t	t		0.1						0.5	
3,3,5,5,8,8-Hexamethyl-7-oxabicyclo[4.3.0]non-1(6)-ene-2,4-dione*	1488																				0.8																			
α-Zingiberene	1492																																						2.5
Eugenol acetate	1493																					4.6																	
Myristicin	1493																																						

		Lauraceae			Myrtaceae																	Pittosporaceae		Poaceae / Gramineae					Rutaceae										Verbenaceae	Zingiberaceae
Components	**RI**	**Cc**	**La[i]**	**Ln**	**Eb[ii]**	**Ebo[ii]**	**Ec[ii]**	**Eci[ii]**	**Ect[ii]**	**Eco[ii]**	**Ed[ii]**	**Ef[ii]**	**Eg[ii]**	**Ep[ii]**	**Epo[ii]**	**Er[ii]**	**Es[ii]**	**Esm[ii]**	**Eu[ii]**	**Ev[ii]**	**Mco**	**Sa**	**Pu1[i]**	**Pu2[i]**	**Cci1[i]**	**Cci2**	**Cci3**	**Cci3 H**	**Cci3 O**	**Cl1**	**Cl2**	**Cl3**	**Cs1**	**Cs2**	**Cs3**	**Cs4**	**Rg1[i]**	**Rg2[i]**	**Rg3**	**Ac[i]**	**Zo**
α-Muurolene	1494				t														t				t	t																
α-*trans*, *trans*-Farnesene	1500																						t	0.2								0.2	0.3							0.5
β-Bisabolene	1500		0.4																				t	0.2					0.1	0.1	0.1									0.4
γ-Cadinene	1500		0.1	0.2	0.1							t							0.1	t									t										0.3	
7-epi-α-Selinene	1500			0.1																																				
trans-Calamene	1505											t											t	0.2																
δ-Cadinene	1505		0.1	0.2	0.1	t						0.1							0.2	t			t	0.1															0.2	
β-Sesquiphellandrene	1508		t																														t							1.3
Kessane*	1517																																							
Elemicin	1525			0.7																																				
α-Calacorene	1525				t																																			
α-Cadinene	1529																																							
Elemol	1530														0.7																									0.3
Germacrene B*	1533								0.1				t								t																			
trans-α-Bisabolene	1536		15.3	0.7																																				
Geranyl butyrate	1544																							t	t	t														
γ-Maaliene*	1544												0.1							0.2																					
trans-Nerolidol	1549		0.3																														0.5						1.4	0.1
β-Caryophyllene alcohol	1550					t		t					t		0.1				t	0.1																				
ar-Turmerol	1551																																							
Spathulenol	1551		t	0.3	0.1	0.3			0.2		0.1	0.2	t	0.1	1.5	0.2			0.2	0.1			t	0.1															4.9	
Dodecanoic acid	1551																																							

		Lauraceae			Myrtaceae																		Pittosporaceae		Poaceae / Gramineae					Rutaceae										Verbenaceae	Zingiberaceae
Components	**RI**	**Cc**	**La[i]**	**Ln**	**Eb[ii]**	**Ebo[ii]**	**Ec[ii]**	**Eci[ii]**	**Ect[ii]**	**Eco[ii]**	**Ed[ii]**	**Ef[ii]**	**Eg[ii]**	**Ep[ii]**	**Epo[ii]**	**Er[ii]**	**Es[ii]**	**Esm[ii]**	**Eu[ii]**	**Ev[ii]**	**Mco**	**Sa**	**Pu1[i]**	**Pu2[i]**	**Cci1[i]**	**Cci2**	**Cci3**	**H**	**O**	**Cl1**	**Cl2**	**Cl3**	**Cs1**	**Cs2**	**Cs3**	**Cs4**	**Rg1[i]**	**Rg2[i]**	**Rg3**	**Ac[i]**	**Zo**
Phenyl ethyl tiglate	1553																																						
Furopelargone A *	1558																																						
β-Caryophyllene oxide	1561		0.1	0.6	0.1	0.2			0.4										0.2		0.1				t	0.6	0.5												7.9	
Globulol	1566				2.5	0.4	0.4	0.7	0.5	0.5	0.1	1.1	1.7	0.4	1.3	0.1	0.1		0.4	4.3			t	0.2																
Viridiflorol	1569		0.1	0.1	0.5		0.1				0.1				0.9	0.1				0.6			0.1	0.1																
trans-Nuciferyl acetate	1571																																					0.7	
Guaiol	1575				0.3		0.2																t	0.2																
Anhydro-oplopanone	1576																																						
Cedrol	1579																																						
Ledol	1580					0.1						0.1		0.1					0.1	0.2																				
Humulene epoxide	1580																																						
Geranyl 2-methyl butyrate	1586																																						
Geranyl isovalerate	1590					t																			t	t	t													
10-epi-γ-Eudesmol	1593																								0.1	0.3	1.6													
UI C	1597																																						
epi-Cubenol	1600																																						
UI D	1609																																						
γ-Eudesmol	1609				0.1		1.2			0.2				0.5	6.4			0.1	0.2																					
epi-α-Cadinol	1616			t	0.1							0.1							0.3				t	t															1.1	0.3
δ-Cadinol	1618			t																																				
α-Muurolol	1618												t						0.1	0.1																				
β-Eudesmol	1620			0.2	0.1	0.1	2.5		t	0.4		0.1		0.6	7.7			1.1	0.1	0.1																				0.3
β-Sinensal*	1622																															0.7	0.7							

		Lauraceae			Myrtaceae																	Pittosporaceae		Poaceae / Gramineae					Rutaceae										Verbenaceae	Zingiberaceae
Components	**RI**	**Cc**	**La**[i]	**Ln**	**Eb**[ii]	**Ebo**[ii]	**Ec**[ii]	**Eci**[ii]	**Ect**[ii]	**Eco**[ii]	**Ed**[ii]	**Ef**[ii]	**Eg**[ii]	**Ep**[ii]	**Epo**[ii]	**Er**[ii]	**Es**[ii]	**Esm**[ii]	**Eu**[ii]	**Ev**[ii]	**Mco**	**Sa**	**Pu1**[i]	**Pu2**[i]	**Cci1**[i]	**Cci2**	**Cci3**	**Cci3 H**	**Cci3 O**	**Cl1**	**Cl2**	**Cl3**	**Cs1**	**Cs2**	**Cs3**	**Cs4**	**Rg1**[i]	**Rg2**[i]	**Rg3**	**Ac**[i]	**Zo**
Valerianol	1623				0.2														0.4																				
Cubenol	1624																																						
Intermedeol	1626																			0.1				t	t	0.1													
α-Cadinol	1626																					0.2	0.4																
UI E	1626																																			5.1	7.1	7.3		
Geranyl valerate	1633																																							
α-Eudesmol	1634					0.1	1.2			0.2		0.1		0.7	5.5			0.2		t																				0.3
α-Muurolol	1634																																							
Anastreptene*	1634										0.1																													
Cadalene	1640																																							
Apiole	1640																																							
UI F	1641																																							
Citronellyl tiglate	1643																																							
Acorenone B*	1645																																							
UI G	1648																																							
α-Bisabolol	1656		0.1																																					
epi-α-Bisabolol	1658																																							
UI H	1662																																							
α-Sinensal*	1667																															0.5	t							
Geranyl tiglate	1671																																							
trans,*cis*-Farnesol	1693																					t	t									1.7								
n-Nonadecane	1900																															0.1								
Palmitic acid	1908																																							
epi-13-Manool	1946																																							

		Lauraceae			Myrtaceae																		Pittosporaceae		Poaceae / Gramineae					Rutaceae										Verbenaceae	Zingiberaceae
Components	**RI**	**Cc**	**La**[i]	**Ln**	**Eb**[ii]	**Ebo**[ii]	**Ec**[ii]	**Eci**[ii]	**Ect**[ii]	**Eco**[ii]	**Ed**[ii]	**Ef**[ii]	**Eg**[ii]	**Ep**[ii]	**Epo**[ii]	**Er**[ii]	**Es**[ii]	**Esm**[ii]	**Eu**[ii]	**Ev**[ii]	**Mco**	**Sa**	**Pu1**[i]	**Pu2**[i]	**Cci1**[i]	**Cci2**	**Cci3**	**H**	**O**	**Cl1**	**Cl2**	**Cl3**	**Cs1**	**Cs2**	**Cs3**	**Cs4**	**Rg1**[i]	**Rg2**[i]	**Rg3**	**Ac**[i]	**Zo**
Falcarinol	2000																																							
Phyllocladene	2006																																							
Abietatriene	2027																																							
Phytyl acetate	2047																																							
Abietadiene	2060																																							
cis-Totarol methyl ether	2175																																							
trans-Totarol	2234																																							
Phytyl acetate 2	2243																																							
n-Tricosane	2300																																							
n-Pentacosane	2500																																							
% Identification		99.6	98.6	98.1	97.9	98.0	99.6	99.0	95.8	99.5	99.9	99.9	98.6	98.4	96.0	99.3	99.9	99.9	99.3	97.2	99.0	99.1	99.0	99.3	99.7	99.6	99.0	90.4	90.0	99.5	99.9	100.0	100.0	100.0	100.0	99.8	94.9	92.9	92.7	83.6	10.0
Monoterpene hydrocarbons		35.4	59.0	22.1	19.9	58.0	37.3	13.2	3.3	14.5	53.5	95.4	19.0	86.9	33.5	32.2	44.1	11.7	67.5	36.1	39.6		74.5	78.7	25.2	38.2	19.5	73.2	0.1	77.0	78.3	69.1	79.4	88.8	97.1	93.5				12.7	41.6
Oxygen-containing monoterpenes		60.8	12.5	61.7	68.5	37.7	56.2	83.7	84.2	82.9	45.3	2.4	77.0	8.5	30.3	66.4	55.7	86.8	25.9	50.5	52.9		22.5	16.7	73.8	60.0	74.2	14.7	88.3	20.0	20.9	30.8	15.2	7.8	2.0	4.0				34.4	49.2
Sesquiterpene hydrocarbons		3.4	21.6	4.8	5.4	1.1	0.5	1.4	7.0	0.7	0.8	0.4	0.9	0.6	8.1	0.3	t		3.9	5.0	1.9	2.1	1.7	2.7	t	0.5	2.7	2.2		2.4	0.3	0.1	1.8	2.7	0.1	0.2				13.4	5.6
Oxygen-containing sesquiterpenes			0.6	1.2	4.0	1.2	5.6	0.7	1.1	1.4	0.3	1.7	1.7	2.4	24.1	0.4	0.1	1.4	2.0	5.6	0.1		0.3	1.0	0.1	0.9	2.2						3.4	0.7						16.0	1.3
Diterpene hydrocarbons																																									
Oxygen-containing																																									

		Lauraceae			Myrtaceae																		Pittosporaceae	Poaceae / Gramineae					Rutaceae										Verbenaceae	Zingiberaceae
Components	**RI**	**Cc**	**La[i]**	**Ln**	**Eb[ii]**	**Ebo[ii]**	**Ec[ii]**	**Eci[ii]**	**Ect[ii]**	**Eco[ii]**	**Ed[ii]**	**Ef[ii]**	**Eg[ii]**	**Ep[ii]**	**Epo[ii]**	**Er[ii]**	**Es[ii]**	**Esm[ii]**	**Eu[ii]**	**Ev[ii]**	**Mco**	**Sa**	**Pu1[i]**	**Pu2[i]**	**Cci1[i]**	**Cci2**	**Cci3**	**H**	**O**	**Cl1**	**Cl2**	**Cl3**	**Cs1**	**Cs2**	**Cs3**	**Cs4**	**Rg1[i]**	**Rg2[i]**	**Rg3**	**Ac[i]**	**Zo**
diterpenes																																								
Phenylpropanoids			t	7.3																	2.5	96.9																0.4	1.2
Polyacetylenes																																								
C11 molecules																																								
C12 molecules																																				0.5	0.1	0.5		
C13 molecules																																							0.1	
N-containing molecules																																0.1								
Fatty acids																																								
Others			4.9	1.0	0.1	t	t	t	0.2	t	t	t	t	t			t	t	t	t	2.0	0.1	t	0.2	0.6	t	0.4	0.3	1.6	0.1	0.4	t	0.1	t	0.8	2.1	94.4	92.8	92.2	6.6	1.1

RI, Calculated retention index relative to C_8-C_{25} *n*-alkanes on the DB-1 column; t, trace (<0.05%); UI = unidentified compounds. *identification based on mass spectra only. [i] - EOs previously tested (Barbosa et al., 2010; Barbosa et al., 2012). [ii] - EOs composition previously reported (Faria et al., 2011).

Annex 2

Eucalyptus from Mata Experimental do Escaroupim (Portugal): evaluation of the essential oil composition from sixteen species

Eucalyptus from Mata Experimental do Escaroupim (Portugal): Evaluation of the Essential Oil Composition from Sixteen Species

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Keywords: *Myrtaceae*, *Eucalyptus* spp., essential oils, cluster analysis, GC, GC-MS, Escaroupim

Abstract

The essential oils isolated from the vegetative aerial parts (mature branches with leaves) of 16 *Eucalyptus* species, grown in Mata Experimental do Escaroupim (Salvaterra de Magos, Portugal) were studied. The essential oils from *E. bosistoana* F. Muell., *E. botryoides* Sm., *E. camaldulensis* Dehnh., *E. cinerea* F. Muell., *E. citriodora* Hook., *E. cordieri* Trabut, *E. dives* Schauer, *E. ficifolia* F. Muell., *E. globulus* Labill., *E. pauciflora* Sieber ex Spreng., *E. polyanthemos* Schauer, *E. radiata* Sieber ex DC, *E. saligna* Sm., *E. smithii* R.T. Baker, *E. urophylla* S.T. Blake and *E. viminalis* Labill. were analyzed by GC and GC-MS, and the percentage composition of the volatiles was used to determine the relationship between the different oil samples by cluster analysis. Cluster analysis showed a high correlation ($S_{corr} \geq 0.80$) among 11 species (*E. bosistoana*, *E. botryoides*, *E. camaldulensis*, *E. cinerea*, *E. cordieri*, *E. globulus*, *E. polyanthemos*, *E. radiata*, *E. saligna*, *E. smithii* and *E. viminalis*), mainly due to their richness in 1,8-cineole (27-83%). The remaining 5 species were dominated by citronellal (36%, *E. citriodora*), piperitone (40%, *E. dives*), limonene and α-pinene (41 and 44%, respectively, *E. ficifolia*), α-pinene (82%, *E. pauciflora*) and α-phellandrene (45%, *E. urophylla*).

INTRODUCTION

Eucalyptus genus, which includes more than 700 species, is mainly used for the production of timber, firewood (as well as charcoal), pulp, and for its essential oils, employed in the pharmaceutical and perfumery industries (Brophy and Southwell, 2002). *Eucalyptus* essential oils are chiefly characterized by their 1,8-cineole (eucalyptol) content, important for the pharmaceutical industry, or by its aroma, in perfumery. It is also valued for its antimicrobial and antiseptic properties, among others (Batish et al., 2008), and is commonly used to alleviate breathing afflictions.

The introduction of eucalyptus in Portugal seems to have been part of a general movement, by the mid-nineteenth century, of ordering exotic plants to embellish parks and gardens. Nevertheless, given the favourable edaphoclimatic conditions, *Eucalyptus globulus* was fast in becoming an unavoidable element of the Portuguese forest (Pereira, 2007; Radich, 2007).

At present, the eucalyptus is the third most representative forest species in Portugal, following cork oak (*Quercus suber* L.) and maritime pine (*Pinus pinaster* Aiton). Portugal, with a paper manufacturing industry since the end of the 14th century, was the first country to manufacture chemical pulp from eucalyptus. The pulp and paper sectors contribute strongly for the Portuguese economy, being the 4th largest net exporter, after textile, leather and wood industries (CELPA, 2008).

In addition to the use of raw *Eucalyptus globulus* wood material in pulp and paper

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industry, its fresh, or dried, leaves are traditionally used in Portugal, in the treatment of several ailments (Salgueiro, 2004), and the dried leaves sole or as one of the ingredients of herbal mixtures are sold in herbal shops throughout the country. The infusion is used externally in the treatment of hair problems, cutaneous ulcers, measles and all-purposes baths. Internally, the infusion is used against round-worms, diabetes, and urinary disorders. Syrup containing eucalyptus infusion, sweetened with honey, is used against cough. Gargles with the leaf infusion water heal mouth wounds, sore throat and bronchial inflammation. Cigarettes made of mashed partially toasted leaves are considered good for respiratory conditions (Salgueiro, 2004).

The Mata Experimental do Escaroupim (Salvaterra de Magos, Portugal), is an area of protected forest tutored by Autoridade Florestal Nacional, which includes an arboretum with an identified, and documented, collection of 125 different eucalyptus species, considered to be the most complete in Europe (Goes, 1977).

Aiming at performing a comprehensive characterization of the essential oils of the existing species in Mata Experimental do Escaroupim, we herewith report a preliminary study on the essential oils isolated from 16 *Eucalyptus* species grown in this arboretum.

MATERIAL AND METHODS

Plant Material

The vegetative aerial parts (mature branches with leaves, from one sample per species) of *E. bosistoana* F. Muell., *E. botryoides* Sm., *E. camaldulensis* Dehnh., *E. cinerea* F. Muell., *E. citriodora* Hook., *E. cordieri* Trabut, *E. dives* Schauer, *E. ficifolia* F. Muell., *E. globulus* Labill., *E. pauciflora* Sieber ex Spreng., *E. polyanthemos* Schauer, *E. radiata* Sieber ex DC, *E. saligna* Sm., *E. smithii* R.T. Baker, *E. urophylla* S.T. Blake and *E. viminalis* Labill. were collected at Mata Experimental do Escaroupim (Salvaterra de Magos, Portugal), in the spring of 2009 and stored at -20°C until extraction.

Isolation of the Essential Oils

The essential oils were isolated by hydrodistillation for 3 h using a Clevenger-type apparatus according to the European Pharmacopoeia method (Council of Europe, 2007). The isolation procedure was run at a distillation rate of 3 ml min^{-1}, and the essential oils were stored at -20°C in the dark until analysis.

Gas Chromatography

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

Gas Chromatography-Mass Spectrometry

The GC-MS unit consisted on a Perkin Elmer Autosystem XL gas chromatograph, equipped with DB-1 fused-silica column (30 m × 0.25 mm i.d., film thickness 0.25 μm; J&W Scientific, Inc.), and interfaced with Perkin-Elmer Turbomass mass spectrometer (software version 4.1, Perkin Elmer, Shelton, CT). Injector and oven temperatures were as

above; transfer line temperature, 280°C; ion source temperature, 220°C; carrier gas, helium, adjusted to a linear velocity of 30 cm s^{-1}; split ratio, 1:40; ionization energy, 70 eV; scan range, 40-300 u; scan time, 1 s. The identity of the components was assigned by comparison of their retention indices, relative to C_9-C_{17} *n*-alkane indices, and GC-MS spectra from a laboratory made library, based upon the analyses of reference oils, laboratory-synthesized components, and commercial available standards.

Statistical Analysis

The percentage composition of the isolated essential oils was used to determine the relationship between the different samples by cluster analysis using Numerical Taxonomy Multivariate Analysis System (NTSYS-pc software, version 2.2, Exeter Software, Setauket, New York) (Rohlf, 2000). For cluster analysis, correlation coefficient was selected as a measure of similarity among all accessions, and the Unweighted Pair Group Method with Arithmetical Averages (UPGMA) was used for cluster definition. The degree of correlation was evaluated according to Pestana and Gageiro (2000) and classified as very high (0.9-1), high (0.7-0.89), moderate (0.4-0.69), low (0.2-0.39) and very low (<0.2).

RESULTS AND DISCUSSION

The essential oil yields of the *Eucalyptus* species studied varied between 0.4% (v/f.w.) in *E. ficifolia* and *E. polyanthemos*, and 5.6% (v/f.w.) in *E. radiata* (Table 1).

The monoterpene fraction was dominant in all the oils analysed (≥64%). In variable amounts, monoterpene hydrocarbons were dominant (54-95%) in *E. botryoides*, *E. dives*, *E. ficifolia*, *E. pauciflora* and *E. urophylla* (Table 1). Oxygen-containing monoterpenes (51-87%) dominated the oils of *E. bosistoana*, *E. camaldulensis*, *E. cinerea*, *E. citriodora*, *E. cordieri*, *E. globulus*, *E. radiata*, *E. saligna*, *E. smithii* and *E. viminalis*. In *E. polyanthemos* the monoterpene hydrocarbons and the oxygen-containing monoterpenes occurred in similar relative amounts (34:30%).

With the exception of *E. polyanthemos* essential oil, in which the sesquiterpene fraction attained 32%, this fraction was ≤11% in the remaining oils (Table 1).

Essential oil cluster analysis showed a high correlation (S_{corr}≥0.80) among 11 species (*E. bosistoana*, *E. botryoides*, *E. camaldulensis*, *E. cinerea*, *E. cordieri*, *E. globulus*, *E. polyanthemos*, *E. radiata*, *E. saligna*, *E. smithii* and *E. viminalis*), mainly due to their high content in 1,8-cineole (27-83%), (Fig. 1). The essential oils isolated from this group of species showed low correlation with the remaining ones (S_{corr}≤0.44) (Fig. 1). *E. pauciflora* and *E. ficifolia* formed another highly correlated cluster (S_{corr}≥0.74), given the high relative amount of α-pinene in both species (82 and 44%, respectively) and the low amount of 1,8-cineole. The essential oils from the remaining three species were dominated by citronellal (36%, *E. citriodora*), piperitone (40%, *E. dives*), and α-phellandrene (45%, *E. urophylla*).

In general, the yield and the essential oil composition, regarding the main components, reported herewith agree with those published by Brophy and Southwell (2002), for the same species. Nevertheless, some differences were noticed, such as the high percentages of *p*-cymene and α-phellandrene in *E. radiata* and *E. urophylla* oils, respectively, detected in the present study. The differences found may reflect the high flexibility of the chemical pathways leading to terpene synthesis reported by Keszei et al. (2008) for the genus *Eucalyptus* and/or the particular environmental growth conditions.

The sole source of eucalyptus essential oil produced in Portugal is *E. globulus*, which has been planted to meet the demands of the large pulp and paper industry. Portuguese eucalyptus oil export rates have progressively decreased due to the increase of labour costs and the severe competition with the low-priced Chinese eucalyptus oil. It is, therefore, fundamental to improve oil quality, by increasing 1,8-cineole content and decreasing α-phellandrene content, as well as to find alternative essential oil sources, in order to attract industries other than the pharmaceutical, namely perfumery- and arising industries such as that of industrial solvents (Brophy and Southwell, 2002).

Within the species studied, *Eucalyptus smithii* was particularly interesting, as it showed both a high oil yield (2.8%) and was 1,8-cineole-rich (83%) and α-phellandrene free. *E cordieri* and *E. globulus* showed a similar essential oil composition but with lower yields.

E. pauciflora essential oil showed potential for use in the solvents industry given the large relative amount of α-pinene (≥80%). *E. dives* showed a high yield (3.3%), and was relatively rich in the oxygen-containing monoterpene, piperitone (40%), which is the main raw material for the production of synthetic menthol and thymol. *E. citriodora* oil showed a rather high citronellal relative amount (36%) and demonstrated the characteristic lemon-scented fresh fragrance of this species, which is commonly used as ingredient of commercial cleaning products.

Essential oil yield and composition screening of other species of the genus *Eucalyptus* from Mata Experimental do Escaroupim is in progress and this knowledge could be used to support models of development of interesting and high-yield producing species that are economically profitable, simultaneously promoting an ecologically responsible and sustainable management of Portuguese forest.

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Tables

Table 1. Percentage composition of the main essential oils components (present in a relative amount ≥5%, in at least one sample), isolated from the aerial parts of 16 *Eucalyptus* species, grown in Mata Experimental do Escaroupim (Portugal). *E. bosistoana* (Ebos), *E. botryoides* (Ebot), *E. camaldulensis* (Eca), *E. cinerea* (Ecin), *E. citriodora* (Ecit), *E. cordieri* (Eco), *E. dives* (Ed), *E. ficifolia* (Ef), *E. globulus* (Eg), *E. pauciflora* (Epa), *E. polyanthemos* (Epo), *E. radiata* (Er), *E. saligna* (Esa), *E. smithii* (Esm), *E. urophylla* (Eu) and *E. viminalis* (Ev).

Components	*Eucalyptus* species															
	RI	*Ebos*	*Ebot*	*Eca*	*Ecin*	*Ecit*	*Eco*	*Ed*	*Ef*	*Eg*	*Epa*	*Epo*	*Er*	*Esa*	*Esm*	*Eu*	*Ev*
α-Thujene	924	t	0.5	t				5.8	t		t	2.2	3.0			1.2	t
α-Pinene	930	14.2	43.2	31.6	7.7	1.2	6.3	1.0	43.5	15.5	82.2	4.2	2.6	40.2	6.7	2.0	12.5
β-Pinene	963	0.4	2.8	0.4	t	1.6	0.1	t	8.8	0.2	0.2	0.2	0.7	0.2	0.1	2.6	0.2
α-Phellandrene	995		5.7	0.8	t		0.1	19.3	t		t	7.8	4.2			45.3	0.1
p-Cymene	1003	0.1	0.6	0.5	0.1	t	1.1	18.5	t	0.7	0.3	6.4	12.8	0.1	0.8	4.0	6.0
1,8-Cineole	1005	59.3	35.0	50.8	67.4	11.4	71.9	t	2.0	70.3	1.4	27.2	47.9	47.9	83.3	22.6	46.4
β-Phellandrene	1005		t					3.5	t			9.1	t			t	
Limonene	1009	4.5	4.3	3.3	5.3	0.2	6.3	0.7	41.1	2.5	3.0	1.3	4.8	3.1	4.0	3.0	5.1
γ-Terpinene	1035	0.1	0.2	t	t	0.1	0.4	0.6	1.7	t		0.8	1.9	t	t	7.9	11.5
Isopulegol	1116					13.4											
Citronellal	1121					35.8											
neo-Isopulegol	1121					5.8											
Terpinen-4-ol	1148	0.3	0.4	t	0.1	0.1	0.5	4.3	t	t	0.1	2.0	9.1	t	0.1	1.3	1.2
α-Terpineol	1159	6.9	1.6	1.2	3.4	0.4	4.4	0.6	0.2	0.6	2.7	0.4	6.3	1.4	1.5	0.3	2.5
Citronellol	1207					12.4					t						
Piperitone	1211							40.2		t	t		0.5				
α-Terpinyl acetate	1334				10.0		4.8			0.7						1.5	
trans β-Caryophyllene	1414	0.1		t	0.6	6.1			0.1	0.2	t	4.8				1.9	
γ-Eudesmol	1609	0.1		1.2			0.2				0.5	6.4			0.1	0.2	
β-Eudesmol	1620	0.1	0.1	2.5		t	0.4		0.1		0.6	7.7			1.1	0.1	0.1
α-Eudesmol	1634		0.1	1.2			0.2		0.1		0.7	5.5			0.2		t
% of Identification		98.1	98.1	99.6	99.0	95.8	99.5	99.9	99.9	98.6	98.4	96.0	99.3	99.9	99.9	99.3	97.2
Grouped components																	
Monoterpene hydrocarbons		19.9	58.0	37.3	13.2	3.3	14.5	53.5	95.4	19.0	86.9	33.5	32.2	44.1	11.7	67.5	36.1
Oxygen-containing monoterpenes		68.5	37.7	56.2	83.7	84.3	82.9	45.3	2.4	77.0	8.5	30.3	66.4	55.7	86.8	25.9	50.5
Sesquiterpene hydrocarbons		5.6	1.2	0.5	1.4	7.0	0.8	0.8	0.4	0.9	0.6	8.1	0.3	t	t	3.9	5.0
Oxygen-containing sesquiterpenes		4.0	1.2	5.6	0.7	1.1	1.3	0.3	1.7	1.7	2.4	24.1	0.4	0.1	1.4	2.0	5.6
Others		0.1	t	t	t	0.1	t	t	t	t	t	t	t	t	t	t	t
Oil yield (%, v/f.w.)		1.8	1.2	1.4	1.6	0.9	1.1	3.3	0.4	1.3	0.8	0.4	5.6	1.0	2.8	0.9	1.1

RI: Retention index relative to C_9-C_{17} *n*-alkanes on the DB-1 column; t: traces (<0.05%).

Figures

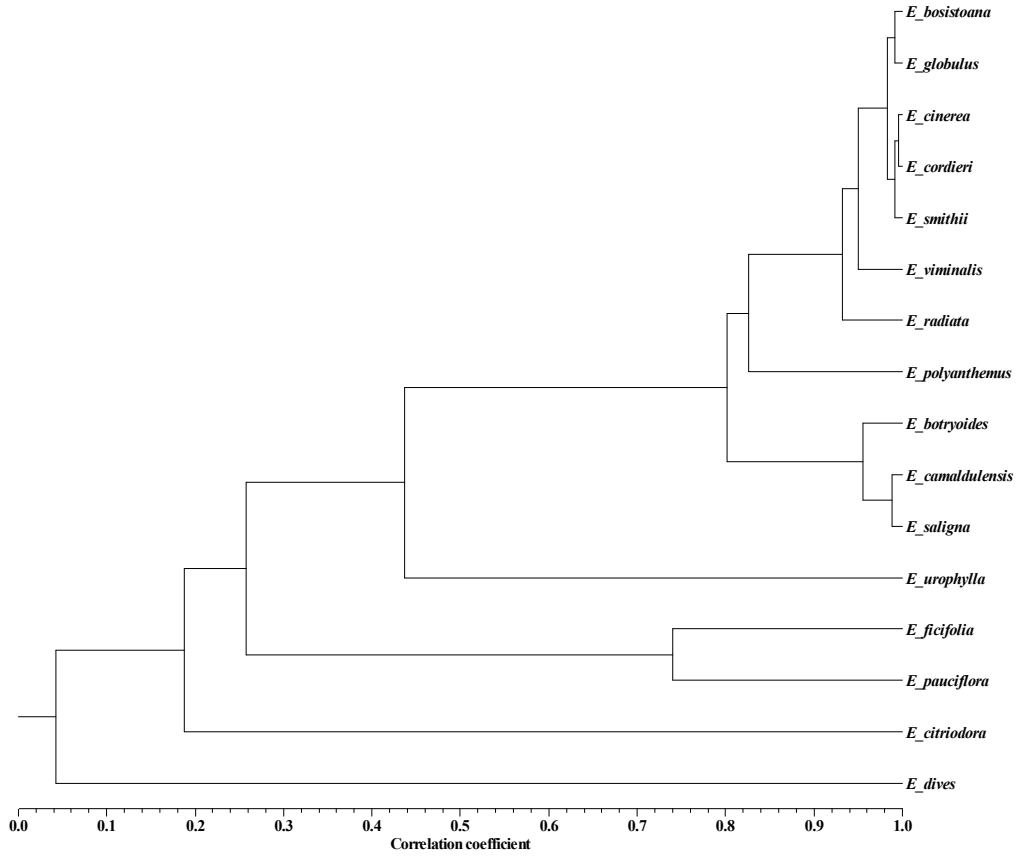

Fig. 1. Dendrogram obtained by cluster analysis of the percentage composition of essential oils from the sixteen *Eucalyptus* species based on correlation and using unweighted pair-group method with arithmetic average (UPGMA).

Annex 3

Supplemmentary data to Chapter 5

Table ST. Percentage composition of the essential oils isolated from 14 species belonging to Amaranthaceae (Am), Apiaceae (Ap), Asteraceae (As), Lamiaceae (L), Myristicaceae (M), Myrtaceae (My), Pinaceae (P), Rosaceae (R), assayed against CRKN egg hatching. For abbreviations and cluster analysis see Table 1 and Fig. 1, respectively.

		Am	Ap		L							M	My	Pe	R
Components	**RI**	**Da**	**Fv2**	**Sv**	**Ll**	**Om**	**So**	**Sm2**	**Thp**	**Thv**	**Thzz**	**Mf**	**Eg**	**Ph**	**Fu**
		_99	**_90**	**_19**	**_87**	**_97**	**_36**	**_97**	**_98**	**_98**	**_99**	**_78**	**_57**	**_50**	**_99**
trans-2-Hexenal	866			0.1											
cis-3-Hexen-1-ol	868			t											
cis-Salvene*	887						t								
trans-Salvene*	893						t								
cis-2-Hexen-1-ol	882			t											
Tricyclene	921			t		t	0.3		t	t				0.1	
α-Thujene	924		t	t		0.8	0.3	0.2	0.8	0.2	1.7	1.0	t	0.3	
3,5-Dimethylene-1,4,4-trimethylcyclopentene	930				1.4										
α-Pinene	930	0.1	1.4	21.2	1.1	0.5	8.0	0.5	0.6	0.6	1.0	1.3	19.7	32.2	
Camphene	938		0.1	0.2	0.2	0.1	7.4	0.1	0.4	0.4	0.9	t	0.1	0.5	
Thuja-2,4(10)-diene	940							t							
n-Heptanol	952						t								
Sabinene	958		t	0.4	t	1.2	0.1		0.2			1t	t	1.5	
1-Octen-3-ol	961				0.1	0.2	t	0.1	2.6	0.1	0.3				t
3-Octanone	961										0.8				
β-Pinene	963		0.3	21.8	0.3	0.6	9.8	0.6	1.9	0.4		0.4	t	2.2	
Dehydro 1,8-cineole	973	t	t		t	0.1		t							
2-Pentyl furan	973		t					t	t						0.1
2,4-Heptadienal	973														t
3-Octanol	974								0.1	t	t				
β-Myrcene	975		0.4	4.2		1.6	2.0	0.9	0.4	1.1	2.3	0.9	0.4	28.7	
α-Phellandrene	995		t	0.1		0.5	0.4	0.2	0.1	0.2	0.4	1.1	t	t	
δ-3-Carene	1000	t	t			t		t	0.1	0.1		t		5.9	
Salicylaldehyde	1000														5.0
Benzene acetaldehyde	1002		t												
α-Terpinene	1002	3.2		t		5.2	0.3	1.1	0.8	1.8	1.5	2.0	t	t	
p-Cymene	1003	6.7	0.1	t	0.3	2.7	0.3	5.7	22.2	21.4	21.8	0.6	0.2	2.4	
1,8-Cineole	1005				15.9		13.0			0.1			65.3		0.2
β-Phellandrene	1005	t	0.7	1.4		1.2	t	0.2	0.7	0.1	0.3	7.4		0.5	
Limonene	1009	0.1	12.1	11.6		0.7	1.4	0.2	0.5	0.5	0.5	5.9	6.7	1.0	0.1
cis-β-Ocimene	1017		0.4	2.9		1.7	1.2	0.2	t	t				t	t
cis-Dehydro-Rose oxide	1020	t													
trans-β-Ocimene	1027		t	7.8		0.4	0.2	t	t	t	t	t		t	t
γ-Terpinene	1035	0.1	0.4	0.1	0.1	13.0	0.4	5.2	6.5	15.9	16.8	3.2	0.4	t	

		Am	Ap		L							M	My	Pe	R
Components	**RI**	**Da**	**Fv2**	**Sv**	**Ll**	**Om**	**So**	**Sm2**	**Thp**	**Thv**	**Thzz**	**Mf**	**Eg**	**Ph**	**Fu**
		_99	**_90**	**_19**	**_87**	**_97**	**_36**	**_97**	**_98**	**_98**	**_99**	**_78**	**_57**	**_50**	**_99**
trans-Sabinene hydrate	1037					0.1	t	t		0.1	0.6	0.3			
2,3,4,5-Tetramethyl-2-cyclopenten-1-enone	1038				1.1										
cis-Linalool oxide	1045				0.6	t	t	t		t					
n-Octanol	1045										t				t
Fenchone	1050		3.9		0.7										
trans-Linalool oxide	1059				0.5		0.1								
2,5-Dimethyl styrene	1059	0.3				0.2		t	0.2	0.1	t			0.1	
Terpinolene	1064		t	0.2		1.7	0.2	0.1	0.1	0.1	0.1	0.7	t	0.1	t
cis-Sabinene hydrate	1066					0.4					t	0.3			
α-Thujone	1073						8.2								
n-Nonanal	1073														0.5
Linalool	1074			0.1	0.6	4.3	1.0	0.5	0.6	1.5	2.2	0.3	0.2	0.4	1.2
2-Methyl butyric acid, isoamyl ester	1074							t							
1,3,8-*p*-Menthatriene	1074	0.1						t	0.1						
β-Thujone	1081						1.4								
trans-*p*-Mentha-2,8-dien-1-ol	1081	0.1					t								
Perillene*	1076		t												
Propionic acid hexyl ester	1079									t					
endo-Fenchol	1085		t	t										0.1	
Albene	1085														
Oct-1-en-3-ol acetate	1086		t		t	0.2									
Myrcenol	1097													0.1	
α-Campholenal	1098				0.2									0.1	
cis-*p*-Mentha-2,8-dien-1-ol	1099	t													
trans-p-2-menthen-1-ol	1099					0.4			t	t		0.5		0.1	
Camphor	1102		t		2.4		7.1			0.1	0.1				t
trans-Pinocarveol	1106				1.0		0.1				t				
allo-Ocimene	1110			0.1		0.5									
cis-*p*-2-Menthen-1-ol	1110					0.3		t		t		0.3			
trans-Limonene oxide	1112		0.1												
cis-Verbenol	1113		t		t	0.1	t	t							
trans-α-Necrodol	1114				1.0										
trans-Verbenol*	1114		0.1			0.1					t				
Menthone	1120					t	0.1		t						
Pinocarvone	1121		t		t										
2-*trans*-Nonen-1-al	1124														t
p-Mentha-1,5-dien-8-ol *	1134		t												
α-Phellandrol*	1134				0.8	0.1								t	

		Am	Ap		L							M	My	Pe	R
Components	**RI**	**Da**	**Fv2**	**Sv**	**Ll**	**Om**	**So**	**Sm2**	**Thp**	**Thv**	**Thzz**	**Mf**	**Eg**	**Ph**	**Fu**
		_99	**_90**	**_19**	**_87**	**_97**	**_36**	**_97**	**_98**	**_98**	**_99**	**_78**	**_57**	**_50**	**_99**
Borneol	1134					0.2	11.5	0.2	1.3	0.7	1.0			t	
NI C *Lavandula luiseri*	1137				1.5										
Lavandulol	1142				0.6										
Rose furan epoxide*	1143	t													
cis-α-Necrodol	1147				1.3										
Menthol	1148					t	0.1		0.1						
Terpinen-4-ol	1148		0.1	0.1		18.2	0.5	0.5	0.9	0.7	0.4	11.2	0.3	2.5	t
p-Cymen-8-ol	1148	0.1						t							
Octanoic acid	1152		t												t
5-Methylene-2,3,4,4-tetramethylcyclopent-2-enone	1152				18.0										
Myrtenal	1153				0.2										
Methyl salicylate	1159								t						85.4
α-Terpineol	1159	0.2	t		0.1	3.1	0.4	0.1	0.2	0.3	t	1.0	1.0	1.0	t
Methyl chavicol	1163		79.2						t						0.6
trans-Dihydrocarvone	1164					0.2					t				
Verbenone	1164				0.6										
Myrtenol	1168						0.1	t							
Hexyl butanoate	1173									t					
n-Decanal	1180														t
cis-Piperitol*	1182	t				0.2		t				0.1			
trans-Piperitol*	1189					0.2						0.4			
trans-Carveol	1189		t		t			t							
Bornyl formate	1199										t				
Cuminaldehyde	1200							t	0.1	t	t				
Lavandulyl formate	1203					t									
Nerol	1206														t
Ascaridole	1209	16.3													
p-Anisaldehyde*	1210		0.1												
Thymol methyl ether	1210					0.1			5.2	0.3					
Pulegone	1210							t		0.1					
Thymoquinone	1210	0.2									t				
Carvone	1210		0.1		t	t	t		0.1						t
Neral	1210						t								
cis-Piperitone epoxide	1211														t
Piperitone	1211											0.1			
Carvotanacetone	1222							t							
2-*trans*-Decenal	1224													0.2	
Carvacrol methyl ether	1224					12.5			2.8	0.1					
Hexyl isovalerate	1225		t												

		Am	Ap		L							M	My	Pe	R
Components	**RI**	**Da**	**Fv2**	**Sv**	**Ll**	**Om**	**So**	**Sm2**	**Thp**	**Thv**	**Thzz**	**Mf**	**Eg**	**Ph**	**Fu**
		_99	**_90**	**_19**	**_87**	**_97**	**_36**	**_97**	**_98**	**_98**	**_99**	**_78**	**_57**	**_50**	**_99**
2-Phenyl ethyl acetate	1228				t										
Geraniol	1236		t	t		0.4			0.1						0.3
Ethyl salicylate	1239														t
Safrole*	1240											40.9			
Linalyl acetate	1245					0.6	0.1								
trans-Anethole	1254		0.5		0.1	0.1	t		t						
Indole	1255							t							
trans-Glycol ascaridole	1258	t													
Cumin alcohol	1260								0.2	0.2					
trans-α-Necrodyl acetate	1265				2.5										
Bornyl acetate	1265			0.3			2.0							0.4	
p-Cymen-7-ol	1265	0.6									t				
NI D *Lavandula luiseri*	1267				2.4										
Nonanoic acid	1273														0.3
Thymol	1275	7.3				0.2	t	0.2	31.7	44.7	0.3				0.3
iso-Ascaridole	1276	51.0													
Lavandulyl acetate	1278				2.3										
cis-α-Necrodyl acetate	1285				1.0										
Carvacrol	1286	6.1	t			16.7	0.1	77.1	1.0	2.5	44.9				0.2
Myrtenyl acetate	1290														
trans-Theaspirane	1300						t	t							
Hexyl tiglate	1316	t													
Thymol acetate	1327								0.1	t					
Eugenol	1327				0.3			t		t		1.4			
α-Terpinyl acetate	1334						0.1			0.2			3.0		
α-Cubebene	1345			t			t	t							
Carvacrol acetate	1348							t			t				
Neryl acetate	1353					0.2									
Decanoic acid	1356														t
Borneol propionate	1361									t					
Geranyl acetate	1370					0.4		t					t	0.1	
Geraniol allyl ether	1371		t												
α-Ylangene	1371						0.2	t							
Heptyl tiglate	1371	t													
trans-β-Damascenone	1372														0.1
α-Copaene	1375						0.3	0.1		t				0.4	
Methyl eugenol	1377			t								2.8			
β-Bourbonene	1379			0.1		t	0.1	0.1	0.4		t				
α-Bourbonene *	1379														
β-Elemene	1388			0.9				t							

		Am	Ap		L							M	My	Pe	R
Components	**RI**	**Da**	**Fv2**	**Sv**	**Ll**	**Om**	**So**	**Sm2**	**Thp**	**Thv**	**Thzz**	**Mf**	**Eg**	**Ph**	**Fu**
		_99	**_90**	**_19**	**_87**	**_97**	**_36**	**_97**	**_98**	**_98**	**_99**	**_78**	**_57**	**_50**	**_99**
α- Gurjunene	1400							t					0.1		
trans-β-Caryophyllene	1414			2.1	0.4	1.6	1.1	3.1	5.7	1.9	1.5			3.6	0.3
β-Copaene *	1426			0.2			0.1	t	0.2						
α-Maaliene	1427							t							
Aromadendrene	1428					0.1	1.4						0.8		
Geranyl acetone	1434								0.1	t					0.1
trans-α-Bergamotene	1434							t							
α-Humulene	1447			0.3		0.1	11.7	0.1	0.2	0.1	t			0.8	t
γ-Thujaplicin*	1447							t							
trans-β-Farnesene	1455								0.1						
trans-β-Ionone	1456														0.1
allo-Aromadendrene	1456						0.1						0.2		
Phenyl ethyl 2-methyl butanoate*	1467													0.2	
Phenyl ethyl 3-methyl butanoate*	1468													0.9	
γ-Muurolene	1469			0.1		0.3	0.4	0.2	0.2	0.1	t				
Octyl tiglate	1469	0.1													
Germacrene-D	1474			14.9					0.2	t	0.2				
β-Selinene	1476						0.1								
Eremophilene*	1480						0.1	t							
γ-Amorphene	1487							0.1							
Viridiflorene	1487						0.5	0.1	0.1						
Bicyclogermacrene	1487			1.2		1.4				t	t				
α-Muurolene	1494			0.4			0.1	t	0.2					0.5	
α-*trans*, *trans*-Farnesene	1500														0.2
β-Bisabolene	1500							0.7	5.6						
γ-Cadinene	1500			0.2			0.2	0.1		0.3	t				
7-*epi*-α-Selinene	1500							0.4							
trans-Calamenene	1505				0.2			t	t	t					
δ-Cadinene	1505			0.8	0.6	0.1	0.5	0.3	0.3	0.2	t			0.2	t
β-Sesquiphellandrene	1508		t												
Elemicin	1525											0.5			
α-Calacorene	1525				0.5		0.1	t		t					
α-Cadinene	1529			0.1											
trans-α-Bisabolene	1536							t							
cis-3-Hexenyl benzoate	1540			0.3					t						
β-Caryophyllene alcohol	1550				0.2										
Dodecanoic acid	1550														0.2
Spathulenol	1551					0.5	0.3	0.3			t				

		Am	Ap		L							M	My	Pe	R
Components	**RI**	**Da**	**Fv2**	**Sv**	**Ll**	**Om**	**So**	**Sm2**	**Thp**	**Thv**	**Thzz**	**Mf**	**Eg**	**Ph**	**Fu**
		_99	**_90**	**_19**	**_87**	**_97**	**_36**	**_97**	**_98**	**_98**	**_99**	**_78**	**_57**	**_50**	**_99**
β-Caryophyllene oxide	1561				0.6	0.2	t	0.2	1.7	0.3	0.3			2.1	t
Globulol	1566				0.4	0.1	t	t					0.7		
Viridiflorol	1569				3.3	t	1.9			t	t				
Guaiol	1575													0.1	
Ledol	1580				1.7									0.9	
Humulene epoxide	1580						0.9			t					
epi-Cubenol	1600							t		0.1					
epi-α-Cadinol	1616			0.1	0.2					0.4				t	
δ-Cadinol	1618					0.2	0.2								
α-Muurolol	1618			0.3	0.2										
Intermedeol	1626													0.1	
α-Cadinol	1626			0.9	6.2			t		t					
α-Bisabolol oxide B	1630														t
NI D *Thymus caespititius*	1662										t				
n-Heptadecane	1700														0.1
Benzyl benzoate	1701			0.3											t
Tetradecanoic acid	1723		t												
Hexadecanal	1776														t
Benzyl salicylate	1790			0.5											
Methyl palmitate	1904							0.2							
Palmitic acid	1908		t												
Phytol acetate	2047	0.3	t												2.1
n-Tricosane	2300														0.5
n-Pentacosane	2500														0.3
% Identification		92.9	100.0	96.3	69.8	96.8	98.5	99.9	97.7	98.0	99.9	94.6	99.1	90.3	98.2
Monoterpene hydrocarbons		10.6	15.9	72.0	4.5	32.6	32.3	15.2	35.6	42.9	47.3	34.5	27.5	75.5	0.1
Oxygen-containing monoterpenes		81.9	4.4	0.5	50.3	59.1	45.9	78.6	44.4	51.6	49.5	14.5	69.8	4.8	2.2
Sesquiterpene hydrocarbons		t	t	21.3	1.7	3.6	17.0	5.3	13.2	2.6	1.7	t	1.1	5.5	0.5
Oxygen-containing sesquiterpenes		t	t	1.3	12.8	1.0	3.3	0.5	1.7	0.8	0.3	t	0.7	3.2	0.1
Phenylpropanoids			79.7	t	0.4	0.1	t	t	t	t		45.6	t		0.6
C13 molecules								t	0.1	t					0.2
Fatty acids			t												0.5
Others		0.4	t	1.2	0.1	0.4	t	0.3	2.7	0.1	1.1		t	1.3	94.0

RI, Calculated retention index relative to C_8-C_{25} *n*-alkanes on the DB-1 column; t, trace (<0.05%); *identification based on mass spectra only.