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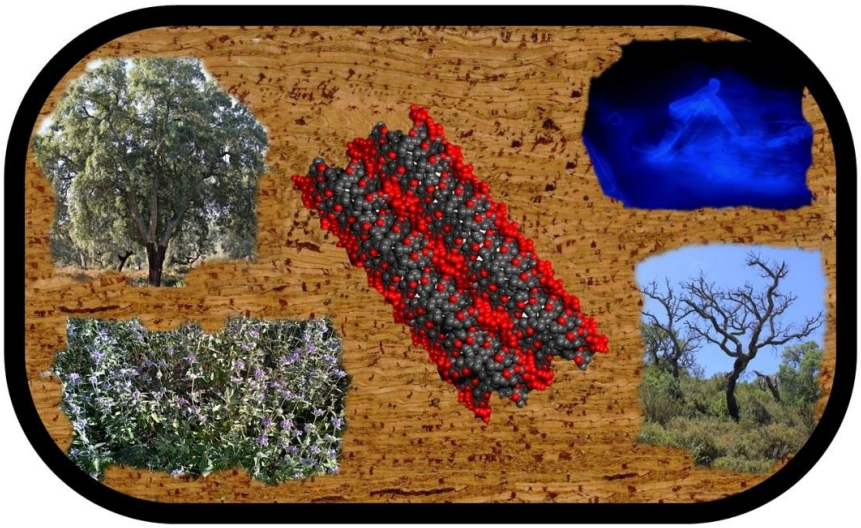
Evaluation of the protective effect of *Phlomis purpurea* against *Phytophthora cinnamomi* in *Fagaceae* and of root metabolites involved



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Evaluation of the protective effect of *Phlomis purpurea* against *Phytophthora cinnamomi* in *Fagaceae* and of root metabolites involved

Author declaration / Declaração de autoria de trabalho

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

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(Dina João Pacheco das Neves)

Dedication / *Dedicatória*

To my beloved family

"Saudade é um sentimento que quando não cabe no coração, escorre pelos olhos." Bob Marley

À memória do meu pai, tia e avós.

Acknowledgements / *Agradecimentos*

This was not a lonely but a rather challenging journey.

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A huge thanks to my family/ *Um enorme obrigada à minha família...*

for all the support, especially in the not so good times. To my mum and parents-in-law, I don't have words to thank you. To my sister and brother and his lovely family thank you for backing me. To my husband, Luís, for always being there for me including helping in greenhouse and field work and the bright discussions about this work, for

his excellent humour and above all, for his unconditional love… To our lovely daughters, Lara and Inês that are children and do not understand my absences…

Thank you to all my friends that gave me motivation and strength throughout this long journey. A special thanks to Daniela Estêvão who always supported me and backed me when I needed the most.

I would like to dedicate this PhD thesis to the memory of my Dad, Custódio Neves, auntie Oliva and grandparents Virgílio and Maria, who passed away during this time. They were great people, who lived for the family and work, and played a big role in my education and in the person I am.

This is part of a poem that makes sense to me right now,

(…)
Ever told your child, we'll do it tomorrow,
and in your haste, not see his sorrow?

Ever lost touch, let a friendship die,
'cause you never had time to call and say hi?

You better slow down, don't dance so fast,
time is short, the music won't last.

When you run so fast to get somewhere,
you miss half the fun of getting there.

When you worry and hurry through your day,
it's like an unopened gift thrown away.

Life isn't a race, so take it slower,
hear the music before your song is over.

By ***David L. Weatherford***

Abstract

Phytophthora cinnamomi Rands devastates natural ecosystems and crops around the world causing enormous economic losses. The "montado" ecosystem is threatened by this highly aggressive pathogen. Much concern involving fungicide use highlighted the need to develop new environmentally friendly means of control.

In this work, *Phlomis purpurea* L., which was recently reported to have activity against *P. cinnamomi* and protect susceptible *Quercus ilex* and *Q. suber* from infection, was further studied.

To determine the protective activity of *P. purpurea* on the infection of susceptible species a greenhouse assay was implemented showing that, when planted next to the host, it was able to significantly reduce the average severity of host's root symptoms. A field assay was set on naturally infested plots to determine the protective effect of this herbaceous plant towards *Q. suber*. *P. purpurea* significantly increased the emergence and survival of *Q. suber.* In both assays *P. purpurea* planted alone was able to completely eliminate *P. cinnamomi*.

In order to study the effectiveness of *P. purpurea* root extracts (PRE) to control *Q. suber* and *Q. ilex* root infection by this oomycete, *in vivo* assays were set up. PRE at 10 mg ml^{-1} significantly inhibited *P. cinnamomi* zoospore infection of both plants. Moreover, PRE was able to elicit a defence response on *Q. suber* roots.

To identify the active principle, bioactivity guided isolation was performed after fractionation of PRE. Isolation, purification and structural characterization led to identification of a novel nortriterpene, phlomispurpentaolone, containing the anti-*P. cinnamomi* activity. Moreover, *P. purpurea* metabolites produced constitutively and upon challenge with *P. cinnamomi* were quantified using LC-MS showing that phlomispurpentaolone is a phytoanticipin.

Another goal was to confirm the resistance of *P. purpurea* to *P. cinnamomi* by histological techniques. *P. purpurea* roots were shown to have a constitutive strengthened exodermis that can act, *per se*, as a physical barrier for the penetration of *P. cinnamomi*.

Keywords: *Phytophthora cinnamomi*, *Phlomis purpurea*, extracts, protection, nortriterpene, metabolomics.

Resumo

Phytophthora cinnamomi Rands é um oomiceta que, tendo uma vasta gama de hospedeiros devasta ecossistemas naturais e culturas em todo mundo, causando enormes prejuízos económicos. O "montado" é um ecossistema agro-silvo-pastoril de alto valor socioeconómico, composto principalmente por sobreiros (*Quercus suber*) e azinheiras (*Quercus ilex* subsp. *rotundifolia*) que estão a ser dizimados por este agente patogénico. O sobreiro reveste-se de particular importância visto que é a árvore de onde se extrai a cortiça. Portugal é o primeiro produtor e exportador de cortiça mas essa posição está em risco dado que os sobreiros estão a ser substituídos por outras árvores mais resistentes a *P. cinnamomi*.

Sendo um agente patogénico do solo dotado de grande capacidade de sobrevivência *P. cinnamomi* é extremamente difícil de erradicar, incluíndo o uso dos fungicidas. A grande preocupação, quer ambiental quer para a saúde, que envolve o uso de fungicidas levou à necessidade de se desenvolverem novos meios ecológicos de controlo.

A marioila (*Phlomis purpurea* L.) é uma herbácea, da família Lamiaceae, espontânea em habitats de *Quercus suber* e *Q. ilex* subsp. *rotundifolia* no sul de Portugal que cresce, sem manifestar sinais de doença, em áreas afetadas pela doença declínio.

Recentemente, foi por nós publicado que o extrato de raízes de *Phlomis purpurea* (PRE) inibe significativamente a produção de todas as estruturas de ciclo da doença bem como a germinação de clamidósporos e de zoósporos. Também foi demonstrado em ensaios de estufa que *P. purpurea* reduz o potencial do inóculo no solo, o que se traduz na capacidade de reduzir a infeção de outras plantas, sugerindo que *P. purpurea* tem potencial para evitar a disseminação da doença. Esta planta é resistente à infeção por este agente patogénico.

Neste trabalho, foram realizados ensaios de estufa para determinar a atividade protetora de *P. purpurea* na infeção de *Quercus canariensis*, *Q. coccifera* e *Castanea sativa* quando é co-plantada com cada uma delas num mesmo vaso. *P. purpurea* reduziu significativamente a severidade média dos sintomas radiculares das plantas hospedeiras.

Foi, também, efetuado um ensaio de campo em parcelas naturalmente infestadas com *P. cinnamomi* para determinar o efeito protetor desta planta em *Q. suber*. Em duas parcelas foram semeadas landes de sobreiro e em outras duas parcelas landes de sobreiro juntamente com *P. purpurea*. Esta planta aumentou significativamente a germinação e consequentemente

a emergência e a sobrevivência de *Q. suber*. Em ambos os ensaios *P. purpurea* isolada foi, por si só, capaz de eliminar completamente o inóculo de *P. cinnamomi* presente no solo à volta das raízes.

De modo a estudar a eficácia do PRE no controlo da infeção das raízes de *Q. suber* e *Q. ilex* por parte deste oomiceta, foram realizados ensaios *in vivo*. O PRE, à concentração de 10 mg ml^{-1}, inibiu significativamente (84.1%) a infeção pelos zoósporos de *P. cinnamomi* em ambas as plantas, não sendo as raízes de *Q. ilex* sequer infetadas. Além disso, o PRE foi capaz de provocar uma resposta de defesa nas raízes de *Q. suber*, como revelado pela redução significativa da proporção de raízes infetadas.

Outro objetivo foi estudar a interacção de *P. purpurea* com *P. cinnamomi* utilizando técnicas histológicas. As raízes de *P. purpurea* infestadas com zoósporos e micélio, após 72 h, não apresentaram sinais de infeção. Também não apresentaram alterações histológicas. Algumas poucas hifas foram detetadas na superfície da epiderme mas não conseguiram penetrar na raiz. As raízes de marioila mostraram ter uma epiderme reforçada, com lignina e taninos, e uma exoderme, composta por celulose e suberina, que pode atuar, por si, como uma barreira física impedindo a penetração de *P. cinnamomi*. Por outro lado, infestou-se *Q. suber* (uma espécie susceptível) com micélio e zoósporos. Após 16-24 h as raízes começaram a mostrar sinais de infeção e após 72 h apresentavam necroses extensas. Após 48 h eram visíveis, microscopicamente, danos nos tecidos e distorção da parede celular com proliferação das hifas tanto inter como intra-celularmente, através da epiderme, do parênquima cortical e cilindro vascular. Porém, as raízes eliciadas com PRE, 24 h antes da infestação com o agente patogénico, apresentavam células epidérmicas e hipodérmicas irregulares com paredes espessas pela deposição de lignina e taninos e, 48 h após a infestação com micélio e zoósporos, as hifas apresentavam-se restritas principalmente aos espaços intercelulares da epiderme, com pequenas incursões no córtex mas não alcançaram o cilindro vascular. Além disso, as raízes de *Q. suber* expostas simultaneamente a PRE e zoósporos, não mostraram lesões macroscópicas após 48 horas e, também não foi observada penetração nos tecidos ao microscópio. Provavelmente, esta proteção foi causada pela atividade antagónica direta do PRE sobre os zoósporos como mostrado previamente *in vitro*.

Um dos objetivos deste estudo foi, ainda, identificar uma substância, eventualmente, responsável pela inibição de *P. cinnamomi*. O PRE foi analisado por HPLC-ESI/MS/MS. As frações de HPLC foram recuperadas e a sua actividade anti-*P. cinnamomi* determinada. A fração com maior atividade revelou a presença de um composto principal com m/z 490,4 $(M+H)^+$, que inibiu completamente o crescimento de micélio de *P. cinnamomi* a uma

concentração de 0,5 mg ml^{-1}. Este composto foi isolado a partir de PRE por cromatografia preparativa em sílica gel com uma pureza de ca 90 % e foi purificado por recristalização Este é um novo nortriterpenoide estruturalmente caracterizado por ressonância magnética nuclear (RMN) 1D e 2D, espectrometria de massa por ionização por electropulverização (ESI/MS), espectroscopia de infravermelho (IR) e difração de raios-X (XRD). Este composto foi denominado phlomispurpentaolone. A uma concentração de 0,1 $mgml^{-1}$, phlomispurpentaolone inibiu 75,7 % do crescimento do micélio de *P. cinnamomi*, cerca de 100 vezes mais do que o PRE.

Com a finalidade de descobrir outros metabolitos que são potencialmente bioativos contra *P. cinnamomi*, plântulas de *P. purpurea* foram inoculadas com zoósporos em 6 tempos (0, 6, 12, 24, 48 e 72 h). Foram, também, preparados controlos e exsudatos das mesmas plantas nos mesmos intervalos de tempo. O material a partir das plantas em cada intervalo de tempo foi reunido (11 x 5 repetições). As raízes e as folhas foram extraídas com MeOH. Lípidos e metabolitos ligeiramente polares foram separados utilizando cromatografia de fase reversa (RPC) com HSS T3 C18. Os exsudados também foram recolhidos e esterilizados por filtração a cada intervalo de tempo, imediatamente submersos em azoto líquido e mantidos a -80 °C. Os metabolitos de *P. purpurea* produzidos, tanto constitutivamente como após infestação com *P. cinnamomi,* foram quantificados utilizando um sistema de cromatografia líquida acoplada a espectrometria de massa (UPLC-MS). Os exsudatos das raízes foram analisados por cromatografia gasosa acoplada a espectrometria de massa (GC-MS) após derivatização. Verificou-se que phlomispurpentaolone é produzido constitutivamente.

Este trabalho de investigação contribuiu para elucidar os mecanismos de defesa de *P. purpurea* contra *P. cinnamomi* bem como o principal composto produzido por esta planta com atividade contra este agente patogénico. A capacidade de erradicar este agente patogénico utilizando uma planta autóctone abre novas perspectivas para o controlo do declínio do sobreiro bem como de outras espécies hospedeiras deste oomiceta.

Palavras-chave: *Phytophthora cinnamomi*, *Phlomis purpurea*, extratos, proteção, phlomispurpentaolone, metabolómica.

Abbreviations

- $[M + H]^+$ positive ion mode
- °C Celsius (Temperature)
- µg, mg, g, Kg microgram, miligram, gram, kilogram
- µl, ml, l microlitre, mililitre, litre
- µM micromolar
- µm, mm, cm micrometer, millimeter, centimeter
- BLAST basic local alignment search tool
- C cortex
- DNA deoxyribonucleic acid
- En endodermis
- Ep epidermis
- ESI MS/MS electrospray ionization mass spectrometry
- ETS effector-triggered susceptibility
- Ex exodermis
- *g* gravitational acceleration, centrifugal force unit
- GC-MS gas chromatography- mass spectrometry
- hpi hours post-inoculation/infestation
- HPLC high performance liquid chromatography
- HR hypersensitive reaction/response
- Hy hyphae
- I infested
- IP inoculum potential
- IR infrared spectroscopy
- ITS internal transcribed spacer
- LC-MS liquid chromatography- mass spectrometry
- m/z mass-to-charge ratio
- mAU absorbance units
- mDa, Da milliDalton, Dalton
- MS, MS/2 Murashige and Skoog culture media, half strength MS

- MSS — mineral salt solution
- NARPH — culture media containing nystatin, ampicillin, rifampicin, pentacloronitrobenzene and hymexazol.
- NCBI — National center for Biotechnology information
- NI — non-infested
- NMR — nuclear magnetic resonance
- OD — optical density
- OPLS-DA — optimized potentials for liquid simulations - discriminant analysis
- P — phloem
- P.c. — *Phytophthora cinnamomi*
- P.p. — *Phlomis purpurea*
- w/v, v/v — mass/volume, volume/volume
- PAMP — pathogen – associated molecular pattern
- PCA — principal component analysis
- PCNB — pentachloronitrobenzene
- PCR — polymerase chain reaction
- ppm — parts per million
- PRE — *Phlomis purpurea* crude root extract
- PRRs — pattern-recognition receptors
- PTI — PAMP-triggered immunity
- rpm — rotation per minute
- s, min, h — second, minute, hour
- SDW — sterile distilled water
- SAR — systemic acquired resistance
- SH — Schenk and Hildebrandt culture media
- T — time
- TLC — thin layer chromatography
- UV — ultraviolet
- V8A — V8 juice, clarified with agar
- V8B — V8 juice, clarified broth
- VC — vascular cylinder
- X — xylem
- XRD — X-ray diffraction
- YMB — yeast mannitol broth

Table of Contents

Thesis objectives

The overall objective of the present work is to develop new approaches to control the highly aggressive phytopathogen *Phytophthora cinnamomi* that has been associated to the decline disease of *Quercus suber* (cork oak) and *Q. ilex* subsp. *rotundifolia* (holm oak). To reach this aim we stick on a second level of objectives which are decisive for the accomplishment of the main one:

I. To determine the protective effect of *Phlomis purpurea* against *P. cinnamomi* in *Fagaceae*;

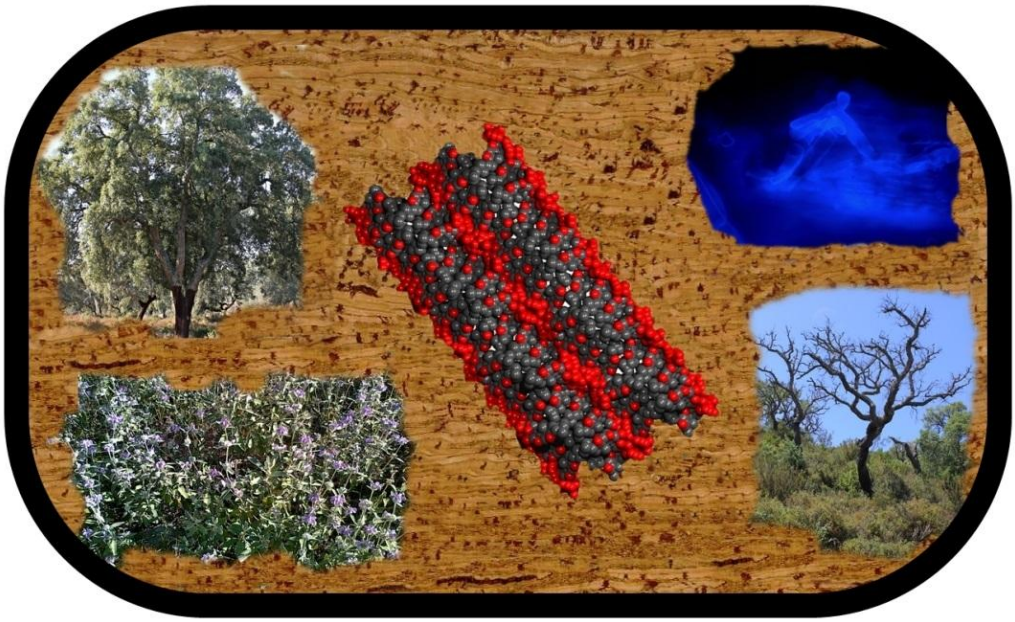
II. To elucidate mechanisms of resistance of *P. purpurea* to *P. Cinnamomi*;

III. To Identify and structurally characterize bioactive substance(s) inhibitory of *P. cinnamomi* life structures;

IV. To establish *P. purpurea* in axenic conditions or in an inert substrate to facilitate the study the metabolites produced constitutively or in condition of stress by infestation with *P. cinnamomi.*

Part I

Evaluation of the protective effect of *Phlomis purpurea* against *Phytophthora cinnamomi* in *Fagaceae.* Glasshouse, in field, in plant and histological studies.



Chapter 1

Introduction and literature review

1.1. Biology, life history and infection process of *Phytophthora cinnamomi*

Many *Phytophthora* species belong to the most aggressive and most important known plant pathogens. The word *Phytophthora* means phyto=plant and phthora=destroyer, hence "plant destroyer". Species of this genera, *ca* 54 in 1996 (Erwin & Ribeiro, 1996) increased to *ca* 100 (Brasier, 2007) and comprises *ca* 150 taxa to date (Crone, 2012; Hansen *et al.*, 2012; Martin *et al.*, 2014) distributed by ten clades (Cooke *et al.*, 2000). They are worldwide known as primary parasites of fine roots and cause devastating diseases in numerous crops, ornamental and native plants.

Phytophthora cinnamomi Rands was first isolated from cinnamon trees by Rands in 1922, in Sumatra although its centre of origin is not consensual. What is, definitely, consensual is its worldwide distribution. The number of *P. cinnamomi* hosts listed by Zentmyer in 1980 was approximately 900, Hardham mentioned 3,000 in 2005, but the number of known host species has now increased to more than 5,000 (Zentmyer, 1980; Erwin & Ribeiro, 1996; Hardham, 2005; Rookes *et al.*, 2008). This pathogen, as well as other species of *Phytophthora*, can be dispersed into new areas by soil, water, seed (Zentmyer, 1980) or infected root material.

Phytophthora cinnamomi is an oomycete that has a growth pattern similar to fungi, but there are several important differences between them: fungi are haploid, hyphae are septate with cell walls containing mainly chitin whereas oomycetes are diploid, hyphae are non-septate with cell walls containing mainly cellulose and β-glucans (Latijnhouwers *et al.*, 2003). Unlike fungi, this oomycete requires an exogenous source of β-hydroxy sterols for sporulation to occur, as it is unable to synthesise sterols endogenously (Erwin & Ribeiro, 1996).

The life history is represented in Figure 1.1, where both sexual and asexual phases are contemplated (see review by Hardham, 2005). *Phytophthora* species are able to survive unsuitable environmental conditions over several years as dormant resting spores (oospores or chlamydospores) in the soil or in infected root tissue (Mccarren, 2006; Jung *et al.*, 2013). When environmental conditions become suitable (high soil moisture, soil temperature > 10 °C) the resting spores germinate by forming sporangia. Sporangia either germinate directly or release 20-30 uninucleate zoospores. Zoospores are wall-less motile cells surrounded only by a plasma membrane, and have two flagellae which allow them to move through water. Zoospores specialized structures for dispersal because they cannot divide nor absorb organic nutrients, are attracted to the soft tissues, by chemotaxis (Morris *et al.*, 1998), electrotaxis (van West *et al.*, 2002) and induced encystment (Gow, 2004) and attach to the root , for example to the root elongation or root hair zones (*ibid*.) where they lose their flagella and encyst.

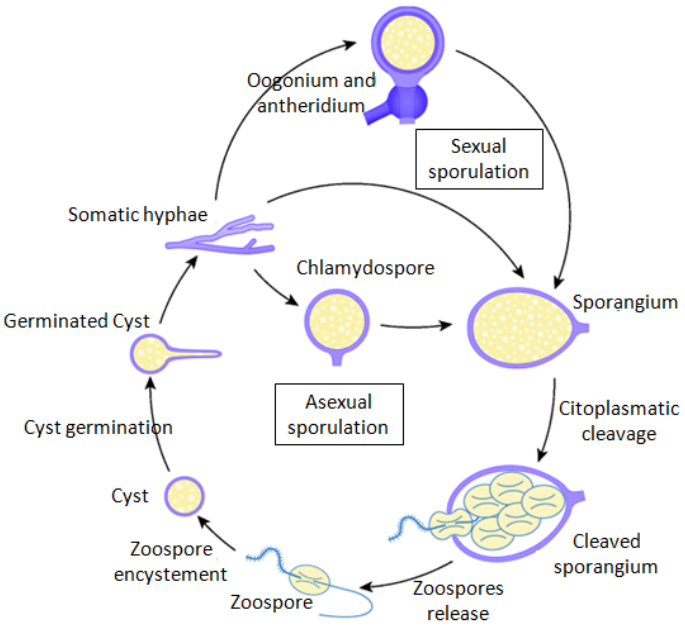


Figure 1.1. Schematic life history of a typical soil-borne Phytophthora, like *P. cinnamomi* (adapted from Hardham, 2005).

Thereafter the cysts produce germ tubes that penetrate the plant (Hardham, 2005). Mycelium, then grows throughout the roots of susceptible hosts and within 48 to 72 hours, new sporangia will form in the root surface (Hardham, 2005). The asexual cycle may be repeated several times, rapidly increasing the amount of inoculum. Within dead plant roots and/or soil, somatic hyphae also produce chlamidospores. They are resistant spores that can germinate, when conditions are favourable, and restart the cycle. This capacity of surviving in the absence of a host represents a competitive advantage (Weste, 1983). This author suggested that *P. cinnamomi* possesses considerable saprophytic ability. Contrarily, McCarren (2006) stated that this oomycete has an extremely limited saprophytic ability except, possibly, under conditions of low microbial competition. The sexual stage requires the presence of opposite mating types (A1 and A2) as this organism is heterothallic (Zentmyer, 1980). The oospores are formed when the male structure, antheridium, fertilizes the oogonium (female). They can germinate and restart the asexual cycle. After infection, the pathogen destroys the young feeder roots, causing rot in susceptible species, preventing water and nutrient absorption.

In very susceptible species death may occur within weeks whereas in moderately susceptible species the plant may not die until one or two years after infection. Resistant species have the ability to prevent further spread of the mycelium.

Phytophthoras can increase and disseminate their inoculum during a relatively short time of favourable environmental conditions. On the other hand, the life cycle has to run million times, and it can take decades of inoculum build-up and progressive fine root destruction before a mature tree begins to show visible crown symptoms (Tsao, 1990). *P. cinnamomi* has a wide host range that ensures its adaptability and survival in ecosystems with high species diversity.

1.2. Global impact of *Phytophthora cinnamomi*

Phytophthora cinnamomi has a broad host range and is widely distributed around the world. The majority of introductions of exotic plant pathogens probably have to do with human activities (Wingfield *et al.*, 2001). In fact, there are three major ways for the introduction of pathogens: 1. direct introduction from the native environment into the foreign environment (e.g. direct reforestation); 2. short term indirect introduction by introduction of the pathogen in a restricted habitat (e.g. nursery) from which it is then released into the broader environment (e.g. forest); 3. long-term indirect introduction, through which an exotic plant pathogen is routinely introduced into a specific habitat (e.g. a plantation, from where it then escapes into the wild). As a result, forests, fruit species, perennial and ornamental species have been negatively impacted by this highly aggressive pathogen: in America, with the decline of fir (*Abies* spp), birch (*Betula* spp), cedar (*Cedrus* spp), chestnut (*Castanea* spp), oaks (*Quercus* spp); in Africa, with the root rot of avocado trees (*Persea americana*); in Europe, with the decline of oaks (*Quercus* spp) and chestnut (*Castanea sativa*); in Australia, with the decline of the *Eucalyptus marginata* forest (Jarrah), just to mention a few examples.

1.3. Impact of *Phytophthora cinnamomi* in the montado ecosystem

Cork oaks (*Quercus suber*), together with holm oaks (*Quercus ilex*), are key species in agro-silvo-pastoral ecosystems called *montado* in Portugal and *dehesa* in Spain which are open woods with low tree density (50–300 trees/ha) supporting rich wildlife populations and simultaneously used by farmers to grow cereals and graze animals. *Montado*-like systems have high conservation and socioeconomic value (reviewed by Gil & Varela, 2008).

Portugal is the first producer of cork, having the largest land area occupied by cork oak: over 700 000 ha. It is also the main exporter and the income from this activity play a relevant role in the country's economy. Its relevance for Portugal is implicit in the election as the national tree and the prohibition of cutting these trees unless authorized by the

‘‘Autoridade Nacional Florestal” (Diário da República – Série A, Decreto –Lei nº169/2001 de 25 de Maio). However, biotic stress and climate change together with changing practices in the wine industry, that are switching to synthetic bottle stoppers, place cork oak woodlands “on the edge of radical change and at clear risk of collapse” (Aronson *et al.*, 2009).

Since the late nineteenth century that episodes of abnormal mortality of cork oaks have been taking place in the Iberian Peninsula (Almeida 1898; Baeta Neves 1949; Natividade 1950). In the 80s of the twenty century there was another abnormal mortality and Portuguese and foreign researchers joined forces to try to discover the cause of this mortality (Brasier et al. 1993). It was verified a decrease in the density of the canopy over time and thought that the reason could be due to the action of pests and diseases attacking the aerial parts of the tree. However, it has been proved that it is a "pseudo-fungus", *Phytophthora cinnamomi*, that infects the young roots who is primarily responsible for this mortality (Brasier *et al.*, 1993). *P. cinnamomi* is an exotic pathogen that infects and destroys the fine roots of trees which function is to absorb water and nutrients, eventually leading to the death of the tree.

Several studies have demonstrated the involvement of *P. cinnamomi* in oak decline in Portugal (Moreira-Marcelino, 2001; Moreira *et al.*, 2006; Caetano, 2007), Spain (Tuset *et al*., 1996; Galego, Perez De Algaba, A. Fernandez-Escobar, 1999; Luque *et al*., 2001;Rodríguez-Molina *et al.,* 2002; Sánchez et al. 2002; Sánchez *et al.* 2003; Rodríguez-Molina *et al.*, 2005; Sanchez *et al.,* 2005)and other countries (Zentmyer, 1980; Brasier *et al.*, 1993; Robin *et al.*, 2001; Thomas *et al.*, 2002).

1.4. Impact of *Phytophthora cinnamomi* in *Castanea sativa*

In Europe, Portugal is one of the most important producers of *Castanea sativa* Mill (chestnut). The first record of the *C. sativa* ink disease caused by *Phytophthora cinnamomi* Rands and/or *P. cambivora* was in Portugal in 1838 (Crandall 1950, cited by Vannini & Vettraino 2001). A resurgence of decline in chestnuts in Europe has been noted since the end of the 20th century. In order to maintain the chestnut production, Asian species resistant to Phytophthora were introduced in the Atlantic area but found to be unsuitable for the foreign environmental conditions (Pereira-Lorenzo *et al.*, 2010). Since the 1950s, resistant hybrid clones between *C. sativa* and the Asiatic chestnuts, mainly *C. crenata*, but also *C. mollissima*, were obtained by Gomes and Taveira in Portugal, Gallastegui and Urquijo in Spain and Schad in France (Pereira-Lorenzo *et al.*, 2010). Clones with a high tolerance to *P. cinnamomi* were selected as rootstocks for high quality fruit cultivars (Vannini & Vettraino, 2001).

Many factors including temperature, soil water content, type and microbiota, determine the infection by *P. cinnamomi* and consequently the severity of the disease and its

rate of spread (Martins *et al.*, 2005, 2010). Dinis *et al.* (2011) studied the pathogenicity of *P. cinnamomi* by stem inoculation in three-year-old *C. sativa* wild rootstock and a hybrid of *C. sativa* x *C. crenata*. Infection led to shoot death in 87.5 % of the *C. sativa* wild plants in 30 days, while all resistant plants (hybrid *C. crenata* x *C. sativa* progeny) survived. Moreover, the infected resistant plants presented a significant increase in the level of total phenols (50 %); whereas in sensitive *C. sativa* plants, no difference in phenol content was observed upon the oomycete infection.

1.5. Biological control methods

Development of disease results from a complex and dynamic interaction between the susceptible host, the pathogen (*Phytophthora cinnamomi*) and a favourable environment (biotic and abiotic factors), the so called disease triangle, a general concept in plant pathology.

Measures that affect the interaction of the host, *P. cinnamomi*, and the environment are effective for the disease control. The critical environment factor in the interaction between *P. cinnamomi* and its hosts is moisture. Temperature is also a key point, since this oomycete is most pathogenic between 25 °C and 30 °C (Zentmyer, 1980). The objective is to set up conditions in which the plant will thrive but the pathogen will not.

There is no method currently available to eradicate this highly aggressive pathogen without destroying the plants. *Phytophthora cinnamomi* is not a true fungus (see section 1.1) and therefore fungicides are not completely efficient. Therefore control measures aim to reduce the spread of the pathogen and to increase plant defence (Aberton *et al.*, 1999).

Various control measures can be implemented: cultural and physical control; chemical control; biological control, including biological control agents (BCAs) and amendments, natural plant products and host resistance; and integrated control. These factors were reviewed by Neves (2007).

Much concern over environmental and human safety issues involving fungicide use, as well as development of resistance, highlighted the need to develop new environmentally friendly means of control. According to Cook and Baker (1983) ‘‘Biological control is the reduction of the amount of inoculum or disease producing activity of a pathogen accomplished by or through one or more organisms other than man’’. Later, Wilson (1997) defined biological control as “the control of a plant disease with a natural biological process or the product of a natural biological process”. It also includes host resistance (constitutive and elicited).

The identification of novel effective microbicide compounds is essential to combat increasing resistance rates. Botanicals represent an extraordinary reservoir of biologically

active molecules. However, most plant species have never been surveyed for biologically active constituents, and novel sources of valuable molecules remain to be discovered.

Plant extracts may be an alternative to currently used fungicides, as they are often active against a limited number of specific target species and biodegrade to non-toxic products; they could lead to the development of new classes of possibly safer disease control agents (Lee *et al.*, 2001). Many preformed and induced antimicrobial substances are known from plants (VanEtten *et al.*, 1994). These are the first line candidates to be considered for use in plant-protection strategies. Moreover, some substances that do not show direct antimicrobial activity might act as resistance inducers or elicitors of the plant's own defences (Goellner & Conrath, 2008).

An increasing number of studies have been made about the use of plant extracts, essential oils and pure active substances against plant pathogens (see review by Neves 2007). Hereinafter a review of the biological control of *Phytophthora* spp by products either "delivered" or "extracted" from plants will be presented.

There are numerous reports on plant extracts exhibiting anti-*Phytophthora* spp properties, particularly against *P. infestans* de Bary, the causal agent of potato and tomato late blight. In Table 1.1, are mentioned some of the botanicals studied from 2001 onwards.

Table 1.1. Anti-*Phytophthora* spp activity of plant extracts, their organic fractions and compounds derived from them.

Botanical	Part used	Extract	Concentration	Compound(s)	*Phytophthora* spp	*Phytophthora* structure	Host	Activity (% of inhibition)	Reference
Piper longum	Fruit	Methanol fraction at 1 $mgml^{-1}$	50 mg of the test sample was dissolved in 0.5 ml of dimethyl sulfoxide (DMSO), followed by diluting it with 49.5 ml of water containing a surfactant Tween 20	n.a.	*P. infestans*	Zoosporangia	Tomato	0	(Lee *et al.*, 2001)
Piper longum	Fruit	Hexane fraction at 1 $mgml^{-1}$	*ibid*.	n.a.	*P. infestans*	*ibid*.	Tomato	40	(Lee *et al.*, 2001)
Piper longum	Fruit	Chloroform fraction at 1 $mgml^{-1}$	*ibid*.	n.a.	*P. infestans*	*ibid*.	Tomato	50	(Lee *et al.*, 2001)
Piper longum	Fruit	Hexane fraction	*ibid*. for 1 $mgml^{-1}$. For the other conc. accordingly.	Pipernonaline at 1, 0.5 and 0.25 $mgml^{-1}$	*P. infestans*	*ibid*.	Tomato	91, 80 and 55, respectively	(Lee *et al.*, 2001)

Botanical	Part used	Extract	Concentration	Compound(s)	*Phytophthora* spp	*Phytophthora* structure	Host	Activity (% of inhibition)	Reference
Curcuma longa	Rhizome	Methanol at 2 $mgml^{-1}$ and 1 $mgml^{-1}$	100 mg of the test sample was dissolved in 0.5 ml of dimethyl sulfoxide (DMSO), followed by diluting it with 49.5 ml of water containing Tween 20	n.a.	*P. infestans*	*ibid*.	Tomato	100 and 91 respectively	(Kim *et al.*, 2003)
Curcuma longa	Rhizome	Ethyl acetate fraction at 2 $mgml^{-1}$ and 1 $mgml^{-1}$	*ibid*.	n.a.	*P. infestans*	*ibid*.	Tomato	100 at both concentrations	(Kim *et al.*, 2003)
Curcuma longa	Rhizome	Ethyl acetate fraction	*ibid*.	Curcumin at 1, 0.5, 0.25 and 0.125 $mgml^{-1}$	*P. infestans*	*ibid*.	Tomato	100, 100, 85 and 57, respectively	(Kim *et al.*, 2003)
Hedera helix	Leaves	50 % aqueous ethanol, applied 2 days before inoculation	20 g fresh weight in 100 ml ethanol	n.a.	*P. infestans*	Sporangia and zoospores	Detached tomato leaves	100	(Röhner *et al.*, 2004)
Paeonia suffruticosa	Leaves	*ibid*.	*ibid*.	n.a.	*P. infestans*	*ibid*.	*ibid*.	100	(Röhner *et al.*, 2004)
Paeonia suffruticosa	fruit	*ibid*.	*ibid*.	n.a.	*P. infestans*	*ibid*.	*ibid*.	100	(Röhner *et al.*, 2004)
Hedera helix	Leaves	50 % aqueous ethanol, applied 2 days before inoculation	20 g fresh weight in 100 ml ethanol	n.a.	*P. infestans*	Sporangia and zoospores	Entire tomato plant	100	(Röhner *et al.*, 2004)

Botanical	Part used	Extract	Concentration	Compound(s)	*Phytophthora* spp	*Phytophthora* structure	Host	Activity (% of inhibition)	Reference
Paeonia suffruticosa	Leaves	*ibid*.	*ibid*.	n.a.	*P. infestans*	*ibid*.	*ibid*.	84	(Röhner *et al.*, 2004)
Heliopsis longipes	Roots	96 % ethanol extract in water (v/v)	0.05, 0.075 and 0.15 $mgml^{-1}$	Affinin	*P. infestans*	Mycelial growth	*In vitro*	52, 100 and 100, respectively	(Molina-Torres *et al.*, 2004)
Rheum rhabarbum	Leaves	Aqueous extracts at 1 %. Tested at 5 %	0.5g of plant powder into 50 ml 0.0125 % Tween 80.	n.a.	*P. infestans*	Mycelial growth	*In vitro*	100	(Stephan *et al.*, 2005)
Rosmarinus officinalis	leaf extracts	20 g of plant into 200 ml of water and autoclaved at 121 °C for 45 min. Volume reduced to 20 ml.	Aqueous extracts tested at 25 %	n.a.	*P.capsici*, *P. megakarya* *P. palmivora*.	Zoospores	*In vitro*	100	(Widmer & Laurent, 2006)
Lavandula officinalis	leaf extracts	20 g of plant into 200 ml of water and autoclaved at 121 °C for 45 min. Volume reduced to 20 ml.	Aqueous extracts tested at 25 %	n.a.	*P.capsici*, *P. megakarya* *P. palmivora*.	Zoospores	*In vitro*	Up to 72, 94 and 98 respectively.	(Widmer & Laurent, 2006)
Rosmarinus officinalis and *Lavandula officinalis*	leaf extracts	n.a.	ibid	Caffeic at 3 $mgml^{-1}$ and rosmarinic acid at 6 $mgml^{-1}$	*P.capsici*, *P. megakarya* *P. palmivora*.	Zoospores	*In vitro*	100	(Widmer & Laurent, 2006)
Phlomis purpurea	roots	50 g chopped roots macerated in 350 ml of 70 %.	Tested at 1 and 5 $mgml^{-1}$	n.a.	*P. cinnamomi*	mycelia	*In vitro*	78.3 and 93.9, respectively.	(Neves, 2007)

Botanical	Part used	Extract	Concentration	Compound(s)	*Phytophthora* spp	*Phytophthora* structure	Host	Activity (% of inhibition)	Reference
Helichrysum sechas	Leaves and flowers	50 g chopped parts macerated in 350 ml of 70 %.	Tested at 1 and 5 and 10 $mgml^{-1}$	n.a.	*P. cinnamomi*	mycelia	*In vitro*	38.8, 76.6 and 86.7 respectively.	(Neves, 2007)
Cupressus benthamii	Leaves	methylene chloride and methanol (1:1 vol/vol) at 300 $mgml^{-1}$	300 $mgml^{-1}$ in sterilized Distilled Water (SDW) containing 0.05 % Tween 20	n.a.	*P. infestans*	Sporangia	*In vitro*	36	(Goufo *et al.*, 2008)
Cupressus benthamii	Leaves	*ibid*.	*ibid*.	n.a.	*P. infestans*	Sporangia	Tomato	92	(Goufo *et al.*, 2008)
Vetiveria zizanioides	Roots	*ibid*.	*ibid*.	n.a.	*P. infestans*	Sporangia	*In vitro*	46	(Goufo *et al.*, 2008)
Vetiveria zizanioides	Roots	*ibid*.	*ibid*.	n.a.	*P. infestans*	Sporangia	Tomato	87	(Goufo *et al.*, 2008)
Allium sativum	Bulb	juice	50 µg ml^{-1}	Allicin	*P. infestans*	Sporangia and zoospores	*In vitro*	*Ca* 82 and 70, respectively	(Portz *et al.*, 2008)
Allium sativum	Bulb	juice	1 $mgml^{-1}$	Allicin	*P. infestans*	Sporangia and zoospores	Tomato leaves	>99[a]	(Portz *et al.*, 2008)
Allium sativum	Bulb	juice	0.5 $mgml^{-1}$	Allicin	*P. infestans*	Sporangia and zoospores	Tomato leaves	96-98[a]	(Portz *et al.*, 2008)
Allium sativum	Bulb	juice	0.2 $mgml^{-1}$	Allicin	*P. infestans*	Sporangia and zoospores	Tomato leaves	84-94[a]	(Portz *et al.*, 2008)
Psoralea corylofolia	Seeds	Methanol at 2 $mgml^{-1}$ applied 1 day before inoculation	Was added to Tween 20 solutions at 5 % (v/v)	n.a.	*P. infestans*	Zoospores	Tomato	*Ca* 95	(Shim *et al.*, 2009)

Botanical	Part used	Extract	Concentration	Compound(s)	*Phytophthora* spp	*Phytophthora* structure	Host	Activity (% of inhibition)	Reference
Psoralea corylofolia	Seeds	Chloroform at 2 $mgml^{-1}$ fraction applied 1 day before inoculation	Dissolved in acetone and added to Tween 20 solutions at 10 % (v/v)	n.a.	*P. infestans*	*ibid*.	Tomato	*Ca* 95	(Shim *et al.*, 2009)
Psoralea corylofolia	Seeds	Chloroform fraction at 0.5 $mgml^{-1}$ and 0.1 $mgml^{-1}$	*ibid*.	Psoralren	*P. infestans*	*ibid*.	Tomato	82 and 29 respectively	(Shim *et al.*, 2009)
Psoralea corylofolia	Seeds	Chloroform fraction at 0.5 $mgml^{-1}$ and 0.1 $mgml^{-1}$	*ibid*.	Isopsoralren	*P. infestans*	*ibid*.	Tomato	84 and 21 respectively	(Shim *et al.*, 2009)
Malus domestica	Fruits	Methanol (70 % aqueous) extract at 3 $mgml^{-1}$	Each solution was then added to Tween 20 (250 µg/ml in distilled water) solution at final concentrations of 5 % (v/v).	n.a.	*P. infestans*	Zoospores	Tomato	65	(Shim *et al.*, 2010)

Botanical	Part used	Extract	Concentration	Compound(s)	*Phytophthora* spp	*Phytophthora* structure	Host	Activity (% of inhibition)	Reference
Persea americana	roots	1 g freeze-dried with N2 and ground. Powder extracted with 10 ml of methanol-chloroform (2:1) shaked for 10 min. Ten ml of distilled water added and phase separation.	Supernatants evaporated and residues dissolved in 500µl absolute ethanol. Tested 50 µl impregnated discs.	stigmastan-3,5-diene at 100 ppm	*P.cinnamomi*	mycelium	*In vitro*	100 (crude extract and compound)	(Sánchez-Pérez *et al.*, 2009)
Annona squamosa	Seeds	Methanol (80 % in water?)	1 and 0.5 $mgml^{-1}$	n.a.	*P. infestans*	Zoospores	Tomato	96.4 and 97.9, respectively	(Dang *et al.*, 2011)
Annona squamosa	Seeds	Ethyl acetate fraction	1 and 0.5 $mgml^{-1}$	n.a.	*P. infestans*	Zoospores	Tomato	98.6 for both	(Dang *et al.*, 2011)
Phlomis purpurea	roots	50 g chopped roots macerated in 350 ml of 70 %.	Tested at 10 $mgml^{-1}$	n.a.	*P. cinnamomi*	Sporangial production and zoospore differentiation and release	*In vitro*	100	(Neves *et al.*, 2014)
Phlomis purpurea	roots	*ibid*.	Tested at 1 and 5 and 10 $mgml^{-1}$	n.a.	*P. cinnamomi*	Zoospore germination	*In vitro*	82.3, 98.2 and 100, respectively	(Neves *et al.*, 2014)
Phlomis purpurea	roots	*ibid*.	Tested at 1 and 5 and 10 $mgml^{-1}$	n.a.	*P. cinnamomi*	Chlamydospore production	*In vitro*	46.7, 100 and 100, respectively	(Neves *et al.*, 2014)
Phlomis purpurea	roots	*ibid*.	Tested at 1 and 5 and 10 $mgml^{-1}$	n.a.	*P. cinnamomi*	Chlamydospore germination	*In vitro*	58.9, 83.3 and 100, respectively	(Neves *et al.*, 2014)

Plants have different ages. See specific articles. [a]Average effectivity according to Abbot.

Recently, work has been done using plants for the suppression of *P. cinnamomi*. Studies, in a glasshouse trial, by D'Souza *et al*. (2004) showed that four out of five Western Australia native *Acacia* species protected the highly susceptible *Banksia grandis* against *P. cinnamomi*. However, in a field trial, only *Acacia pulchella* significantly protected *B. grandis,* from *P. cinnamomi* infection, up to a year after inoculation. D'Souza *et al.* (2005) increased the number of legume species tested to 15 and assessed their potential to control *P. cinnamomi*. Moreover *A. pulchella*, *A. extensa*, *A. lateriticola*, *A. drummondi*, *A. stenoptera* and *A. alata* all reduced soil inoculum levels. However, *P. cinnamomi* is able to asymptomatically infect the roots of *A. lateriticola* and *A. drummondi*. Tolerant hosts can be a continuous source of inoculum, so these species do not seem to have the potential to be used for biological control.

Acacia pulchella has been shown to be resistant to *P. cinnamomi* infection (Tippett & Malajczuk, 1979) and appeared to reduce infection of susceptible *Eucaliptus marginata* (jarrah) grown together with it in pots (Shea *et al.*, 1976). In a glasshouse trial, seedling exudates were shown to lyse *P. cinnamomi* chlamidospores and degenerate them (Jayasekera, 2006). However, it was found that the presence of roots of *A. pulchella*, enhances the production of oospores (Jayasekera *et al.*, 2007), which is the most resistant *P. cinnamomi* structure (Jung *et al.*, 2013).

Phlomis purpurea has been shown to be resistant to *P. cinnamomi* infection (Neves, 2007) and when grown together with *Quercus ilex* and *Q. suber* were shown to significantly protect these susceptible species against *P. cinnamomi* infection (Neves *et al.*, 2014). Moreover *P. purpurea* reduced the soil inoculum potential in glasshouse trials, indicating the ability to reduce root infection by the pathogen (*ibid*.).

Amending the soil with *Brassica juncea* and *B. napus* resulted in an immediate suppression of the inoculum potential and infective ability of *P. cinnamomi*. Soils amended with *B. juncea* reduced the incidence of *Lupinus angustifolius* infection by 25 % (Dunne, 2004). These species, belonging to the *Brassicaceae* family, synthesize significant amounts of glucosinolates (GSLs) which showed to be relatively inactive against microorganisms (Brown & Morra, 2005). However, GSLs are hydrolysed by the myrosinase enzyme (present endogenously in Brassica tissues) to release a range of products including isothiocyanates (ITCs) which have broad biocidal activity (Brown & Morra, 2005) including activity against oomycetes (Smith & Kirkegaard, 2002; Dunne, 2004).

It is known that plants produce primary and secondary metabolites that are economically important organic compounds (Balandrin *et al.*, 1985). Secondary metabolites are also known to possess, among others, antimicrobial activity (*ibid*.). Nevertheless, only a few products derived from plants are used commercially to fight plant pathogens

[(e.g. neemazal, a product from *Azadirachta indica* (neem); Timorex, a product from *Melaleuca alternifolia* (tea tree)]. Human concerns about health and environment issues are demanding bioproducts of plant origin for organic or traditional agricultures to keep our environment clean and safer for humankind and animals. Hopefully the future will see the increased development of successful plant protection strategies based on botanicals.

1.6. *Phlomis purpurea* and suppression of *Phytophthora cinnamomi*

The genus Phlomis is described in the Kew gardens webpage (Kew gardens).

Phlomis spp are often found in open shrub lands of the Mediterranean region. Four of them are native of the Iberian Peninsula: *P. crinita*, *P. herba-venti*, *P. lychnitis* and *P. purpurea* (Algieri *et al.*, 2013). *P. purpurea* L. (purple phlomis, "marioila" in Portuguese and "matagallo" in Spanish) is a herb that can grow to a small shrub (up to 2.0 m height) with purple/pink inflorescences, hence the name "purpurea" (Figure 1.2). It is usually found in disturbed areas on the edge of thickets in dry, rocky areas, on clay, marl and calcareous soils. It is also part of the understory of *Quercus suber* (cork oaks). In Portugal, it only occurs in central and southern mainland territory, being particularly abundant in the Arrábida natural park (Novais *et al.*, 2004) and the Algarve. It is, also present in the south of Spain and NW Africa. Flowering takes place from March to June. Propagation is by cuttings or seed (*ibid*.).

Figure 1.2. *Phlomis purpurea* (purple phlomis) during the flowering stage.

Phlomis spp have been used for many decades as phytomedicines with stimulant, tonic, wound healer, pain reliever, anti-inflammatory, among other properties (Amor *et al.*, 2009). These properties are supported by the more than 150 different compounds produced by this genus (Liu *et al.*, 2006; Amor *et al.*, 2009; Hussain *et al.*, 2010a; Li *et al.*, 2010; Deng *et al.*, 2011). *P. purpurea* has various medicinal uses in Portugal. It is used as an infusion to treat

abdominal pain, as cardiotonic, antidiarrheal, digestive, gastric analgesic, anti-inflammatory, anthelmintic, emetic, for sea–sickness, for cold' prevention, renal antispasmodic, for bladder aliments, hepatic protector, for stomach ulcers, for gastritis and its olive oil decoction is used for fever, cough and colds (Novais *et al.*, 2004).

In Spain, its aerial infusion is frequently used to treat prostate and liver complaints (González-Tejero *et al.*, 1995). The leaves were used traditionally as detergent to wash kitchen utensils, which indicate the natural presence of saponins.

Some *Phlomis* spp have antimicrobial activities against bacteria (Kyriakopoulou *et al.*, 2001; Calis *et al.*, 2005; Hussain *et al.*, 2010b; Li *et al.*, 2010; Ulukanli & Akkaya, 2011) and fungi (Demirci *et al.*, 2009; Li *et al.*, 2010) but, as far as we know, no studies were done, up to now, on anti-oomycete, namely anti- *P. cinnamomi* activity from *Phlomis* spp, apart from ours. A revision of the suppression of this oomycete by *P. purpurea* and its extracts is made in section 1.6 including Table 1.1.

1.7. Plant resistance mechanisms

1.7.1. Pathogen lifestyles and possible host responses

Plants are continually exposed to a vast number of potential pathogens and, as a result, have evolved diverse defence mechanisms to recognise and overcome the invading pathogens. The resistance or susceptibility of a plant to a particular pathogen depends on two interrelated factors: (i) the substrate requirements of the pathogen and (ii) the response of the plant to the pathogen (Guest & Brown, 1997). All plant pathogens can be divided into three classes according to their lifestyles – necrotrophs, biotrophs and hemibiotrophs. Necrotrophs and biotrophs are distinguished by their different substrate requirements. Necrotrophs kill the plant cells before colonising and feeding on them (Agrios, 1997). They can have a wide host range or only one or few hosts. Biotrophs on the other hand, grow and reproduce in living plant tissue. They obtain nutrients from the living cells (*ibid*.). Consequently, they must establish a compatible cellular relationship with their hosts. They mostly grow between the cells of their host and may penetrate host cell walls but not host cell membranes (Guest & Brown, 1997). The pathogen develops without eliciting the host's defence responses or by spreading before the plant activates them (*ibid*.). Hemibiotrophs have intermediate life-styles. They initially derive their nutrients biotrophically but once infection is established, they switch to a necrotrophic stage. This type of lifestyle is observed in species of the Oomycete genera *Phytophthora* and *Pythium* (Agrios, 1997). *Phytophthora cinnamomi* is considered a necrotroph by same authors (Hardham, 2007; Rookes *et al.*, 2008), a biotroph (Crone, 2012) or

a hemibiotroph (Engelbrecht & van den Berg, 2013), meaning that the pathogen has evolved a range of lifestyles allowing it to persist in an ecosystem independently of the presence of susceptible hosts or not.

Plants that are resistant restrict or retard the development and reproduction of the majority of individual pathogen propagules that attack them. Resistance may be expressed in many ways, from the inhibition of propagule germination and penetration, the killing of pathogens before establishment, to the restriction or retardation of colony development and reproduction once the pathogen has established (Guest & Brown, 1997). Resistance may occur at the varietal level (race or cultivar specific resistance) or at the species or genus level (non-host resistance). In addition, resistance may be a quantitative phenotype (partial resistance) with a partial reduction in disease severity (Kamoun *et al.*, 1999).

The host-parasite-environment interaction is mediated by a complex interchange of signals. Plants respond to pathogen attack by erecting a highly coordinated series of molecular, cellular and tissue-based defence barriers (Guest & Brown, 1997). All plants have the capacity to activate these defences. However, if they are activated too little, too late or in the wrong place they will fail to restrict the pathogen and the plant will be susceptible (*ibid*.). Pathogens respond by escaping or suppressing plant defence responses or by rendering these responses powerless, for example by detoxifying plant antibiotics (*ibid*.).

The interaction of pathogen lifestyle and host response leads to five possible outcomes, if environmental conditions favour infection (*ibid*.):

- No relationship is established when the plant and the pathogen ignore each other. The plant is a non-host.
- A plant is antagonistic to the pathogen when it secretes inhibitory compounds into its environment that prevent pathogen development e.g. asparagus and marigolds secrete substances into the rhizosphere that are toxic to nematodes and are a useful protection against nematodes when inter-planted with nematode susceptible species such as tomato. In this relationship, the pathogen fails to develop and has no observable effect on the metabolism of the host plant.
- The pathogen is antagonistic to the plant and usually causes the death of the plant.
- Mutual antagonism between plant and pathogen which causes the death of both.
- Mutual adjustment leads to a compatible cellular relationship between the two. Symbiotic relationships between mycorrhizal fungi and plant roots and endophytic fungi and bacteria are examples.

1.7.2. Non-host resistance

Non-host resistance (NHR), shown by an entire plant species to a specific parasite or pathogen, is the most common and durable form of plant resistance to disease-causing organisms (Heath, 2000; Mysore & Ryu, 2004; Nürnberger & Lipka, 2005). Plants that are resistant to all isolates of a pathogen are termed non-host plants. It has been proposed that NHR against bacteria, fungi and oomycetes can be classified into two types: type I and type II. Type I NHR does not produce any visible symptoms (necrosis) whereas type II is always associated with rapid localised necrosis (hypersensitive response – HR – a programmed cell death pathway). The type of NHR response activated is dependent on both the plant species and the pathogen species. The same plant species can show type I NHR against one pathogen species and type II resistance against another pathogen species (Mysore & Ryu, 2004). Type I NHR relies on passive or preformed defences (Osbourn, 1996, 2001; Heath, 2000; Mysore & Ryu, 2004; Nürnberger & Lipka, 2005; Fan & Doerner, 2012), including physical barriers such as cytoskeleton and/or chemical strategies such as secondary metabolites and antimicrobial proteins (Broekaert *et al.*, 1995). These preformed antibiotics are also called phytoanticipins (VanEtten *et al.*, 1994) as opposed to phytoalexins which are low molecular weight, antimicrobial compounds that are both synthesised *de novo* by and accumulated in plants after exposure to stressors (Paxton, 1981; Hammerschmidt, 1999; Pedras *et al.*, 2008). Type II NHR relies on active or induced defence responses, probably by the production of detoxifying enzymes occurring when pathogens overcome preformed and general elicitor-induced plant defence responses. Specific pathogen elicitors are then recognised by the plant and this triggers plant defence leading to HR (Osbourn, 1996; Mysore & Ryu, 2004). This will prevent the further spread of the pathogen from the infected cell. However, HR has been described in both host-specific and in NHR and it is unclear whether non-host HR is controlled by the same regulators of cell death that are responsible for host HR. Non-host interactions are, therefore, often referred to as displaying “non-host HR” or “HR-like” cell death (Mysore & Ryu, 2004; Nürnberger & Lipka, 2005). Type II NHR has been described, for instance in *Acacia pulchella*, *Juncus bufonius*, *Triticum aestivum* and *Zea mays* plants against *Phytophthora* species including *P. cinnamomi* (Tippett & Malajczuk, 1979; Hinch *et al.*, 1985; Wetherbee *et al.*, 1985; Cahill *et al.*, 1989; Reiter *et al.*, 2004; Rookes *et al.*, 2008; Allardyce *et al.*, 2012).

Defence activation requires pathogen detection, which can occur outside or inside the plant cell, using a two-branched innate immune system (Jones & Dangl, 2006). The first recognises and responds to molecules common to many classes of microorganisms, including non-pathogens. The second responds to pathogen effectors, either directly or through their effect on host targets (*ibid.*). The first line of detection resides at the cell surface and involves

recognition of pathogen-associated molecular patterns (PAMPs) that activate pattern-recognition receptors (PRRs) in the host, resulting in a downstream signalling cascade that lead to PAMP-triggered immunity (PTI) (Jones & Dangl, 2006; Oßwald *et al.*, 2014). Successful pathogens have evolved effectors to overcome PTI resulting in effector-triggered susceptibility (ETS). Plants in turn possess a second line of defence: acquired resistance (R) proteins that recognise specific effector molecules or their effects on host cell components activating effector-triggered immunity (ETI). The pathogen uses new mechanisms to avoid ETI, resulting in a new ETS phase. Consequently, by natural selection, new resistance (R) proteins can arise and ETI can be activated again and immunity is restored. As an "arms race" this process can be repeated indefinitely (Jones & Dangl, 2006; Oßwald *et al.*, 2014).

1.7.3. Resistance to *Phytophthora cinnamomi* at the cellular level

Resistance to *P. cinnamomi* at the cellular level has been described. The first study with a field resistant host, *Acacia pulchella*, revealed that root host cells appeared to respond with HR to infection by accumulation of phenolic compounds in the necrotic cells and wall appositions close to the invading hyphae (Tippett & Malajczuk, 1979). However, studies by Weste and Cahill (1982) revealed that after 24 h infestation of *A. pulchella* with *P. cinnamomi* zoospores, no root necrosis, water soaked tissue, leaf wilt or chlorosis were seen; just scattered small brown flecks. Moreover, no sporangia, chlamydospores or hyphae were observed on the roots. Although no macroscopic lesions were seen in *A. pulchella*, hyphae associated with necrotic cortical cells were lysed, but others penetrated the stele 16 h after infestation (*ibid*.).

High constitutive levels of phenolic compounds and lignin were found in *P. cinnamomi*-resistant clonal lines of *Eucalyptus marginata* and both type of compounds increased after infestation (Cahill *et al.*, 1993). Although lignin deposition increased over time in *Zea mays* roots infested with *P. cinnamomi* this was not associated with attempted hyphal penetration as the pattern of accumulation was similar in both control and infected tissue (Allardyce, 2011).

Root histology and microtechniques are used to study the changes at cellular level and characterise responses to abiotic and biotic factors. A study of cellular and histological changes in 13 plants ranging from fully susceptible to fully resistant, on the basis of their symptoms shown in the field, revealed that *P. cinnamomi* penetrated the roots of all species. Root growth stopped within 24 h of infection but restarted within 48 h in resistant species. In resistant species, lesions were contained before spreading to the hypocotyl. In all plant species there was deposition of phenolic materials, granulation of the cytoplasm, shrinkage of the

protoplasts and cell wall distortion and disruption. Cell wall lignification and formation of callose papillae were frequently observed in the resistant species but also occurred in some susceptible ones. These changes followed a gradient with the fully susceptible and fully resistant types representing the extremes (Cahill *et al.*, 1989). Papillae (callose deposits) were also observed by electron microscopy in root tissues of *Z. mays* in response to *P. cinnamomi* hypha in some, but not all, regions of contact. Papillae did not completely encase the hypha or stop hyphal growth (Hinch *et al.*, 1985). In this plant, callose deposition was detected at all examined time-points (Allardyce, 2011).

The function of the roots of terrestrial plants, besides anchorage, is to take up water and nutrients. At the same time, roots must avoid toxic compounds and infection by soil borne pathogens (Hose *et al.*, 2001). Specific unicellular tissues, the exodermis and the endodermis, allow roots to establish and maintain this selectivity. The exodermis is a unicellular cell layer located at the outer surface of the root directly below the root epidermis. The endodermis is a unicellular cell layer separating the central cylinder of the root from the cortex. Both tissues are characterised by specific cell wall modifications. In early developmental stages, the anticlinal radial walls exhibit Casparian bands, composed of the polymers suberin and lignin. There may also be a deposition of cellulosic secondary walls (Hose *et al.*, 2001). A typical root is comprised of an outer cortex bounded on the outside by an epidermis and on the inside by an endodermis.

Although *Z. mays* is considered a resistant monocot model for *P. cinnamomi*, in the epidermal layer, hyphae grow intercellularly through the middle lamella (Hinch *et al.*, 1985). Within the outer cortex intercellular growth and intracellular growth also occurs. In the inner cortex, growth is preferentially intercellular through the air spaces but in the stele intracellular growth is common (Wetherbee *et al.*, 1985).

1.8. References

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Chapter 2

Can *Phlomis purpurea* protect susceptible species under controlled conditions?

The introduction has been omitted, as a general introduction was presented in Chapter 1.

2.1. Abstract

Phlomis purpurea plants were shown to protect susceptible *Quercus ilex* subsp. *rotundifolia* and *Q. suber* (Neves *et al.*, 2014) against *Phytophthora cinnamomi* infection and to reduce the inoculum potential in glasshouse trials, indicating the ability of *P. purpurea* to reduce root infection by the pathogen. Now, three more experiments were performed to evaluate the protective action of *P. purpurea* towards other *Quercus* spp, namely *Q. canariensis* and *Q. coccifera* and the highly susceptible *Castanea sativa. P. purpurea* was able to significantly reduce the average severity of root symptoms in the susceptible plants, when planted together. In addition, *P. purpurea* reduced the soil inoculum potential in two out of these three experiments.

Planted alone, *P. purpurea* was able to eliminate all the *P. cinnamomi* propagules present in the soil after 16 weeks (Experiment 2). However, once the roots are infected, *P. cinnamomi* recovery is not significantly different from the infested host roots both from plants planted together with *P. purpurea* or planted alone.

2.2. Material and Methods

2.2.1. Plant material

Plant material for pathogenicity tests was obtained from different places (Table 2.1).

Table 2.1. Origin and age of plants/seedlings[1] used for pathogenicity tests (Experiments 1, 2 and 3)

Species	Origin†	Age	Experiment
Castanea sativa	Plants from nursery - Spain	18-month-old	1
	Seeds from Trás-os-Montes	12-month-old	2
	Seeds from Trás-os-Montes	3-week-old	3
Quercus canariensis	Plants from nursery - Spain	27-month-old	1
Quercus coccifera	Plants from nursery - Spain	12-month-old	1
	Seeds from Monchique -Algarve	12-month-old	2
Phlomis purpurea	Plants from nursery - Spain	12-month-old	1
	Seeds from Moncarapacho - Algarve	6-month-old	2
	Seeds from Moncarapacho - Algarve	14 month-old	3

[1] Plants and *Castanea sativa* seeds were kindly offered by "Viveros de la Junta de Andalucía - San Jerónimo" nursery at Seville, Spain and by Professor José Laranjo (UTAD), Portugal, respectively.

Castanea sativa (n=200)*, Q. coccifera* (n=300)*,* were sown in alveolar trays. Fifty well trays were disinfected with sodium hypochlorite at 10 % for 24 h and then washed with tap water. The substrate, a mixture of potting soil and vermiculite (1:1), was analysed and confirmed to be *P. cinnamomi* free. After three months, the germinated seeds were transplanted to 4-litre plastic pots (13 x 13 x 24 cm). The germination rate varied from species to species, being 98 % in *Q. coccifera* and 60 % in *C. sativa*. *P. purpurea* seeds were pre-germinated (up to 54 % germination rate) and sown in PVC tubes (see Chapter 5– Figure 5.2). After 6 months the survival rate was 91 %. All seedlings had distinct genotypes and *P. purpurea* seeds were collected from a number of different plants growing in the wild in order to ensure genetic diversity.

2.2.2. *Phytophthora cinnamomi* isolates

Pure stock cultures of each of the *P. cinnamomi* isolates-PA37 and PA45-both mating type A2 were tested for pathogenicity. They were isolated in the Algarve region (southern Portugal) from *Quercus suber* roots at Lagos and from soil associated with declining *Q. suber* stands at S. Brás de Alportel, respectively. Isolation was carried out using 3-8-day-old hydromorphic soft leaflets of *Q. ilex* or *Q. suber* floated over flooded soil (Moreira-Marcelino, 2001). Infected brownish or discoloured leaflets, which normally appeared after 2 to 5 days, were blotted on sterile filter paper to remove excess water, cut into small pieces and plated onto selective NARPH medium [Corn meal agar (17 gl^{-1}) amended with Nystatin (0.03 gl^{-1}), Ampicillin (0.25 gl^{-1}), Rifampicin (0.10 gl^{-1}), Pentachloronitrobenzene (0.10 gl^{-1}) and Hymexazol (50 mgl^{-1})] (Rodríguez *et al.*, 2004). Petri dishes were incubated at 24 °C in the dark and examined daily under the light microscope for *Phytophthora*–like hyphae. After *ca* 48 h, *P. cinnamomi* isolates were transferred onto V8 agar (V8A). [V8A was prepared by adding to 330 ml of V8 juice (Campbell Soup Co., Camden, NJ), 4.5 g of calcium carbonate ($CaCO_3$) and stirred for 30 min. Culture media was transferred to 1,000 ml centrifuge flasks and centrifuged at 2,500 x g for 15 min at 20 °C . The supernatant was then, poured into a new flask without disturbing the pellet. The cleared V8-juice (V8 broth) was then, diluted 10 fold with distilled water; 15 g of agar per 1000 ml was added and autoclaved at 121 °C for 20 min. After sterilisation, the culture media was poured into Petri dishes, allowed to solidify at room temperature and kept at 4 °C]. The colonies obtained were identified morphologically under the light microscope and using a molecular identification method (Coelho *et al.*, 1997). Culture maintenance was on V8A.

2.2.3. Inoculum preparation

Axenic cultures of PA 37 and PA 45 were grown in the dark at 24 °C on V8A. After 4 to 5 days, 5 mm agar plugs taken from the edge of individual colonies were transferred into a 90 mm Petri dish containing V8® broth (V8B). Isolates were grown for 4 weeks at 24 °C, to allow each isolate to produce abundant infective structures. Inoculum suspensions were prepared by mixing the content of six agar plates previously washed and free of agar with 600 ml of sterile deionised water in a blender (Philips twister) for 3 min at high speed. *P. cinnamomi* suspensions for plant and soil infestation were prepared in water because they are easier to prepare, and are as effective as inoculum from culture media (Sánchez *et al.*, 1998; Sanchez *et al.*, 2005; Caetano, 2007).

The volume of water suspension inoculum was calculated to have sufficient to inoculate all plants/seedlings and/or infest soil apart from the controls.

2.2.4. Soil and plant infestation

For infestation, plants/seedlings were taken out of their containers and the root ball homogeneously covered with the inoculum of *P. cinnamomi* isolates PA37 and PA45. They were then transferred into 4-litre plastic pots (13 x 13 x 30 cm) containing potting soil (PlantaFlor, Humus verkausfs, GmbH, D-49377 Vechta, Germany) and river sand−1:1 for experiment 1 and vermiculite (1:1) for experiment 2 and 3. This mixture was screened for the presence of *P. cinnamomi*, which was confirmed to be absent.

One hundred millilitres of water suspension inoculum was added to each plant or to the soil, in the case of soil infestation without plants/seedlings. All controls were treated in the same way, except that inoculum was substituted by sterile water. To avoid contamination, controls were treated first. The replicate pots for each species, isolate and control were gathered in plastic trays and placed in a net shelter. To avoid cross contamination, infested and control pots were placed in different trays. After one week, every tray (40 x 30 x 24 cm), including the controls, was filled with tap water and plants/seedlings submitted to soil waterlogging two days per week. The water level in the trays was maintained at 5 cm below the soil surface, by adding water each time that plants/seedlings were submitted to waterlogging, as described by Sánchez *et al.* (1998). Maximum and minimum temperatures were recorded every day.

In Experiment 1, the average temperature was 21.6 °C (average of maximum temperature was 28.2 °C and average minimum temperature of 15 °C). The experiment lasted for 8 weeks for *C. sativa*, 12 weeks for *Q. coccifera* and 13 weeks for *Q. canariensis* plants.

In Experiment 2, the average temperature was 14.0 °C (average of maximum temperature was 20.3 °C and average minimum temperature of 7.8 °C). The experiment lasted for 16 weeks.

In Experiment 3, the average temperature was 13.9 °C (average of maximum temperature was 20.1 °C and average minimum temperature of 7.6 °C). The total experiment lasted for 15 weeks, although *C. sativa* had been infested for only 5 weeks.

2.2.5. Pathogenicity tests and protective effect of *Phlomis purpurea* against *Phytophthora cinnamomi* under controlled conditions - Experimental design

2.2.5.1. Pathogenicity tests and influence of *Phlomis purpurea* on *Castanea sativa*, *Quercus canariensis* and *Quercus coccifera* plants[2] infested with *Phytophthora cinnamomi* (Experiment 1)

Six replicate pots per plant species were mock infested by adding sterile distilled water (condition 1, hereinafter C1). A second group consisted of one of each plant species planted between two *P. purpurea* plants also mock infested (condition 2, hereinafter C2). Another group consisted in six replicate pots per plant species infested with a mixture of *P. cinnamomi* isolates (condition 3, hereinafter C3). A fourth group consisted of one of each plant species infested with *P. cinnamomi* and planted between two *P. purpurea* plants (condition 4, hereinafter C4). The experiment was repeated once.

The *C. sativa* controls in Experiment 2.1 showed that the plant material from the nursery was contaminated with *Phytophthora cambivora* so it was decided to repeat the experiment with plantlets obtained from seeds.

Quercus canariensis is an uncommon oak in the Iberian Peninsula, and we could only get a few seeds from Monchique and Córdoba (Spain) which were not enough to perform an experiment, so it was decided to exclude this species from the Experiment 2.

2.2.5.2. Pathogenicity tests and influence of *Phlomis purpurea* on *Castanea sativa and Quercus coccifera* seedlings infested with *Phytophthora cinnamomi* (Experiment 2)

Ten replicates per plant species were prepared. The experiments were conducted using the same design as in Experiment 1, i.e. a total of 40 seedlings per species, except for

[2] It was decided to call "plants" to the material that came from the nursery, as there is no information about the plant propagation (seeds or cuttings).

P. purpurea for which 100 plants were used. The infestation was carried out as described above. The experiment was repeated once.

2.2.5.3. Pathogenicity tests and influence of *Phlomis purpurea* planted in soil infested with *Phytophthora cinnamomi* 10 weeks before transferring *Castanea sativa* plantlets (Experiment 3)

Ten pots per seedling species/condition were prepared. Another two pots without seedlings were also prepared. Thirty two pots with infested soil and 32 control pots were placed in trays containing two pots each, under a net shelter. From these, 20 infested and 20 control pots had two *P. purpurea* seedlings planted in each pot (Figure 2.1). Ten weeks later, pre-germinated *C. sativa* were transferred to the control or previously infested soil in pots, alone or with *P. purpurea*. Another 10 pots were prepared to infest 10 *C. sativa* seedlings (simultaneously to the plantation) to evaluate the effect of *P. cinnamomi* inoculum on pre-germinated seedlings. The experiment lasted for 5 weeks after planting *C. sativa* because the infested seedlings started to die. The experiment was repeated once.

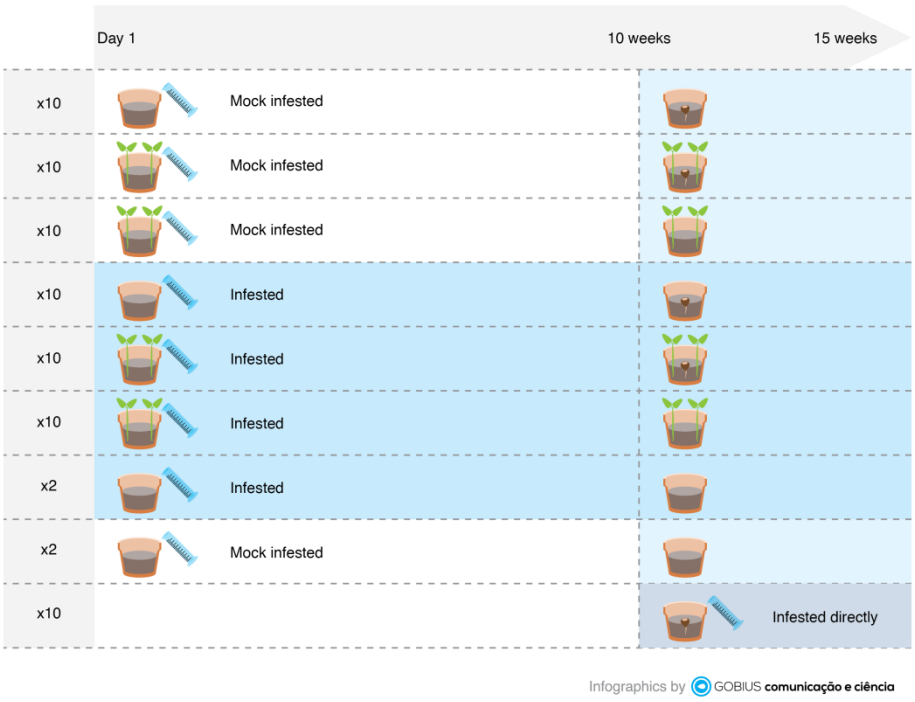

Figure 2.1. Experimental design of Experiment 3 showing the infestation of soil and of 14-month-old *Phlomis purpurea* seedlings with *Phytophthora cinnamomi* 10 weeks before the introduction of the highly susceptible 3-week-old *Castanea sativa*. *C. sativa* seedlings were also infested simultaneously to the plantation (Infested directly). Plant replicates are shown in the first column.

2.2.6. Assessment of foliar and root symptoms

The seedlings were removed from their pots and the foliar and root systems were evaluated: soil was removed from the roots and the roots washed with tap water and kept in water for 2 to 6 h until they were evaluated. The severity of aerial and root symptoms was assessed for each plant on a 0-4 scale (Table 2.2). The aerial part of the plant was evaluated according to the percentage of leaves with yellowing, necrosis or defoliation and the roots by the amount of roots lost, when compared with controls, and percentage of necrotic roots.

Table 2.2. Scale to evaluate the severity of symptoms in the plants/seedlings.

Severity of symptoms	Percentage of affected tissue (root or aerial)
0	0 % = without symptoms
1	1-33 %
2	34-66 %
3	67-99 %
4	100 % = dead plant

Intermediate values were used according to the severity of symptoms: 0.3 = weak, 0.5 = moderate, 0.7 = strong (Trapero & Jiménez, 1985; Sánchez *et al.*, 1998). Plants/seedlings were kept with their roots wrapped in a wet absorbent paper in the dark at 4°C , until needed, but no longer than 12 days (Streito *et al.*, 2002).

2.2.7. *Phytophthora cinnamomi* re-isolation from roots

Confirmation of infection of root tissue by *P. cinnamomi* was assessed by re-isolation of the pathogen. Roots from infested and control plants/seedlings were plated on NARPH medium after being washed under running tap water for 1-2 hours, aseptically cut transversally into small segments (5-8 mm long) and blotted on sterile filter paper. For each plant, six to eight groups of root fragments were laid equidistantly on the medium surface in three to five Petri dishes. Each group comprised of 3–10 fragments according to their diameter so that each group occupied an identical area.

For each plant, *ca* 5 Petri dishes were used and sealed with Parafilm®. The plates were incubated at 24 °C in the dark for 2-3 days. Colonies isolated from the tissues were classified on the basis of cultural characteristics and identified by the morphology of vegetative and reproductive structures under a light microscope (Sánchez *et al.*, 1998).

2.2.8. Inoculum potential of soil

Two different methods were used, alone or in parallel, to isolate *P. cinnamomi* from soil. After removing plants/seedlings from pots, the soil from control and infested plants/seedlings was analysed for *P. cinnamomi* inoculum population.

Method 1 is soil extension onto NARPH selective medium. Two and a half grams of soil, taken from several places in each sample of soil, were placed in an Erlenmeyer containing 50 ml 0.2 % sterile agar solution and homogenised with a magnetic stirrer. Once the mixture was homogeneous, 1 ml was placed into each of 20 Petri dishes containing NARPH culture media. Plates were incubated in the dark at 24 °C. After 24 hours, the plates were washed under running tap water to eliminate the soil and incubated in the dark at 24 °C for another 2 to 3 days (D'Souza *et al.*, 2004; Rodríguez *et al.*, 2004). *P. cinnamomi* population was expressed as colony forming units per gram of soil (c.f.u. g soil^{-1}).

Method 2 is the serial dilution end-point (Tsao 1960) and is commonly used to estimate the disease potential of *Phytophthora* spp. in soil. The soil is diluted with sterilized soil or sand in a series. Soil samples are covered with distilled water and leaves from susceptible host(s) are incubated at a suitable temperature in the water above the soil as baits to attract zoospores (see section 2.2.2.). From each group of plants/seedlings, 200 ml (Experiment 1) and 1000 ml (Experiment 2) soil subsamples were used. Isolates were identified by watching colony growth patterns and morphological features. When classical identification was ambiguous, ITS-rDNA sequences were generated according to Cooke *et al.* (2000). Sequence analysis of ITS regions allowed distinguishing morphologically similar *Phytophthora* species.

2.2.9. DNA extraction, PCR amplification and sequencing

Agar plugs cut from the growing margin of fresh cultures were inoculated into V8B so as to produce sufficient mycelium for DNA extraction. After incubating 3 days at 24 ^{o}C, the mycelium was washed in SDW, the residual agar removed and the mycelium blotted dry in sterile filter paper and freeze dried for storage at -20 ^{o}C.

Genomic DNA from freeze-dried and milled, with a pestle and a mortar in the presence of liquid nitrogen, mycelium was extracted using the DNeasy plant mini kit (Qiagen, Hilden, Germany) according to the manufacturer protocol. Ten microliters of DNA of 40 samples were used to perform PCR using primers for the ITS region. The primers were ITS 4: 5'-TCC TCC GCT TAT TGA TAT GC-3' and ITS 6: 5'-GAA GGT GAA GTC GTA ACA AGG-3'. The cycling parameters

were: denaturation at 94 °C for 2 min, followed by 35 cycles of 30 s at 94 °C, 45 s at 55 °C and 60 s at 72 °C and ended with a final elongation step at 72 °C for 5 min. Amplification products were checked by agarose gel electrophoresis and samples were sent to Macrogen (sequence service provider) for sequencing with ITS 4 and ITS 6 primers. The sequences were compared to other ITS sequences using the BLAST tool in NCBI (http://www.ncbi.nlm.nih.gov/) database.

2.2.10. Determination of aerial plant parts, secondary roots and principal root weights

The weight of the aerial plant parts, secondary roots and principal root was determined (Mettler Toledo, AG204) in experiments 2 and 3, to find any significant differences between the conditions. Fresh weight was determined, since for *P. cinnamomi* recovery from roots, these cannot be dried.

2.2.11. Statistical analysis

Data were analysed by One-Way ANOVA, followed by Fisher's protected least significant difference (LSD) test or independent T-test at $P \leq 0.05$ (Steel & Torrie, 1985) with the software package "PASW statistics 18" (IBM software, 2009 SPSS Inc. Hong Kong). The data was normally distributed and homogeneity of variance was confirmed using Levene's test (Levene, 1960). Statistical comparisons were made between the different conditions within the same plant species.

The independent variables were the plant species under the four conditions (C1, C2, C3 and C4) mentioned under section 2.2.5.1. The dependent variables were the evaluation of aerial and root symptoms, the percentage of *P. cinnamomi* re-isolated from roots, the number of *P. cinnamomi* colonies that grew out from soil in NARPH media, the weight of the aerial plant parts, the weight of the principal root, the weight of the secondary roots, the weight of total roots and root length, when applicable.

2.3. Results and discussion

2.3.1. Pathogenicity tests and influence of *Phlomis purpurea* on *Castanea sativa*, *Quercus canariensis* and *Quercus coccifera* plants infested with *Phytophthora cinnamomi* (Experiment 1)

To evaluate the effect of *Phytophthora cinnamomi* in potential hosts and the effect of *Phlomis purpurea* in the eventual protection of these host species, pathogenicity tests were performed by infesting the plants with liquid inoculum (Sánchez *et al.*, 2002). The plants were infested with a mixture of two *P. cinnamomi* isolates (PA 37 and PA 45). Plants were submitted to waterlogging to induce favourable conditions for disease development (Sánchez *et al.*, 2002; Caetano, 2007). To evaluate the protective effect of *P. purpurea* against *P. cinnamomi* infection, potential hosts were planted with two *P. purpurea* plants in the same pot.

The pathogenicity of *P. cinnamomi* towards *C. sativa*, *Q. canariensis* and *Q. coccifera* was evaluated by the re-isolation of the pathogen from the plant roots by plating the roots onto NARPH and by assessing the foliar and root symptoms. The comparison was made between control (non-infested, C1) and infested (C3) plants.

The influence of *P. purpurea* was evaluated by the re-isolation of the pathogen from the host roots that grew together, in the same pot, with purple phlomis, by assessing the foliar and root symptoms and by determining the soil inoculum potential. The protective effect was determined by the comparison between infested alone (C3) and infested plants grown together with *P. purpurea* plants (C4). A comparison between control (C2) and infested (C4) both grown together with *P. purpurea* plants was also done to evaluate the effect of three plants growing in the same pot. Moreover, *P. purpurea* was always evaluated using the same parameters.

The average temperature was 21.6 °C (average of maximum temperature was 28.2 °C and average minimum temperature of 15 °C). The experiment lasted for 8 weeks for *C. sativa*, 12 weeks for *Q. coccifera* and 13 weeks for *Q. canariensis* and *P. purpurea* plants.

2.3.1.1. Assessment of foliar and root symptoms

The aerial symptoms of all plants were evaluated according to the percentage of leaves with yellowing, necrosis or defoliation; and the roots by the amount of roots and percentage of necrotic roots (Sánchez *et al.*, 2002) at the end of the experiment.

Castanea sativa plants started to decline around 4 weeks after the assay started. After 8 weeks many plants were dead, so it was decided to evaluate these plants first (Figure 2.2).

Four infested *C. sativa* died and two grown together with *P. purpurea* also died. The root system was severely affected in infested plants and there was no significant difference between plants both grown alone and together with *Phlomis purpurea* (Table 2.3). In this assay *C. sativa* was shown to be highly susceptible to *P. cinnamomi*. There was a very strong significant positive correlation (r=0.883; *P*<0.0001) between the average severity of aerial symptoms and the average severity of root symptoms[3]. Plants infested both grown alone and infested together with *P. purpurea* had average symptom severities of 3.2 and 3.3 respectively. This is not surprising as it is largely documented the susceptibility of *C. sativa* to *P. cinnamomi* (Vettraino *et al.*, 2001, 2005). In Portugal, *P. cinnamomi* was first isolated from severely declining *C. sativa* stands affected with the ink disease, together with *P. cambivora* (Lopes-Pimentel, 1947).

Quercus coccifera and *Q. canariensis* plants were evaluated at weeks 12 and 13, respectively. There was a significant difference between the root symptoms of non-infested and infested *Q. canariensis* (*P*=0.001) suggesting that *P. cinnamomi* is pathogenic to *Q. canariensis*. The root symptoms of infested *Q. canariensis* planted together with *P. purpurea* (C4) were not significantly different from the non-infested (control, C2), and were significantly less than the infested plants grown alone (C3), meaning that *P. purpurea* was able to significantly protect *Q. canariensis* against *P. cinnamomi* infection (Table 2.3). However, the aerial parts of infested *Q. canariensis* planted together with *P. purpurea* were not significantly less affected than the infested ones. This was also observed in other pathogenicity tests of *Quercus suber* (Neves, 2014). There was a significant moderate positive correlation (r=0.492; *P*=0.015) between the average severity of aerial symptoms and the average severity of root symptoms[4].

The aerial and root symptoms of infested *Quercus coccifera* plants (C3) are significantly higher than the non-infested plants (C1) suggesting that *P. cinnamomi* is also pathogenic to this plant (Table 2.3). The aerial and root parts of *Q. coccifera* infested plants planted together with *P. purpurea* (C4) had significantly less severe symptoms than the infested plants grown alone (C3).

Phytophthora cinnamomi is a soil-borne pathogen that affects roots, and the most conclusive evaluation is from the root system. The use of aerial symptoms to evaluate infection should be done with caution as they may be highly unspecific, especially in

[3] Appendix I.I.IV.
[4] Appendix I.I.VIII.

glasshouse trials involving water logging. It is, usually, necessary to have a very extensive root lesion to observe the aerial symptoms (Erwin & Ribeiro, 1996).

Both *Q. canariensis* and *Q. coccifera* seem to be moderately susceptible to this oomycete when compared to *C. sativa*, which is highly susceptible. *C. sativa* seems to be more susceptible to *P. cinnamomi* infection than *Q. suber* and *Q. ilex*, but to compare the susceptibility these plants should be tested in the same experiment, as the environmental conditions are known to influence the infection.

Furthermore, when the roots of non-infested plants are compared (C1 versus C2), the plants grown alone (C1) have a better evaluation, although not significant, than plants together with *P. purpurea* (C2) probably due to competition for space due to the reduced volume of the pot. When the roots of non-infested plants grown together with *P. purpurea* are compared with infested plants grown together with *P. purpurea* (C2 versus C4), in *C. sativa*, there is a significant difference but in *Q. canariensis* and in *Q. coccifera* plants there is no significant difference suggesting that *P. purpurea* significantly protected these two oaks.

The assessment of *P. purpurea* aerial and root systems in infested and non-infested (Table 2.3) confirmed former work showing that *P. purpurea* is not susceptible to *P. cinnamomi* (Neves, 2007; Neves *et al.,* 2014).

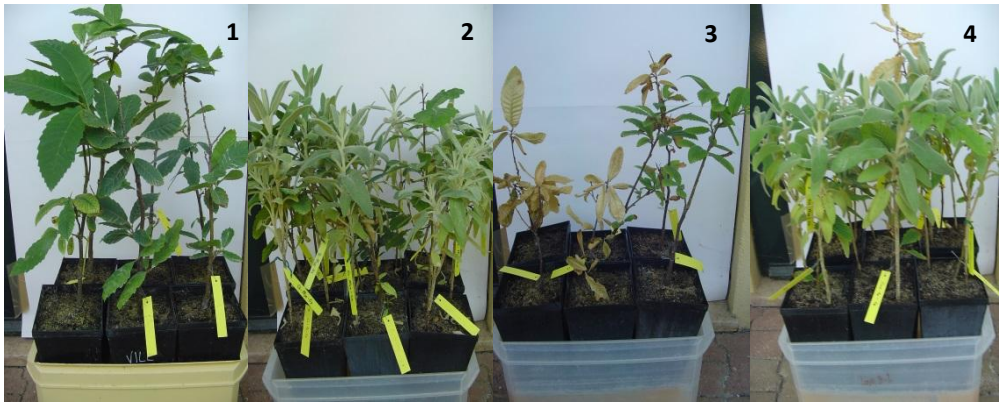

Figure 2.2. Experiment do evaluate the influence of *Phlomis purpurea* (12 month-old) on *Castanea sativa* (18 months-old) plants infested with *Phytophthora cinnamomi* for 8 weeks. Conditions: 1- *C. sativa* control plants; 2- *C. sativa* control plants together with *P. purpurea*; 3- *C. sativa* plants infested; 4- *C. sativa* plants infested together with *Phlomis purpurea*.

Table 2.3. Assessment of the pathogenicity, by evaluation of symptom severity, of *Phytophthora cinnamomi* towards *Castanea sativa, Quercus canariensis*, *Q. coccifera* and *Phlomis purpurea,* under controlled conditions*,* and of the effect of the presence of *P. purpurea* on symptom severity[5].

Species	Condition[§]	Average of symptom severity [†]	
		Aerial	Root
Castanea sativa	C1: Non-infested (NI) (control)	1.6±0.7 [a]	1.0±0.7 [b]
	C2: NI (control) + *P. purpurea*	1.9±0.9 [a]	1.2±1.0 [b]
	C3: Infested	3.2±1.2 [b]	3.2±1.2 [d]
	C4: Infested + *P. purpurea*	3.0±1.0 [b]	3.3±0.6 [d]
Quercus canariensis	C1: Non-infested (NI) (control)	0.8±0.2[a]	0.4±0.1 [a]
	C2: NI (control) + *P. purpurea*	1.1±0.2 [a]	0.7±0.2 [a]
	C3: Infested	1.7±0.2 [b]	1.6±0.9 [b]
	C4: Infested + *P. purpurea*	1.9±0.2 [b]	1.0±0.6 [a]
Quercus coccifera	C1: Non-infested (NI) (control)	0,5±0.2 [a]	1.0±0.3 [a]
	C2: NI (control) + *P. purpurea*	0.4±0.2 [a]	2.0±0.7 [b]
	C3: Infested	1.8±0.6 [b]	2.7±0.2 [c]
	C4: Infested + *P. purpurea*	0.9±0.2 [a]	2.0±0.9 [b]
P. purpurea	C1: Non-infested (NI) (control)	0.6±0.1 [a]	0.4±0.2 [a]
	C3: Infested	0.5±0.1 [a]	0.3±0.0 [a]

Statistical comparison made between the different conditions for each species. Figures followed by the same letters are not significantly different at $P \leq 0.05$, using Fisher's Least Significant Difference Test (LSD).

[§] Pathogenicity test [comparison between non-infested (control) (C1) and infested (C3) plants]. Protective effect [comparison between infested (C3) and infested + *P. purpurea* (C4) plants]

†Average symptom severity (± standard deviation). Scale for symptom severity: 0=without symptoms; 1=<33 %; 2=34–66 %; 3=67–99 %; 4=dead root or aerial part. In between values were used according to the severity of symptoms: 0.3 = weak, 0.5 = moderate, 0.7 = strong. *P. cinnamomi* was a mixture of isolates PA 37 and PA 45.

2.3.1.2. *Phytophthora cinnamomi* re-isolation from plant roots

The re-isolation of *P. cinnamomi* from infested roots is a proof that roots were infected and consequently the plant is a host for *P. cinnamomi*, either tolerant or susceptible.

Phytophthora cinnamomi was recovered, in a high percentage, from the roots of all infested plants grown alone (C3) except from *P. purpurea*. In *Q. canariensis*, the percentage of *P. cinnamomi* recovery was as high as in *C. sativa*. In a previous study, a trunk inoculation experiment was conducted (Belbahri *et al.*, 2006) using *Phytophthora polonica* a species isolated from *Alnus glutinosa* stands. *Q. canariensis* were inoculated with *P. cinnamomi* as a positive control based on the supposition that *P. cinnamomi* is a well-known pathogen for oaks. *P. polonica* caused lesion lengths to *Q. canariensis* less than *ca* 10 % when compared to *P. cinnamomi* although data were not shown. Gómez-Aparicio et al. (2012) also studied the pathogenicity of *P. cinnamomi* towards *Q. canariensis* in the field concluding that "*Q. canariensis* seems to act as a reservoir without showing any apparent disease symptoms".

[5] Appendix I.I.

However, in our study *Q. canariensis* showed moderate aerial and root symptoms (3 out of 6 plants had an average of root symptom severity ≥ 2) (Figure 2.3) and *P. cinnamomi* was recovered from 91.3 % of roots. Pathogenicity involve two components: the virulence (synonym of aggressiveness, quantifiable by the amount of disease) and the parasitic ability, which is the result of the reproductive ability and the specific pathogenicity, that describes the behavior of an isolate on specific hosts (reviewed by Robin and Desprez-Loustau 1998).

To the best of our knowledge, this is the first pathogenicity trial, using the whole plant, showing that *P. cinnamomi* is pathogenic to *Q. canariensis*.

Figure 2.3. Experiment to evaluate the pathogenicity *Phytophthora cinnamomi* on 27-month-old *Quercus canariensis*, after 13 weeks. A. Non-infested (control) and B. Infested *Q. canariensis*.

Phytophthora cinnamomi was also recovered from infested plants grown together with *P. purpurea* (C4) (Table 2.4). Nonetheless, the recovery of *P. cinnamomi* was significantly less (almost half) from *C. sativa* and *Q. canariensis* when planted with *P. purpurea* (C4), suggesting a protective effect of *P. purpurea* towards the infection of these plants by *P. cinnamomi* (Table 2.4). This corroborates previous findings by Neves (2007) who used the susceptible *Q. ilex* to evaluate the protective effect of *P. purpurea* on *P. cinnamomi* infection. *P. cinnamomi* was re-isolated from 100 % of *Q. ilex* infested roots, but only from 36.8 % of *Q. ilex* infested roots planted together with *P. purpurea.* Thus, the association of *P. purpurea* with a susceptible host growing in a close environment appears to reduce the infection of host

roots. This is not so evident in *Quercus coccifera* towards which *P. purpurea* had only a slightly protective effect (Table 2.4), although the average of symptom severity was identical in conditions C2 and C4 (Table 2.3).

A single colony of *P. cinnamomi* was isolated from one fragment of *P. purpurea* infected roots, resulting in 2.7 % recovery. This was probably due to soil particles attached to the roots, since *P. cinnamomi* was never isolated from *P. purpurea* roots (Neves, 2007).

Table 2.4. Re-isolation of *Phytophthora cinnamomi* from the roots of *Castanea sativa, Quercus canariensis*, *Q. coccifera* and *Phlomis purpurea.*

Species	Condition	Re-isolation of *P. cinnamomi* from roots (%)[†]
Castanea sativa	C1: NI	0.0
	C2: NI + *P. purpurea*	0.0
	C3: I	90.0
	C4: I + *P. purpurea*	48.6
Quercus canariensis	C1: NI	0.0
	C2: NI+ *P. purpurea*	0.0
	C3: I	91.3
	C4: I + *P. purpurea*	50.0
Quercus coccifera	C1: NI	0.0
	C2: NI + *P. purpurea*	0.0
	C3: I	78.9
	C4: I + *P. purpurea*	72.5
Phlomis purpurea	C1: NI	0
	C3: I	2.7[*]

[†]Percentage of re-isolation of *P. cinnamomi* from roots, representing the percentage of groups of root fragments with *P. cinnamomi* colonies.
*A small colony in one fragment in a total of 37 groups of fragments.
NI- non infested; I- infested.

2.3.1.3. Inoculum potential of soil

The inoculum potential (IP), was defined as "the energy of growth of a parasite, or fungus, available for infection of a plant or host, or colonization of a substrate, at the surface of the plant organ to be infected, or substrate to be colonized" (Holliday 1989). In the present work it was assumed that when all the environmental conditions are favourable to the interaction between host and pathogen, the IP is the pathogen's capacity to cause infection and would be proportional to the amount of inoculum.

The determination of soil inoculum potential was performed using two methods namely the soil extension on NARPH medium (method 1) and dilution end-point method (method 2) (see section 2.2.8). A comparison between the two methods is presented in Table .2.5.

Using method 1, *P. cinnamomi* was not recovered from the soil of the *C. sativa* NI (C1), as expected, but *P. cambivora* was isolated and confirmed by sequencing. *P. cinnamomi* was recovered from the infested soil both with the host plant alone (condition 3) and grown together with *P. purpurea* (condition 4) but was not recovered from the NI host with *P. purpurea* (C2). However, soil inoculum potential was higher in infested soils where *C. sativa* and *Q. canariensis* grew together with *P. purpurea* (C4). These results are contrary to observations in soils where infested *P. purpurea* plants grew next to infested *Q. ilex* or *Q. suber* plants, and in which the inoculum amount was significantly reduced (P=0.002 and P=0.015, respectively) compared with infested soil associated with *Q. ilex* or *Q. suber* plants grown alone (Neves *et al*., 2014). *P. cinnamomi* was not recovered from the soil of *Q. canariensis* controls (NI) and *Q. coccifera* (C 1, 2, 3 and 4) and *P. purpurea* (C1 and C3).

Jayasekera (2006) used method 1, to show that soil infested with *P. cinnamomi* under 3 varieties of resistant *Acacia pulchella* yielded fewer colonies on agar (mean number of colonies *ca* 1 g^{-1} soil) than the susceptible *A. urophylla* (mean number of colonies *ca* 4 g^{-1} soil). Using the same method no propagules were obtained in infested soil under *P. purpurea* plants (Table 2.5), showing this species a more efficient capacity than *A. pulchella* to eliminate *P. cinnamomi* propagules.

Using the dilution end-point method (method 2), like in method 1 and as expected, *P. cinnamomi* was not recovered from soil associated with *C. sativa* NI (C1 and C2) but *P. cambivora* was recovered. *P. cinnamomi* was recovered from *C. sativa*, *Q. canariensis* and *Q. coccifera* grown alone and infested (C3) and together with *P. purpurea* and infested (C4) using all volumes of soil excepted in C4 in which it was not recovered from 50 ml (Table 2.5). *P. cinnamomi* was also recovered in *P. purpurea* associated soil (C3) using 200 ml of soil but not in 100 ml of soil, meaning that *P. purpurea* was able to reduce the soil inoculum potential but not eliminate all propagules. In general, *P. cinnamomi* was detected in 50 ml of infested soil associated with susceptible species, but not in 50 ml of infested soil associated with susceptible species planted together with *P. purpurea*. These results are not consistent with the ones from method 1. In method 1, only two and half grams of soil were used and if *P. cinnamomi* is not homogeneously dispersed in the soil, it may not be present in that soil sample, explaining the discrepancy in results between the two methods. In method 2, eighty times more soil was used. Also, method 2, using baits is more sensitive than the soil extension in selective medium as is shown in the case of *Q. coccifera* associated infested soil, where no propagules could be recovered using method 1. In method 2, a single propagule of *Phytophthora* spp., which germinates can produce many zoospores and infect several baits in a soil sample and therefore cannot be expressed as amount of inoculum present in soil.

Table 2.5. Recovery of *Phytophthora cinnamomi* from soil, using method 1 – soil extension on NARPH medium and method 2 – baitings with *Quercus suber* leaves.

Species	Condition	*Phytophthora cinnamomi* recovery from soil			
		Method 1* (c.f.u. g^{-1} soil)	Method 2** Soil (ml) 200	100	50
Castanea sativa	C1: NI	0[1]	(-)[1]	(-)[1]	(-)[1]
	C2: NI + *P.p.*	0	(-)[1]	(-)[1]	(-)[1]
	C3: I	1	(+)	(+)	(+)
	C4: I + *P.p.*	15	(+)	(+)	(-)
Quercus canariensis	C1: NI	0	(-)	(-)	
	C2: NI+ *P.p.*	0	(-)		
	C3: I	2	(+)	(+)	(+)
	C4: I + *P.p.*	5	(+)	(+)	(-)
Quercus coccifera	C1: NI	0	(-)		
	C2: NI + *P.p.*	0	(-)		
	C3: I	0	(+)	(+)	(+)
	C4: I + *P.p.*	0	(+)	(+)	(-)
Phlomis purpurea	C1: NI	0	(-)		
	C3: I	0	(+)	(-)	

Conditions 1: Control (NI) plants without *Phlomis purpurea*; 2: Control (NI) plants together with *Phlomis purpurea* (*P.p.*); 3: Plants infested (I) without *Phlomis purpurea*; 4: Plants infested (I) together with *Phlomis purpurea*. *Two and half grams of soil per treatment were analysed. ** Two hundred grams of soil per treatment were analysed. [1)] *Phytophthora cambivora* detected by ITS sequences. (+) - *Phytophthora cinnamomi* detected; (-) - *Phytophthora cinnamomi* not detected.

The DNA of the cultures isolated from NI *C. sativa* soil samples were extracted amplified and sequenced (see section 2.2.9). *Phytophthora cambivora* was identified by ITS sequences. This infestation with *P. cambivora* clearly came from the nursery. The amount of propagules was probably not enough to cause symptoms on the roots, since the average root symptom was low (Table 2.3). A study by Martín *et al*. (2012) showed that under optimal conditions for infection there needs to be more than 6.1 c.f.u. g^{-1} of *P. cinnamomi* in the soil to produce *Q. ilex* and *Q. suber* disease. The same is probably true for *P. cambivora*. *P. cambivora* is the most aggressive species to *C. sativa* after *P. cinnamomi*, and is responsible for the ink disease in chestnut stands in Europe (Vettraino *et al.*, 2001, 2005). Finding *P. cambivora* in our chestnut samples is not surprising as infected nursery stock has been found all around the world (Hansen *et al.*, 1979; Zentmyer, 1980; Hardy & Sivasithamparam, 1988; Jung & Blaschke, 2004; Sanchez *et al.*, 2005; Jung, 2012) including Portugal (M. Jung and T. Jung, personal communications). Most nurseries have the ideal conditions for the development of soil-born oomycetes: periodic waterlogging (caused by excessive irrigation) and a warm environment. It would be advisable to screen the soil and the plants before they are planted off the field, otherwise dissemination of these pathogens would result in serious implications for the

environment namely the decline of many forest trees and crops. As a matter of fact it has been happening for years out of control.

Although these results suggest the high potential of *P. purpurea* for the control of this pathogen, this experiment was repeated with soil and seedlings that were *Phytophthora spp* free (Experiment 2).

2.3.2. Influence of *Phlomis purpurea* on *Castanea sativa and Quercus coccifera* seedlings infested with *Phytophthora* cinnamomi (Experiment 2)

As *P. cambivora* was isolated from the control *C. sativa* plants, it was decided to repeat the experiment with seedlings from a safe source. Therefore, *C. sativa* and *Q. coccifera* seedlings were obtained from seeds prepared by us. In this experiment *P. purpurea* seedlings were 6-month-old. We could not get enough *Q. canariensis* seeds, so this species was excluded in this experiment. This assay lasted 16 weeks, twice the time compared with experiment 1, since no major aerial symptoms were found before in the seedlings. This discrepancy might have been due to the health status of the seedlings and the temperature. The usual temperature range for *P. cinnamomi* growth is 5 to 34 °C being the optimal temperature 20 to 32.5 °C (EPPO, 2004). In experiment 1, the average temperature was 21.6 °C (average of maximum temperature was 28.2 °C and average minimum temperature of 15 °C) that are more favourable to *P. cinnamomi* infection than the temperatures of experiment 2, which average temperature was 14.0 °C (average of maximum temperature was 20.3 °C and average minimum temperature of 7.8 °C) and therefore symptoms take longer to develop. Shew and Benson (1983) showed that the optimal soil temperature infection of *Abies frasieri* (fraser fir) by *P. cinnamomi* was between 16 and 25 °C and at 25 °C all seedlings became infected and died within 41 days.

2.3.2.1. Assessment of foliar and root symptoms

The results of the evaluation of foliar and root symptoms, in this experiment and in experiment 1 hereinabove confirmed that *P. cinnamomi* is pathogenic for *C. sativa* (Marçais *et al.*, 1996; Robin & Desprez-Loustau, 1998; Maurel *et al.*, 2001). *P. purpurea* significantly protects *C. sativa* from infection by the pathogen as shown by the average severity of root symptoms (Table 2.6). There was a significant moderate positive correlation (r=0.519; *P*=0.001) between the average severity of aerial symptoms and the average severity of root symptoms[6].

[6] Appendix I.II.IV

Phytophthora cinnamomi is pathogenic for *Q. coccifera* as it was previously shown in Experiment 1 (Table 2.3), although in this experiment it did not caused major root symptoms, probably due to the temperature as explained in 2.3.2. *P. purpurea* was not able to significantly protect this plant from *P. cinnamomi* infection. There was a highly significant positive correlation (r=0.614; *P*<0.0001) between the average severity of aerial symptoms and the average severity of root symptoms in these *Q. coccifera* seedlings[7] (Table 2.6).

Again, there were no significant differences between *P. purpurea* control and infested seedlings.

Table 2.6. Pathogenicity of *Phytophthora cinnamomi* isolates towards *Castanea sativa*, *Quercus coccifera*, and *Phlomis purpurea* seedlings and *in* situ protective effect of *P. purpurea* against *P. cinnamomi*.

Species	Condition[§]	Average of symptom severity [†]	
		Aerial	Root
Castanea sativa	C1: NI	1.7±0.8 [a]	0.3±0.1 [a]
	C2: NI + *P.p.*	2.0±0.9 [a,b]	0.3±0.1 [a]
	C3: I	2.9±1.3 [b]	1.3±1.0 [b]
	C4: I + *P.p.*	2.5±0.3 [a,b]	0.5±0.3 [a]
Quercus coccifera	C1: NI	0,8±0.3 [a,b]	0.5±0.2 [a]
	C2: NI + *P.p.*	0.5±0.2 [a]	0.4±0.3 [a]
	C3: I	1.3±0.2 [b]	0.9±0.7 [a,b]
	C4: I + *P.p.*	1.1±0.5 [b]	1.1±0.5 [b]
P. purpurea	C1: NI	0.7±0.5 [a]	0.1±0.2 [a]
	C3: I	1.0±0.4 [a]	0.5±0.3 [a]

Statistical comparison made between the different conditions for each species. Figures followed by the same letters are not significantly different at *P*≤0.05, using Fisher's Least Significant Difference Test (LSD).
Conditions 1: Control (NI) seedlings without *Phlomis purpurea*; 2: Control (NI) seedlings together with *Phlomis purpurea* (*P.p.*); 3: Seedlings infested (I) without *Phlomis purpurea*; 4: Seedlings infested (I) together with *Phlomis purpurea*.
[§] Pathogenicity test (comparison between control and inoculated seedlings). Protective effect (comparison between inoculated and inoculated + *P. purpurea* treated seedlings).
†Average symptom severity (± standard deviation). Scale for symptom severity: 0=without symptoms; 1=<33 %; 2=34–66 %; 3=67–99 %; 4=dead root or aerial part. In between values were used according to the severity of symptoms: 0.3 = weak, 0.5 = moderate, 0.7 = strong. *P. cinnamomi* was a mixture of isolates (PA 37 and PA 45).

2.3.2.2. *Phytophthora cinnamomi* re-isolation from roots

Phytophthora cinnamomi was recovered from *C. sativa* and *Q. coccifera* roots of infested seedlings grown both in the presence (C4) and absence of *P. purpurea* (C3). This means that once the pathogen is present it will infect the host in spite of the presence of *P. purpurea*. The oomycete was never recovered from the roots of control seedlings (C1 and 2) or *P. purpurea* seedlings (C1 and 3) (Table 2.7).

[7] Appendix I.II.VIII

In *C. sativa* and *Q. coccifera* seedlings there was a significant moderate positive correlation (r=0.378; *P*=0.016[8] and r=0.473; *P*=0.006[9], respectively) between the average severity of root symptoms and the percentage of *P. cinnamomi* recovered from roots, meaning that the lower the recovery, less root symptoms.

Table 2.7. Re-isolation of *Phytophthora cinnamomi* from the roots of *Castanea sativa, Quercus coccifera* and *Phlomis purpurea.*

Species	Condition	*P. cinnamomi* root isolation (%)[†]
Castanea sativa	C1: NI	0.0±0.0 [a]
	C2: NI + *P.p.*	0.0±0.0 [a]
	C3: I	31.8±37.8 [b]
	C4: I + *P.p.*	25.5±33,1 [b]
Quercus coccifera	C1: NI	0.0±0.0 [a]
	C2: NI + *P.p.*	0.0±0.0 [a]
	C3: I	26.0±25.4 [b]
	C4: I + *P.p.*	69.9±31.2 [c]
Phlomis purpurea	C1: NI	0.0±0.0 [a]
	C3: I	0.0±0.0 [a]

Statistical comparison made between the different conditions for each species. Figures followed by the same letters are not significantly different at *P*≤0.05, using Fisher's Least Significant Difference Test (LSD).
[†]Percentage of re-isolation of *P. cinnamomi* from roots, representing the percentage of groups of root fragments with *P. cinnamomi* colonies. Average of *P. cinnamomi* re-isolation percentage (± standard deviation).
Conditions 1: Control (NI) seedlings without *Phlomis purpurea*; 2: Control (NI) seedlings together with *P.purpurea* (*P.p.*); 3: Seedlings infested (I) without *P.purpurea*; 4: Seedlings infested (I) together with *P. purpurea*.

2.3.2.3. Inoculum potential of soil

In this experiment, *P. cinnamomi* was never isolated from soil where control (NI) seedlings were growing. It was not isolated from infested (I) soil where *P. purpurea* seedlings were growing alone either. *P. cinnamomi* was isolated from 63 ml of artificially infested soil where *C. sativa* seedlings were growing and from 31 ml of soil where *Q. coccifera* seedlings were growing. Below that soil volume no *P. cinnamomi* could be recovered (Table 2.8). These results are in reasonable agreement with the results obtained from experiment 1 (Table 2.5). However, in contrast with the results from Experiment 1, in infested soil where *P. purpurea* was growing with the susceptible seedlings, *P. cinnamomi* was recovered from a lower amount of soil than when the corresponding seedlings were growing alone (Table 2.8). The age of *P. purpurea* (6-month-old versus 12-month-old in experiment 1) used in this experiment might have been crucial as the root development might not be enough to reduce the inoculum

[8] Appendix I.II.IV
[9] Appendix I.II.VIII

potential. An effect of plant age was demonstrated with *A. pulchella*. Four-year-old seedlings were more efficient to suppress *P. cinnamomi* than one-year-old seedlings (Jayasekera, 2006).

Table 2.8. Semi-quantification, using the dilution end-point method, of *Phytophthora cinnamomi* in soil artificially infested. (+) = Presence; (-) = absence of *P. cinnamomi* in soil.

Species	Condition	Volume of soil (ml)						
		1000	500	250	125	63	31	16
Castanea sativa	C1: NI	-	-	-	-	-	-	-
	C2: NI + *P.p.*	-	-	-	-	-	-	-
	C3: I	+	+	+	+	+	-	-
	C4: I + *P.p.*	+	+	+	+	+	+	-
Quercus coccifera	C1: NI	-	-	-	-	-	-	-
	C2: NI + *P.p.*	-	-	-	-	-	-	-
	C3: I	+	+	+	+	+	+	-
	C4: I + *P.p.*	+	+	+	+	+	+	+
Phlomis purpurea	C1: NI	-	-	-	-	-	-	-
	C3: I	-	-	-	-	-	-	-

Conditions 1: Control (NI) seedlings without *Phlomis purpurea*; 2: Control (NI) seedlings together with *Phlomis purpurea* (*P.p.*); 3: Seedlings infested (I) without *Phlomis purpurea*; 4: Seedlings infested (I) together with *Phlomis purpurea*.

2.3.3.4. Determination of plant's aerial, secondary roots and principal root weight

In *C. sativa* seedlings there was a significant difference between the control and infested seedlings for aerial part weight, principal root and total root weight, but not for secondary root weight (Table 2.9). Secondary roots are the ones that are primarily infected by the pathogen reducing the plant ability to absorb water and nutrients (Zentmyer, 1980). There was no significant difference between infested seedlings grown alone (C3) and infested seedlings grown together with *P. purpurea* (C4) although, the weight of the later was generally higher, except for the weight of the principal root that had a higher weight in the infested seedlings grown together with *P. purpurea* (C4), similar to that of the control seedlings grown together with *P. purpurea* (C2) (Table 2.9). There was a significant moderate negative correlation (r=-0.429; *P*=0.006) between the average severity of aerial symptoms and the weight of the aerial part[10], indicating that these variables are inversely proportional, as expected (Tables 2.6 and 2.9). There was a highly significant negative correlation between the average severity of root symptoms (Table 2.6) and the weight of the principal root(r=-0.539; *P*<0.0001) [11], and a moderate significant negative correlation between average severity of root symptoms and the total root weight (r=-0.434; *P*=0.005)[12], but not with the secondary root weight (Table 2.9). It would be interesting if we could correlate the visual root symptoms with

[10] Appendix I.II.IV
[11] Appendix I.II.IV
[12] Appendix I.II.IV.

secondary root's weight, as these are the main roots affected by the pathogen. However, necrotic roots do not weigh less than healthy ones, so unless a big portion of those roots are lost there will not be a significant difference in weight. We have to bear in mind that a statistical correlation does not always mean causation.

In *Q. coccifera* seedlings there was no significant difference in the weight of the aerial part, of the principal root and of total root between the control and infested seedlings, but there was a difference in the secondary root's weight (Table 2.9). The infested roots (C3 and C4) weighed less than the controls (C1 and C2) (Table 2.9), corresponding to same trend of symptom severity (Table 2.6). There was a significant moderate negative correlation (r=-0.483; *P*=0.005) between the average severity of aerial symptoms and the weight of aerial parts[13], so the weight is inversely proportional to the symptom, as expected. There was also a significant moderate negative correlation between the average severity of root symptoms and the weight of secondary roots (r=-0.481; *P*=0.005)[14], so a lower average severity of root symptoms results in a higher weight of secondary roots. There was no significant difference in the weight of aerial parts, of the principal root, of secondary roots and total root weight between the control and infested *P. purpurea* seedlings (Table 2.9).

Table 2.9. Plant´s aerial and root weight.

Species	Condition	Weight (g)†			
		Aerial	Principal Root	Secondary roots	Total roots
Castanea sativa	C1: NI	13.0±1.8 ^{b,c}	10.7±2.1 ^{c}	9.5±3.1 ^{a}	20.2±4.3 ^{b}
	C2: NI + *P.p.*	10.5±6.2 ^{a,b}	8,5±1.4 ^{b}	10.8±3.2 ^{a}	19.3±4.8 ^{a,b}
	C3: I	9.4±4.6 ^{a,b}	6.3±1.7 ^{a}	9.6±2.5 ^{a}	15.8±3.6 ^{a}
	C4: I + *P.p.*	8.3±2.5 ^{a,b}	8.6±2.2 ^{b}	9.8±3.0 ^{a}	18.4±4.1 ^{a,b}
Quercus coccifera	C1: NI	2.6±1.3 ^{a}	4.1±1.8 ^{a}	3.2±1.6 ^{b}	7.2±3.2 ^{a}
	C2: NI + *P.p.*	2.5±0.9 ^{a}	3.7±1.0 ^{a}	3.3±1.7 ^{b}	7.0±2.4 ^{a}
	C3: I	2.2±0.5 ^{a}	3.8±1.0 ^{a}	1.8±0.4 ^{a}	5.6±1.3 ^{a}
	C4: I + *P.p.*	2.5±1.1 ^{a}	4.4±2.2 ^{a}	1.5±0.9 ^{a}	6.0±2.8 ^{a}
Phlomis purpurea	C1: NI	2.1±1.8 ^{a}	0.4±0.2 ^{a}	0.8±0.7 ^{a}	1.2±0.8 ^{a}
	C3: I	2.1±1.3 ^{a}	0.2±0.1 ^{a}	0.7±0.3 ^{a}	0.9±0.3 ^{a}

†Average weight (± standard deviation).

Statistical comparisons were made between the different conditions within the same plant species. For each column, values followed by the same letter means that they do not differ significantly (P<0.05), using Fisher's Least Significant Difference Test (LSD).

Conditions 1: Control (NI) seedlings without *Phlomis purpurea*; 2: Control (NI) seedlings together with *P. purpurea* (*P.p.*); 3: Seedlings infested (I) without *P.purpurea*; 4: Seedlings infested (I) together with *P.purpurea*.

[13] Appendix I.II.VIII.

[14] Appendix I.II.VIII.

The initial weight of each plant part could not be determined, for obvious reasons, and the determination of the weight at the end of the trial will only give us a bias as seedlings, even having the same age, had different sizes and consequently different weight. Moreover, the plantation in the same pot of the plant to be tested next to two *P. purpurea* seedlings leads to competition for space and therefore to a decrease in the plant weight (Table 2.9). The use of this morphometric evaluation is not consensual: Scott *et al*. (2012) did not found significant differences in the weight of *Eucalyptus* spp. plant parts including fine roots, all roots, stems or leaves, between seedlings infested with *P. cinnamomi* isolates and the control; Vettraino *et al*. (2003) found that *P. cinnamomi* caused damage to the lateral roots of *Juglans regia* (English walnut) whose weight was significantly different from the controls, albeit there was no difference in the weight of the above-ground parts. Caetano (2007) reported that root weight is not an adequate method to evaluate the symptom severity, as it does not reflect the root functionality. Some trees can indeed survive secondary root reduction without appreciable crown symptoms (Crombie *et al*. 1987, cited in Vettraino *et al*. 2003) even though their water status and nutrition are affected (Maurel *et al.*, 2001).

As *P. purpurea* was able to significantly protect the highly susceptible *C. sativa* from *P. cinnamomi* infection by reducing significantly the root symptom severity and by reducing the percentage of *P. cinnamomi* re-isolated from the roots, we hypothesized that *P. purpurea* exudes compounds with anti-*P. cinnamomi* activity which take a certain time to eliminate the propagules. Nevertheless, because the seedlings were planted at the same time the activity of *P. purpurea* exudates was insufficient to eliminate all the propagules as the pathogen immediately infects the hosts. Would *P. purpurea* be able to eliminate *P. cinnamomi* inoculum, if planted in advance of susceptible hosts? To answer this question, another greenhouse assay was done using the highly susceptible *C. sativa*.

2.3.3. Influence of *Phlomis purpurea*, planted in advance of 10 weeks in soil infested with *Phytophthora cinnamomi*, on protection of *Castanea sativa* (Experiment 3)

To determine the influence of *P. purpurea* on *P. cinnamomi* inoculum and consequently on the protection of *C. sativa*, soil was infested with *P. cinnamomi* in advance of 10 weeks in pots with two 19-month-old *P. purpurea* seedlings. The same amount of pots with and without *P. purpurea* seedlings was also prepared with infested soil. Moreover, non-infested pots with and without *P. purpurea* seedlings were also prepared. After 10 weeks, three-week-old pre-germinated *C. sativa* seedlings were then planted in pots containing

P. purpurea seedlings (infested and non-infested) and infested and non-infested soil only. At the same time another group of *C. sativa* seedlings were infested to evaluate the effect of *P. cinnamomi* inoculum on pre-germinated seedlings (see Figure 2.1). The last seedlings started to die one week after planting. The average temperature was 13.9 °C and the total experiment lasted for 15 weeks, although *C. sativa* had been infested for only 5 weeks.

2.3.3.1. Assessment of foliar and root symptoms

Castanea sativa seedlings, infested with *P. cinnamomi* inoculum at the time of plantation started to die just one week after start of assay. The amount of inoculum was possibly excessive as it was the same amount used for 18-month-old (Experiment 1) and 12-month-old (Experiment 2) plantlets.

After five weeks, 8 out 10 seedlings in pre-infested soil were dead, so it was decided to evaluate these seedlings immediately, i.e. 15 weeks after soil infestation. The age of the *C. sativa* and the inoculum density were key factors, in this case. Inoculum density and plant age have, in relation to disease severity, a positive and negative correlation respectively (Raftoyannis & Dick, 2002).

Castanea sativa in pre-infested soil in which *P. purpurea* had been growing for 10 weeks displayed significantly lower severity symptoms compared to the infested plantlets grown alone, demonstrating a protective effect of *P. purpurea* (Table 2.10).

Non-infested and infested *P. purpurea* seedlings planted with *C. sativa* seedlings did not show any symptoms of disease and there were no significant differences between them (Table 2.10).

Table 2.10. Evaluation of the protective effect of *Phlomis purpurea* when planted 10 weeks before *Castanea sativa,* against *Phytophthora cinnamomi*. Evaluation of aerial and root symptom severity.

Species	Condition[§]	Average of symptom severity [†]	
		Aerial	Root
Castanea sativa	C1: NI	0.5±1.0 [a]	0.4±0.3 [a]
	C2: NI + *P. purpurea* pre-planted	0.3±0.4 [a]	0.9±0.5 [a]
	C3: In pre-infested soil	3.3±1.5 [c]	3.8±0.4 [c]
	C4: In pre-infested soil + *P. purpurea*	1.9±1.8 [b]	1.9±1.6 [b]
	C5: Planted and infested simultaneously	4.0±0.0 [c]	4.0±0.0 [c]
Phlomis purpurea	C1.1: NI *P. purpurea* 1	1,8±1.0 [c]	0.5±0.4 [a,b]
	C1.2: NI *P. purpurea* 2	1.0±0.5 [b,c]	0.5±0.6 [a,b]
	C2.1: NI *P. purpurea* 1 + *C. sativa*	0.8±0.7 [b]	0.5±0.3 [a]
	C2.2: NI *P. purpurea* 2 + *C. sativa*	0.7 ±0.4 [b]	0.4±0.3 [a]
	C3.1: Infested *P. purpurea* 1	1.0±0.9 [b,c]	0.9±1.0 [b]
	C3.2: Infested *P. purpurea* 2	1.1±0.6 [b,c]	0.4±0.2 [a]
	C4.1: Infested *P. purpurea* 1 + *C. sativa*	0.4±0.2 [a]	0.3±0.3 [a]
	C4.2: Infested *P. purpurea* 2 + *C. sativa*	0.3±0.2 [a]	0.3±0.3 [a]

Statistical comparisons were made between the different conditions within the same plant species. For each column, values followed by the same letter means that they do not differ significantly ($P<0.05$), using Fisher's Least Significant Difference Test (LSD).

[§] Both *Phlomis purpurea* seedlings were evaluated separately (*P. purpurea* 1 and *P. purpurea* 2). *C. sativa* – 8-week-old pre-germinated seedlings; *P. purpurea* – 19-month-old.

[†] The scale used to evaluate the severity of symptoms was: 0 = without symptoms, 1 = 1-33 %, 2 = 34-66 %, 3 = 67-99 %, 4 = 100 % of affected tissue (dead plant). In between values were used according to the severity of symptoms: 0.3 = weak, 0.5 = moderate, 0.7 = strong. *P. cinnamomi* was a mixture of isolates PA 37 and PA 45.

2.3.3.2. *Phytophthora cinnamomi* re-isolation from roots

Although there was a significant reduction of symptom severity (Table 2.10) and no propagules were found in *P. purpurea* infested soil where *P. purpurea* was grown alone, at the end of the experiment (Table 2.11), *P. purpurea* was not able to significantly reduce the root infection of *C. sativa* seedlings when planted 10 weeks in advance. Probably, more than 10 weeks are needed to eliminate all the propagules. In experiment 2 no propagules were recovered from soil after 16 weeks of *P. purpurea* growing.

There was no recovery of *P. cinnamomi* from non-infested *C. sativa* or from infested and non-infested *P. purpurea* seedlings planted alone and with *C. sativa* (apart from 1 propagule that could be explained by contaminated soil on the surface of the root), confirming that *P. cinnamomi* does not infect *P. purpurea* (Table 2.11).

There were no significant differences in the recovery of *P. cinnamomi* from *C. sativa* roots between all conditions of infested soil (C2, C3 and C4).

2.3.3.3. Inoculum potential of soil

To determine the amount of *P. cinnamomi* present in the soil, only method 1 was used since there were no young host leaves available for baiting when the experiment was run. Soil was analysed 21 weeks after infestation. The presence of *P. purpurea* (C4) significantly reduced the amount of *P. cinnamomi* present in the soil when compared with the soil where plantation and infestation was simultaneously (C5) and did not differ significantly from the control (C1) nor from the pre-infested soil (C3) (Table 2.11).

Table 2.11. Evaluation of the protective effect of *Phlomis purpurea* when planted 10 weeks before the plantation of *Castanea sativa* against *Phytophthora cinnamomi* infection. *P. cinnamomi* recovery from plant roots and soil.

Species	Condition	*Phytophthora cinnamomi*	
		Root recovery (%)[†]	Soil recovery [†] (CFU g^{-1} soil)
Castanea sativa	C1: Control	**0.0±0.0** [a]	**0.0±0.0** [a]
	C2: Control + *P. purpurea* pre-planted	**0.0±0.0** [a]	**0.0±0.0** [a]
	C3: In pre-infested soil	**100±0.0** [b]	**1.8±0.8** [b]
	C4: In infested soil + *P. purpurea*	**99.6±1.3** [b]	**0.3±0.5** [a,b]
	C5: Planted and infested simultaneously	**100±0.0** [b]	**9.8±3.2** [c]
Phlomis purpurea	C1.1: Control *P. purpurea* 1	**0.0±0.0** [a]	**0.0±0.0** [a]
	C1.2: Control *P. purpurea* 2	**0.0±0.0** [a]	**0.0±0.0** [a]
	C2.1: Control *P. purpurea* 1 + *C. sativa*	**0.0±0.0** [a]	**n.a**
	C2.2: Control *P. purpurea* 2 + *C. sativa*	**0.0±0.0** [a]	**n.a**
	C3.1: Infested *P. purpurea* 1	**0.0±0.0** [a]	**0.0±0.0** [a]
	C3.2: Infested *P. purpurea* 2	**0.0±0.0** [a]	**0.0±0.0** [a]
	C4.1: Infested *P. purpurea* 1 + *C. sativa*	**0.2±0.7** [a]	**n.a**
	C4.2: Infested *P. purpurea* 2 + *C. sativa*	**0.0±0.0** [a]	**n.a**

Statistical comparisons were made between the different conditions within the same plant species. For each column, values followed by the same letter means that they do not differ significantly ($P<0.05$), using Fisher's Least Significant Difference Test (LSD).
[†] Average ± standard deviation. "n.a" – "Non-aplicable", because it was the same soil from *C. sativa* pots.
[§] Both *Phlomis purpurea* were evaluated (*P. purpurea* 1 and *P. purpurea* 2). *C. sativa* – 8-weeks-old pre-germinated seedlings; *P. purpurea* – 19-months-old.

Phytophthora cinnamomi was not recovered, using method 1 (soil extension in selective medium) either from the two control pots as expected or from the two infested soil pots without seedlings. This could indicate the inability of *P. cinnamomi* to proliferate in the absence of plant material and consequently the inability of propagule survival for long periods of time. Anderson (2006) showed that infective propagules of *P. cinnamomi* could not be detected by baiting in infested soil after 12 weeks incubation at 25 °C, though they have been detected by PCR. When the soil was heated at 30 °C for 12 weeks the pathogen could not be

detected by either baiting or PCR. Our experiment lasted 15 weeks and the average maximum temperature was 20.1 °C might have contributed to the non-recovery of viable *P. cinnamomi*. However, after 10 weeks infestation the propagules were still viable and able to infect *C. sativa* pre-germinated seedlings (Table 2.11).

These results raise the question whether *P. purpurea* exudes substances with anti-*P. cinnamomi* activity or simply, in the absence of a host, the propagules will be unviable. However, it was demonstrated that *P. purpurea* was able to significantly protect *Q. suber* and *Q. ilex* from *P. cinnamomi* infection and to reduce the inoculum potential in glasshouse trials, indicating the ability to reduce root infection by the pathogen (Neves *et al.*, 2014). Moreover, in Experiment 1 (Table 2.4), *P. purpurea* significantly reduced the amount of recovery of *P. cinnamomi* from infested soil where *C. sativa* and *Q. canariensis* were grown and from their infested roots as well as from *Q. coccifera* roots.

2.3.3.4. Determination of the weight of aerial part, and of secondary and principal roots of seedlings

At the end of the experiment the weight of *C. sativa* seedlings (8 weeks-old) and *P. purpurea* aerial and root parts in each condition was recorded (Table 2.12). *C. sativa* seedlings planted and infested simultaneously (C5) had the smallest aerial part, lowest secondary and principal root's weights and were significantly different from the controls (C1 and C2). The weight of all plant parts from *C. sativa* seedlings infested and planted together with *P. purpurea* (C4) was higher but not significantly different from infested seedlings in the absence of *P. purpurea* (C3). The root length of the *C. sativa* control seedlings (C1) was significantly higher than that of the infested seedlings (C3), and showed no significant difference from the control together with *P. purpurea* (C2) and infested together with *P. purpurea* (C4) (Table 2.12).

There was a significant moderate negative correlation between the average severity of aerial symptoms and the aerial weight (r=-0.486; $P<0.0001$)[15], as in Experiment 2. A significant moderate negative correlation was found between the average severity of root symptoms and the weight of the principal root (r=-0.387; $P=0.001$)[16] and the weight of secondary roots (r=-0.461; $P<0.0001$)[17].

[15] Appendix I.III.XXVII.
[16] Appendix I.III.XXVIII.
[17] Appendix I.III.XXVIII.

Table 2.12. Protective effect of *Phlomis purpurea* against *Phytophthora cinnamomi* infection when planted 10 weeks before *Castanea sativa,* assessed by the weight of seedlings aerial part and by the weight and length of roots.

Species	Condition[§]	Weight[†] (g)			Root length (cm)
		Aerial	Principal Root	Secondary roots	
Castanea sativa	C1: Control	2.6±1.9 [c]	1.7±0.4 [b]	1.2±1.5 [c]	25.3±4.6 [b]
	C2: Control+*P.p.* pre-planted	2.3±1.3 [b,c]	1.6±1.0 [b]	0.8±0.9 [b,c]	24.8±8.6 [b]
	C3: In infested soil	1.3±1.2 [a,b]	1.2±0.7 [a,b]	0.3±0.3 [a,b]	19.3±5.0 [a]
	C4: In infested soil+ *P. p.*	1.5±0.9 [b,c]	1.3±0.4 [a,b]	0.3±0.2 [a,b]	20.7±3.4 [a,b]
	C5: Planted and infested sim.	0.5±0.4 [a]	1.0±0.4 [a]	0.1±0.2 [a]	22.0±5.4 [a,b]
Phlomis purpurea	C1.1: Control *P. purpurea* 1	1.9±0.9 [a]	1.2±0.3 [a,b]	4.1±1.9 [a,b]	26.3±4.4 [a]
	C1.2: Control *P. purpurea* 2	2.5±1.7 [a,b]	1.3±0.7 [a,b]	4.6±3.0 [b]	29.7±6.2 [a,b]
	C2.1: Control *P.p.* 1 + *C. sativa*	6.9±2.2 [c]	1.0±0.4 [a,b]	4.4±2.2 [b]	30.6±5.4 [a,b]
	C2.2: Control *P.p.* 2 + *C. sativa*	6.0±3.6 [c]	1.3±0.9 [a,b]	5.1±2.4 [b]	29.2±5.4 [a,b]
	C3.1: Infested *P. purpurea* 1	4.7±2.7 [b,c]	1.6±1.0 [b]	4.7±3.5 [b]	34.9±10.5 [b]
	C3.2: Infested *P. purpurea* 2	3.9±1.8 [b]	0.9±0.5 [a]	1.8±1.2 [a]	33.3±9.9 [b]
	C4.1: Infested *P.p.*1 + *C. sativa*	7.4±2.9 [c]	1.2±0.6 [a,b]	5.2±3.1 [b]	32.1±5.1 [a,b]
	C4.2: Infested *P.p.*2 + *C. sativa*	7.3±3.2 [c]	1.4±0.6 [a,b]	4.5±1.9 [b]	32.0±6.2 [a,b]

[§] Both *Phlomis purpurea* were evaluated (*P. purpurea* 1 and *P. purpurea* 2). *C. sativa* – 8-weeks-old pre-germinated seedlings; *P. purpurea* – 19-months-old. C5: Planted and infested simultaneously.

[†] Average±Standard deviation. Statistical comparisons were made between the different conditions within the same plant species. For each column, values followed by the same letter means that they do not differ significantly ($P<0.05$), using Fisher's Least Significant Difference Test (LSD).

2.3.4. Conclusion

Several factors affect the outcome of an experiment (Table 2.13). The results from different methods for the detection of the pathogen (method 1 - soil extension onto NARPH selective medium and method 2 -the serial endpoint dilution using baits), in some cases, did not match. False negatives in soil extension onto NARPH selective medium occurred, being method 2 more reliable, although no method is fully reliable (O'Brien *et al.*, 2009).

Table 2.13. The effect of *Phlomis purpurea* planted together with *Castanea sativa*, *Quercus canariensis* and *Quercus coccifera* infested plants/seedlings with *Phytophthora cinnamomi*.

Experiment	*Phlomis purpurea* together with			Evaluation Decrease of…
	Castanea sativa†	*Quercus canariensis*†	*Quercus coccifera*†	
1	X	✔	✔	Root symptoms
	✔	✔	✔	*Phytophthora cinnamomi* isolation from roots
	✔	✔	✔	Soil inoculum potential- Method 1
	✔	✔	✔	Soil inoculum potential- Method 2
2	✔		X	Root symptoms
	X✔		X	*Phytophthora cinnamomi* root isolation
	X		X	Soil inoculum potential- Method 2
3	✔			Root symptoms
	X			*Phytophthora cinnamomi* root isolation
	X✔			Soil inoculum potential- Method 1

†✔ - Significant protection; X – Not significant protection; X✔ - Decrease, but not significant.

As stated by Grant & Byrt (1983) temperature affects the growth of *P. cinnamomi*: "even in susceptible species the degree to which root tissue is colonised is temperature dependent". The temperature under the net shelter where the plants/seedlings were placed was within the limits of development for this oomycete in all experiments. Caetano (2007) evaluated the optimal temperature for the growth of dozens of strains of *P. cinnamomi* isolated from *Q. suber* and Q. *ilex* stands and concluded that for most of them it lies between 26 and 30 °C. Shew and Benson (1983) demonstrated that optimal temperatures for *P. cinnamomi* infection of *Abies fraseri* (fraser fir) were between 16 and 25 °C and for host mortality, between 19 and 25 °C. The temperatures were more favourable to *P. cinnamomi* in Experiment 1, hence the susceptible *C. sativa* started to die earlier when compared to Experiment 2. Factors other than temperature affect the experiments in the glasshouse such as the age of the plants/seedlings and the amount of inoculum (Raftoyannis & Dick, 2002). The age of the plants were shown to affect the outcome of the interaction plant-pathogen. Three-week-old *C. sativa* seedlings (Experiment 3) were even more susceptible than older seedlings/plants (Experiments 1 and 2). Seedlings/plants were submitted to waterlogging two times per week. This condition favours the pathogen growth, root infection and symptom expression and therefore has a negative effect on plant health (Zentmyer, 1980; Sánchez *et al*. 1998, 2005), due to a reduction in oxygen supply and in the nutrient uptake. The low level of

root symptoms showed by the control compared with the infested plants, in all experiments, suggests that flooding was not responsible for the symptoms showed by infested plants as previously demonstrated by Sánchez *et al*. (2005). The soil in Experiment 1 was potting soil and river sand (1:1) and was more compact and consequently more difficult to drain than soils in experiments 2 and 3 where potting soil and vermiculite (1:1) were used. This might have also contributed to the exacerbation of symptoms in the *C. sativa* plants.

Disease results from a complex and dynamic interaction between three factors: the host (plant), the pathogen (*P. cinnamomi*) and the environment (biotic and abiotic factors), i.e. a conducive soil. Soil can vary from very conducive to highly suppressive to *P. cinnamomi*. A *P. cinnamomi* suppressive soil can be defined as "a soil in which disease incidence and severity remain low, despite the presence or introduction of *P. cinnamomi*, a susceptible host plant and favourable environmental conditions" The ability of soil to suppress *P. cinnamomi* can be altered by several factors such as soil composition and pH, and the presence of suppressive microbiota (Erwin & Ribeiro, 1996; Cahill *et al.*, 2008). It would be interesting to know how *P. purpurea* manages to modify a conducive to a highly suppressive soil, i.e., reduces the soil inoculum potential.

Other similar glasshouse assays using native resistant flora to determine their protective activity towards susceptible species, have been reported before. D'Souza *et al*. (2004) demonstrated that four out of five Western Australia native *Acacia* species protected the highly susceptible *Banksia grandis* against *P. cinnamomi*. Jayasekera (2006) determined the inoculum potential of infested soil under 3 varieties of *Acacia pulchella* and *A. urophylla*, field resistant and susceptible species, respectively. The mean number of colonies per gram of soil was significantly reduced in soil under *A. pulchella*. Moreover, *A. pulchella* seedling exudates were shown to lyse *P. cinnamomi* chlamidospores and degenerate them (Jayasekera, 2006).

The natural ecosystem is more complex than glass-house-conditions. However, the experimental conditions−temperature, flooding and amount of inoculum−were artificially imposed in order to create much more favourable conditions to hasten *P. cinnamomi* infection. Theoretically, one could expect to achieve control more easily in the field conditions.

The use of pioneer shrubs as nurse plants is a technique that improves the establishment of woody seedlings in harsh environmental conditions, such as the Mediterranean-type ecosystems, thus facilitating reforestation success (Castro *et al.*, 2002, 2004; Gómez-Aparicio *et al.*, 2004). Planting *P. purpurea* together, or even before sowing *Q. ilex* and *Q. suber*, could potentially create a protective environment, not only by reducing the solar radiation through the canopy of shrubs, but as a mean of reducing *P. cinnamomi*

propagules and consequently infection, enabling the susceptible plants to become more easily established and survive in infested areas.

Recapitulating, the results of our experiments support the view that *P. purpurea* significantly protects the plants tested from *P. cinnamomi* infection when planted together with the susceptible hosts, by reducing the soil inoculum potential. These can be achieved by the exudation of metabolites that alter the soil pH or microbiota creating unfavourable conditions to *P. cinnamomi* development.

The appendices can be found at https://meocloud.pt/link/047230bb-31f7-43e0-8a04-5d7c3a20a6ae/Appendices_Chapter%202.docx/ (see CD in attachment).

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Chapter 3

Determination of the protective effect of *Phlomis purpurea* towards *Quercus suber* in two naturally infested areas

The introduction has been omitted, as a general introduction was presented in chapter 1.

3.1. Abstract

A field trial was established to determine the protective effect of *Phlomis purpurea* (purple phlomis) on *Quercus suber* (cork oak) in soils naturally infested with *Phytophthora cinnamomi*. Acorns from a tree (Alt 05) belonging to a susceptible family were chosen based on the presupposition that if *P. purpurea* is able to significantly protect a susceptible cork oak then it would be able to protect trees from more tolerant families.

The emergence of *Q. suber* seedlings in two plots (A1 and B1) without *P. purpurea* was significantly lower (A1=21.5 % and B1=8 %) than in two plots (A2 and B2) in which acorns were co-planted with *P. purpurea* (A2=37.5 %; and B2=49 %) showing that this plant contributed to the emergence success.

At the end of the trial, the survival of *Q. suber* was 27.9 % in plot A1 and 18.8 % in plot B1. In plots A2 and B2 the survival was 26.7 % and 46.9 % respectively.

The survival of *P. purpurea,* at the end of the trial, was 84 % in plot A2 and 46 % in plot B2. *P. purpurea* seedlings with well-developed root system, planted alone, were able to completely eliminate *P. cinnamomi*. However purple phlomis did not significantly reduced the soil inoculum potential when planted together with *Q. suber* seedlings.

3.2. Materials and methods

3.2.1. Selection and sampling of areas for the presence of *Phytophthora cinnamomi*

Areas of land were selected at Serra do Caldeirão (*Barranco-do-Velho*) in the Algarve region, Portugal on the basis of a widespread decline in cork oaks. Plots (100 m^2) with minimum slopes, without depressions and with a small amount of vegetation were chosen. After removal of the organic litter layer, soil samples were taken at a depth of *ca* 20 cm, from four locations in each plot, thoroughly mixed, and the presence of *P. cinnamomi* determined (Chapter 2, section 2.2.3.3). In the area at *Menta* (37º13'58''N 7º54'12''W) (hereinafter called area A) *P.* cinnamomi was not isolated whereas in area "PTS" near *Cortelha* (37º14'51''N 7º56'53´´W) (hereinafter called area B) this oomycete was present. During sowing and planting (section 3.2.4) more samples were taken from area A and analysed for the presence of *P. cinnamomi*.

3.2.2. Plant material

Quercus suber acorns were collected during November and December 2011, from a tree (ALT 05) in *Almonte*, *Huelva*, Spain. That tree was identified as one of the most susceptible, among 64 families using morphometric evaluations, in glasshouse and field trials (Cravador *et al.*, 2009). Seeds were stored at low temperature (4 ºC) until they were sown.

Phlomis purpurea were pre-germinated from collected seeds (*Moncarapacho*, Portugal) and then planted in 1 L containers when they were 4-6 months old. One year old plants were used.

3.2.3. Experimental design

Two areas, each with two plots, were sown manually with *Quercus suber* acorns, in ten rows and ten columns one metre apart from each other, making 100 m^2. In one area, soil was naturally infested with *Phytophthora cinnamomi* whereas in the other area *P. cinnamomi* was not recovered from soil and was considered a control area. In each area, *Q. suber* were sown in one plot (control) and in the other plot, *Q. suber* were sown between two *Phlomis purpurea* (Figure 3.1). The experiment lasted for 25 months and was repeated once.

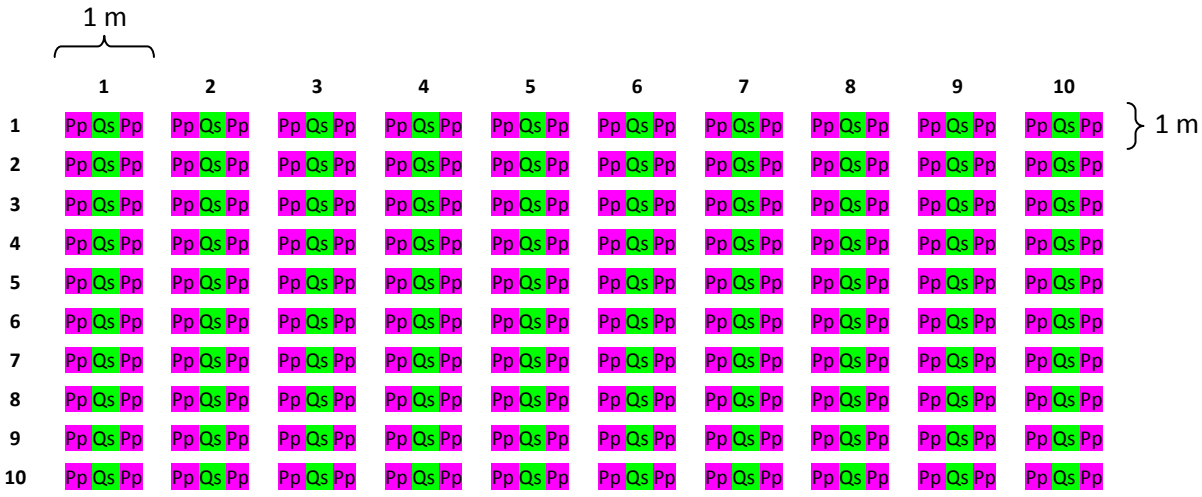

Figure 3.1. Plot with purple phlomis (Pp = *Phlomis purpurea* seedlings; Qs = *Quercus suber* acorns).

3.2.4. Sowing *Quercus suber* and planting *Phlomis purpurea*

In January 2012, a field trial was established in 2 areas of land (A and B) each with 2 plots. Plots without *P. purpurea* seedlings were designated 1 and with *P. purpurea*,

2. Hereinafter, area A, without *P. purpurea* is called "A1" and with *P. purpurea* "A2" whereas area B, without *P. purpurea* is called "B1" and with *P. purpurea* "B2".

The plots were roughly ploughed; the soil raked to remove broken bushes and weeds, and holes were made manually with a hole-digger apparatus. In each plot, holes were dug approximately 30-40 cm deep and 20 cm in diameter, and 1 m apart from each other, in 10 rows with 10 holes each, making a total of 100 holes. Sampling from the area initially found *P. cinnamomi*-free was made at this depth (30-40 cm). One liter of a mixture of peat and vermiculite (1:1) was placed inside every hole. Two *P. purpurea* seedlings were carefully taken out of their containers and placed inside each hole and two *Q. suber* acorns were sown manually, between the two seedlings (Figure 3.1). The control plots only received two *Q. suber* acorns per hole. The *P. purpurea* roots and acorns were covered with soil and watered with tap water. The first three months were very dry (February was considered the driest since 1931 - IPMA) and seedlings had to be watered with *ca* 2 L per hole, every two weeks. As the following spring and summer continued to be very dry, the seedlings were watered monthly.

The soil is classified as sandy-clay-loamy[18]. The climate is of Mediterranean type, with hot and dry summers. Mean minimum and maximum temperatures in 2012 were 14.5 °C (monthly minimum of 5.9 °C) and 23.1 °C (monthly maximum of 30.1 °C), respectively. In 2013, the mean minimum and maximum temperatures were 11.4 °C (monthly minimum of 4.0 °C) and 23.0 °C (monthly maximum of 32.1 °C), respectively. The annual rainfall was low in 2012 but the exact amount is unknown due to a failure of meteorology station in September, November and December. In 2013 the annual rainfall was 489.8 mm (IPMA).

3.2.5. Protective activity of *Phlomis purpurea* towards *Quercus suber* sown in naturally infested areas

3.2.5.1. Determination of *Quercus suber* emergence

The emergence of *Q. suber* seedlings was assessed by visual inspection, by the presence of the aerial part (shoot), in the four plots, 4 months after sowing and then monthly or bi-monthly during 2012. In the following year, emergence was assessed in January, April,

[18] Appendix II.I.

May, June, July, August and November. Two acorns were sown per hole, so if only one germinated and emerged the emergence was 50 % in that hole.

3.2.5.2. Determination of *Quercus suber* and *Phlomis purpurea* survival

The determination of survival was based on emerged seeds for *Q. suber.* If two seeds emerged and only one survived, the survival was accounted as 0.5. The determination of survival was based on seedlings planted for *P. purpurea*.

3.2.6. *Quercus suber* and *Phlomis purpurea* evaluation

After 25 months, all *Q. suber* from the control plots, 12 *Q. suber* and *P. purpurea* planted together and 12 *P. purpurea* seedlings growing where no *Q. suber* was present were harvested (the aim was to have a control with *P. purpurea* only). Forty to fifty centimetre holes were dug to remove the seedlings with the root system intact. All tools were disinfected with 10 % sodium hypochlorite and rinsed with distilled water in between seedling removal. Seedlings were evaluated according to their foliar and root symptoms as described in Chapter 2 (section 2.2.6).

3.2.6.1. *Phytophthora cinnamomi* re-isolation from roots

The isolation of *P. cinnamomi* from roots was performed according to the procedure described in Chapter 2 (section 2.2.7).

3.2.6.2. Inoculum potential of soil

The inoculum potential of soil was determined according to the procedure described in Chapter 2 (section 2.2.8), using the soil (10 g) extension on NARPH medium (method 1) and baits in 1000 ml of soil (method 2). All negatives were repeated.

Soil from PTS area was collected by *Associação de Produtores Florestais da Serra do Caldeirão* and chemical analysis was performed by *Direção Regional de Agricultura e Pescas do Algarve*[19].

[19] Appendix II.I.

3.2.7 Statistical analysis

Data were analysed by analysis of variance (One-Way ANOVA), followed by Fisher's protected least significant difference (LSD) test or independent T-test at $P \leq 0.05$ (Steel & Torrie, 1985) and contingency analysis (Pearson's correlations). The data was normally distributed and homogeneity of variance was confirmed using Levene's test (Levene, 1960). The software package "PASW statistics 22" (IBM software, 2009 SPSS Inc. Hong Kong) was used. Statistical comparisons were made between the different conditions (*Q. suber* alone, *Q. suber* planted with *P. purpurea* and *P. purpurea* alone) with the dependent variables: *Q. suber* emergence, *Q. suber* and *P. purpurea* survival, *Q. suber* and *P. purpurea* average of symptom severity of aerial and root parts, *P. cinnamomi* re-isolation from roots of both seedling species and soil inoculum potential.

3.3. Results and discussion

3.3.1. Selection and sampling of areas for the presence of *Phytophthora cinnamomi*

Two areas of land (A and B) each with 2 plots were selected to determine the protective effect of *Phlomis purpurea* against *Phytophthora cinnamomi* infection. In area A (Figure 3.2), *P. cinnamomi* was not detected and was chosen to be a control area, whereas area B (Figure 3.3) was found to be naturally infested with *P. cinnamomi*. Plots without or with *P. purpurea* seedlings were designated 1 and 2, respectively. Hereinafter, area A, without *P. purpurea* is called "A1" and with *P. purpurea* "A2" whereas area B, without *P. purpurea* is called "B1" and with *P. purpurea* "B2".

Control plots received two *Q. suber* acorns per hole, whereas plots with *P. purpurea* two acorns were sown together with 2 *P. purpurea* seedlings, per hole. As there was no control with *P. purpurea* only, holes in which no emergence of *Q. suber* occurred the *P. purpurea* were considered as a control.

Figure 3.2. Panoramic view of area A, at *Menta* (37º13'58''N 7º54'12''W).

Figure 3.3. Panoramic view of area B, at "PTS" near *Cortelha* (37º14'51''N 7º56'53´´W).

The soil collected during sowing and planting in area A (found negative for the presence of *P. cinnamomi,* intended to be a control) was again analysed and *P. cinnamomi* was now found to be present, although this area was devastated by a huge fire in 2004. This raises the question of how many samples are needed to show that *P. cinnamomi* is absent from an area. This question was answered by Davison & Tay (2005) that studied the presence of this oomycete from sites in the South-West of Western Australia. With a probability of 0.05 it varied from 40 samples taken from 5 m of a vegetation boundary to 271 samples from the centre of a suspected dieback site. This area had no trees, just a few shrubs and weeds that were probably hosts for *P. cinnamomi*. No appropriate area could be found free from *P. cinnamomi,* so we could not have a negative control area but instead, worked in two naturally infested areas. McLaughlin *et al*. (2007) evaluated the effect of fire on the survival of *P. cinnamomi* during two low-intensity prescribed fires. *P. cinnamomi* was recovered from all 70 soil aliquots buried at 10 cm depth, showing that prescribed fires had minimal impact on the survival of *P. cinnamomi* in soil. On sites with a mean soil pH> 3.5 and sandy-loamy to clayey soil texture *Phytophthora* spp. were commonly isolated from rhizosphere soil (Jung *et*

al., 2000). Moreira & Martins (2005) studied the impact of site factors on the cork oak decline by *P. cinnamomi* in Portugal and found that the incidence of *P. cinnamomi* and the impact of the decline were higher in shallow soils, evolved from schist bedrocks, with a high content of clay and silt. Algarve presented the highest incidence of the oomycete (73 %) (*ibid*.) Moreover, soils with low mineral content, such as phosphorous seemed to favour infection (*ibid*.). That is the case with these soils[20]

3.3.2. Protective activity of *Phlomis purpurea* towards *Quercus suber* sown in naturally infested areas

To determine the protective activity of *Phlomis purpurea* towards *Quercus suber* sown in naturally infested areas, two areas, each with two plots, were sown manually with *Quercus suber* acorns, in ten rows and ten columns one metre apart from each other (100 holes), making 100 m^2. Plots A1 and B1 were the controls and only received two *Q. suber* acorns per hole (100 holes, 200 acorns). In plots A2 and B2, two *P. purpurea* seedlings were planted together (one at each side) with two acorns (100 holes, 200 acorns, 200 *P. purpurea*).

3.3.2.1. Determination of *Quercus suber* emergence

Acorns were sown, during winter, in naturally infested areas. However, rainfall was too scarce and seedlings had to be watered. Seed germination depends on both internal and external conditions. The most important external factors include temperature, water, oxygen and sometimes light or darkness.

The emergence of *Q. suber* started in May 2012, 3-4 months after sowing, and was evaluated throughout the following months[21] In a sub-humid Mediterranean forest, *Q. suber* emergence usually starts 70-80 days after sowing (see for instance, Urbieta *et al*. 2008) which is in agreement with our results. When one seed germinated and the aerial part emerged, it was recorded as one emergence. As two seeds were sown per hole, it was checked whether the aerial part belonged to one or two seeds. When one seedling died and a new emergence occurred later it was considered an emergence from the other seed. However, apparently dead seedlings (by shoot dieback) can recover and re-sprout after summer (Urbieta *et al.*, 2008).

[20] Appendix II.I.
[21] Appendices II.II, II.III, II.IV, II.V.

The average of *Q. suber* emergence (mean ± standard deviation) in "A1" was significantly lower (0.4 ± 0.6) than in "A2" (0.8 ± 0.8) ($t_{(177)}$=-3.196, *P*=0.002)[22]. Also, *Q. suber* emergence in "B1" was significantly lower (0.2 ± 0.4) than in "B2" (1.0 ± 0.8) ($t_{(152)}$=-9.274, *P*≤0.0001)[23] A greater number of seedlings emerged in October-November 2012 in area A-22 in plot A1 and 41 in plot A2. In area B, the majority of seedlings emerged earlier, in June 2012-7 in plot B1 and 52 in plot B2. However, seedlings emerged all year round and after 1 year, in January 2013, 15 seedlings emerged in plot B2. The dry conditions did not favour seed germination and in October 2012 the 58.2 mm rainfall (IPMA) might have contributed to the new emergences.

The use of nurse shrubs (shade) and irrigation are frequently used to increase the success of Mediterranean *Quercus* spp. plantations. However, there is some controversy about the combined effects of these treatments on plant performance. Castro-Díez *et al*. (2006) showed that moderate shade and daily summer watering enhance the performance of Mediterranean *Quercus* seedlings. Urbieta *et al*. (2008) concluded that the probability of *Q. suber* germination exponentially declined as soil volumetric water content of the wet season increased and light availability also increased.

We realised that acorns can be a feast for predators (rodents, hares and wild boar) and although seeds were buried we cannot neglect that fact. However, we could only find traces of predators' presence sporadically. No signs of dug acorns were seen. Herrera (1995) found that single buried *Q. suber* acorns experienced the lowest predation under open or dense shrub (52 % and 0 %, respectively) when compared with seeds placed on the ground surface. It was impractical to examine each hole for the presence of the two acorns, because it would have serious implications on the survival of germinated acorns. However two or three holes were examined in "B1" and non-germinated and germinated acorns with completely necrotised radicles were found.

An assay with acorns with different origins was set up at *Serra do Caldeirão*, in 2003, and the germination (supposedly emergence) determined 6 months after sowing (Moreira *et al.*, 2007). The emergence varied according to origins and plots but on average it was 61.3 %. In another study (Carvalho, 2008) the emergence varied between 0 and 28 %. The acorn's emergence registered by Herrera (1995) under open shrub was 38 %. These values are higher when compared with those observed in our study (A1=21.5 % and B1=8 %). However, it is worth remembering that the seeds we used were collected from a tree (Alt 05) that has

[22] Appendix II.V.
[23] Appendix II.VI.

been characterised to be one of the most susceptible to *P. cinnamomi* infection (Cravador *et al.*, 2009). In fact, a field trial using acorns from different families showed that, from Alt 05, 14 out of 30 acorns (46.7 %) germinated (*ibid*.). This discrepancy may be due to the drought, since, as explained previously the rainfall was almost absent and the watering was not enough to cover the seedlings needs.

The emergence of *Q. suber* seedlings in the two plots (A1 and B1) without *P. purpurea* was significantly lower (A1=21.5 % and B1=8 %) than in two plots (A2 and B2) in which acorns were co-planted with *P. purpurea* (A2=37.5 %; and B2=49.0 %) showing that *P. purpurea* contributed to the emergence success.

3.3.2.2. Determination of *Quercus suber* and *Phlomis purpurea* survival

The most vulnerable stages for most plant species are seed germination and seedling establishment. Resource limitation, competition, herbivory and pathogens, namely *Phytophthora cinnamomi*, may jeopardize their survival.

At the end of the trial, the survival of *Q. suber* in plots without *P. purpurea* was 27.9 % in plot A1 and 18.8 % in plot B1. In plots with *P. purpurea*, A2 and B2 the survival was 26.7 % and 46.9 % respectively.

The average of *Q. suber* survival after 2 years (mean ± standard deviation) in "A1" was not significantly different (0.3 ± 0.4) than in "A2" (0.3 ± 0.4) ($t_{(88)}$=-0.242, P=0.810)[24]. However, the survival in "B1" was significantly different (0.2 ± 0.4) than in "B2" (0.6 ± 0.7) ($t_{(30)}$=-2.574, P=0.015)[25].

The drought may be responsible for the reduced survival, because the emergence was significantly higher in the plots with *P. purpurea* (A2 and B2). It is worth to mention that an association of local producers (Associação dos produtores florestais da Serra do Caldeirão) sowed acorns at the same time and the emergence was close to zero (J. Albuquerque, personal communication). *Q. suber* and *P. purpurea* seedlings growing together had to compete for water, and the outcome may be unfavourable to both species. The presence of *P. cinnamomi* at both areas may have decrease the survival. The rain or presence of water, even scarce, and the mild temperatures are favourable for *P. cinnamomi* infection. The fine roots get infected and the absorbance of water and nutrients are reduced. Consequently plants get debilitated and even more vulnerable to the pathogen. When drought occurs, the mortality increases.

[24] Appendix II.VII
[25] Appendix II.VIII

Corcobado *et al*. (2014) demonstrated, in greenhouse assays, that drought events determined performance of two-year-old *Quercus ilex* seedlings and rendered them more vulnerable to *P. cinnamomi* infection. Other factors may have influenced the survival, both biotic and abiotic, as we cannot control them, in an open field.

In a 3 year trial at *Serra do Caldeirão*, Moreira *et al*. (2007) found 12.3 % *Q. suber* mortality in the first year (87.7 % survival) doubling over the following two years (*ca* 75.4 % survival). However, Herrera (1995) found that 97.8 % of the acorns sown were dead after one year (2.2 % survival) and none survived two years after sowing. These authors had highly different results (87.7 % versus 2.2 % survival). Herbivory is the principal cause attributed, by Herrera (1995) to the low survival. To protect the seedlings from direct exposure to the sun and from predators, protective tubes were placed around each *Q. suber* seedling in May 2013, 1 year after germination of the first *Q. suber* seedlings. Herrera (1995) reported that despite this protection shoots were eaten and out of the 49 buried acorns which produced a shoot, only two seedlings were alive one year after germination. This confirms the low natural *Q. suber* regeneration seen in nature.

The survival of *P. purpurea* at the end of the trial was 84.0 % (16.0 % mortality) in plot A2 and 46.0 % (54.0 % mortality) in plot B2. We found that, at plot B2, some *P. purpurea* were cut at the stem base, possibly, by hares, as *P. purpurea* were not protected (Figure 3.4).

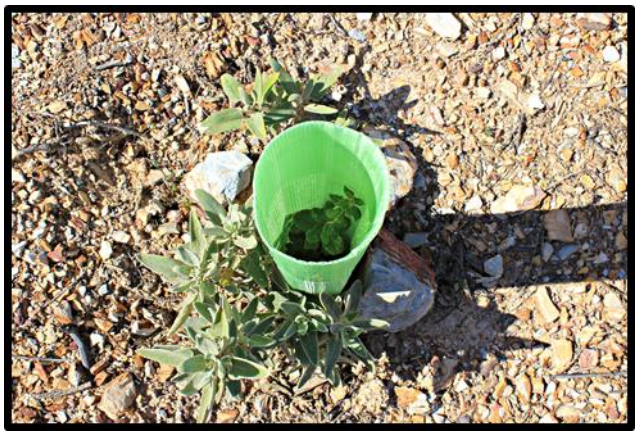

Figure 3.4. Protection of *Quercus suber* seedlings from direct exposure to the sun, and herbivory, with aerated tubes. *Phlomis purpurea* was planted outside the tubes.

3.3.3. *Quercus suber* and *Phlomis purpurea* evaluation

At the end of the trial, all *Q. suber* (12 at plot A1 and 3 at plot B1) and associated soil from the control plots, twelve holes with *Q. suber* sown with *P. purpurea* and twelve holes

containing only *P. purpurea* seedlings (see section 3.3.1.) were removed from plots A2 and B2 to evaluate the root system, aerial part and to detect the presence of the pathogen.

The analysis of variance showed that in area A, although the aerial parts of *Q. suber* sown with *P. purpurea* (plot 2) were significantly healthier than *Q. suber* alone (plot 1) there was no significant difference between the *Q. suber* roots of the 2 plots (Table 3.1)[26]. This might have been due to the low amount of *P. cinnamomi* inoculum present in the soil, as this area was initially considered negative for the oomycete.

In area B, the aerial and root parts of *Q. suber* sown with *P. purpurea* (plot 2) were significantly healthier than *Q. suber* sown alone[27] (plot 1). This confirms that *P. purpurea* significantly protected *Q. suber* in the field (Table 3.1).

There was no significant difference between *P. purpurea* seedlings growing alone or planted with *Q. suber* in both areas (Table 3.1) as shown also in Chapter 2.

Table 3.1. Evaluation of aerial and root symptom severity in *Quercus suber* seedlings grown alone, planted with *Phlomis purpurea* and in *P. purpurea* grown alone.

Species	Condition	Area A "Menta"		Area B "PTS"	
		Average of symptom severity ††			
		Aerial	Root	Aerial	Root
Quercus suber	Alone	2.3±1.0 ^{d}	2.4±1.3 ^{b}	1.6±0.9 ^{b}	3.0±0.5 ^{c}
	Planted with *P. purpurea*	1.4±0.7 ^{c}	2.1±1.0 ^{b}	0.7±0.6 ^{a}	1.9±1.2 ^{b}
Phlomis purpurea	Alone (PP1)	0.1±0.1 ^{a}	0.1±0.2 ^{a}	0.4±0.2 ^{a}	0.4±0.3 ^{a}
	Alone (PP2)	0.3±0.3 ^{a,b}	0.3±0.3 ^{a}	0.5±0.2 ^{a}	0.5±0.2 ^{a}
	Planted with *Q. suber* (PP1)	0.4±0.2 ^{a,b}	0.4±0.3 ^{a}	0.4±0.3 ^{a}	0.4±0.3 ^{a}
	Planted with *Q. suber* (PP2)	0.5±0.3 ^{b}	0.5±0.3 ^{a}	0.4±0.3 ^{a}	0.6±0.8 ^{a}

††The scale used to evaluate the severity of symptoms was: 0 = without symptoms, 1 = 1-33 %, 2 = 34-66 %, 3 = 67-99 %, 4 = 100 % of affected tissue. In between values were used according to the severity of symptoms: 0.3 = weak, 0.5 = moderate, 0.7 = strong. Average of symptom severity ± standard deviation.
Statistical comparisons were made between the different conditions. For each column, values followed by the same letter means that they do not differ significantly (P<0.05), using Fisher's Least Significant Difference Test (LSD).

3.3.3.1. *Phytophthora cinnamomi* re-isolation from roots

The assessment of the frequency of re-isolation of *P. cinnamomi* from roots gave a hint on the extent at which *P. purpurea* was efficient in protecting *Q. suber* from infection (see Chapter 2, section 2.2.7). In area A the percentage of *P. cinnamomi* isolated from the roots of *Q. suber* sown with *P. purpurea* was significantly less than the percentage of

[26] Appendix II.X.
[27] Appendix II.XV.

P. cinnamomi isolated from the roots of *Q. suber* sown alone (35.3±33.9 and 53.8±40.9, respectively)[28] and in area B, the same trend occurred (14.3±21.9 and 44.4±50.9, respectively)[29] (Figure 3.5). *P. cinnamomi* was never isolated from the roots of *P. purpurea*. This shows the potential of *P. purpurea* to protect *Q. suber* and confirms the results obtained in the greenhouse experiments (Chapter 2, Table 2.13).

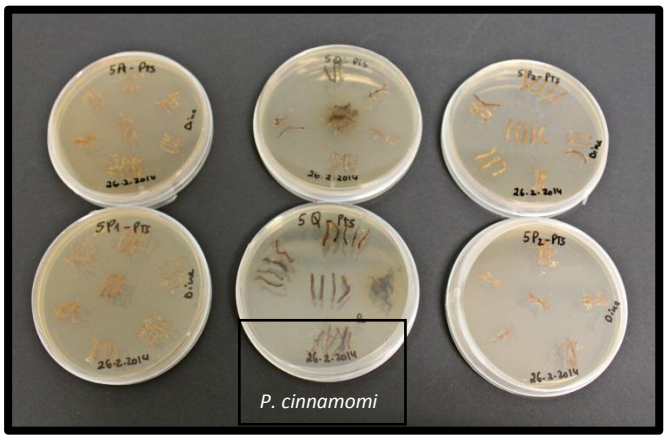

Figure 3.5. Attempt to isolate *Phytophthora cinnamomi* from the roots of *Quercus suber* seedlings and *Phlomis purpurea* plants, growing together in stand B (PTS). In this sample, *P. cinnamomi* only grew in 1 out of 11 groups of *Q. suber* root fragments and was absent in all *P. purpurea* plants.

3.3.3.2. Inoculum potential of soil

Two methods were used to determine soil inoculum potential. Method 1 was soil (10 g) extension on NARPH medium and method 2 used baits in 1000 ml of soil (see Chapter 2, section 2.2.8).

Using method 1, it was shown that *P. purpurea* alone significantly inhibited (P<0.05) *P. cinnamomi* in naturally infested soils in both areas[30]. Although the recovery of *P. cinnamomi* from soil where *Q. suber* was sown together with *P. purpurea*, in area A was approximately half of that obtained in soil with *Q. suber* alone, there was not a statistically significant difference between the two[31] (Table 3.2). In area B, the sample 11[32], was an outlier, and the average without this outlier would have been 1.5, but all samples were considered in this test, hence no significant difference was found. Using method 2, there is a significant difference between *P. purpurea* alone and the other conditions in area A[33], but in area B the difference

[28] Appendix II.IX. and Appendix II.XI.
[29] Appendix II.XIV. and Appendix II.XVI.
[30] Appendix II.IX. and Appendix II.XIV.
[31] Appendix II.XIII.
[32] Appendix II.XIV.
[33] Appendix II.XII.

was not statistically significant[34] (Table 3.2). Once more, it was shown that method 2 is more sensitive, so more accurate, as discussed in Chapter 2. In method 1, it is possible to quantify the colonies [number of colony forming units (C.F.U.)] whereas in method 2 if one propagule is present it can infect and consequently cause necrosis of several leaves, so it is difficult to quantify the amount of inoculum, unless a semi-quantitative approach is used by using the serial dilution end-point (Chapter 2, section 2.2.8) Several authors have used method 1 to detect the presence of *P. cinnamomi* in naturally (Rodríguez et al. 2004; Jayasekera 2006; Caetano 2007) or artificially infested soil (Jayasekera, 2006; Neves *et al.*, 2014). However, false-negative isolations may lead to mis-diagnosis of the presence of *P. cinnamomi*.

Table 3.2. Recovery of *Phytophthora cinnamomi* from soil, using method 1 – soil extension on NARPH medium and method 2 – baitings with *Quercus suber* leaves.

Species	Condition	Area A "Menta"		Area B "PTS"	
		***Phytophthora cinnamomi* recovery from soil**			
		Method 1* (c.f.u. g^{-1} soil)	**Method 2****	**Method 1* (c.f.u. g^{-1} soil)**	**Method 2****
Quercus suber	Alone	16.4±24.6 [b]	100±0.0 [b]	2.7±4.6 [b]	100±0.0 [a]
	Sown with *P. purpurea*	8.2±8.7 [b]	100±0.0 [b]	4.67±11.4 [b]	91.7±28.9 [a]
Phlomis purpurea	Alone PP1 and PP2	1.3±4.3 [a]	75.0±45.2 [a]	0.3±0.9 [a]	66.7±49.2 [a]

*Ten grams of soil per sample were analysed. ** One litre of soil per sample was analysed.

Jayasekera (2006) showed that, in three areas, with different soil types, during the summer, soil extracts beneath *A. pulchella*, showed a high level of suppression of *P. cinnamomi*-less than 10 % of the baitings yielded *P. cinnamomi* colonies. In our work, the infected leaves (baits) were not counted, since it is not an accurate method of quantifying the amount of inoculum (T. Jung, personal communication) and method 1 was used in an attempt to quantify the *P. cinnamomi* propagules.

Soil from the rhizosphere of purple phlomis seedlings that developed larger root systems showed to be oomycete free using method 1 and 2 (Figure 3.6) whereas soil associated with purple phlomis that had less developed root system (Figure 3.7) were found to be *P. cinnamomi* free (apart from two seedlings) using method 1, but using method 2, *P. cinnamomi* was recovered from all samples.

[34] Appendix II.XVII.

Figure 3.6. Field trial with naturally infested soil in which soil associated with *Phlomis purpurea* growing alone, showing ample-developed root system were found to be *Phytophthora cinnamomi* free, by soil spread and bait methods.

Figure 3.7. Field trial with naturally infested soil in which soil associated with *Phlomis purpurea* growing alone, showing less developed root system were found to be *Phytophthora cinnamomi* free by soil spread (except the 4th from the left), but all were oomycete positive using the bait method.

These results confirm the potential of *P. purpurea* with a large root system to eliminate *P. cinnamomi* inoculum potential. Similarly, Jayasekera (2006) demonstrated that suppression of *P. cinnamomi* under *A. pulchella* is dependent on the age of the plants. There was no significant difference in the recoveries of *P. cinnamomi* from mycelium incubated under the extracts from soils of one-year-old *A. pulchella* and the control whereas no *P. cinnamomi* was recovered from soils under four-year-old stands (*ibid*.).

As stated before, the chemical composition of these soils is highly conducive for *P. cinnamomi*[35] and the determination of soil inoculum potential before setting up the trial, using the baiting method, revealed that PTS soil was heavily infested since all the leaves were necrotic after 24 h.

[35] Appendix II.I.

Our results support the hypothesis that *P. purpurea* roots exude compounds that are inhibitory to *P. cinnamomi*, similar to the *P. purpurea* root extracts that are inhibitory to *P. cinnamomi* propagules (Neves *et al.*, 2014). The anti-*P. cinnamomi* activity was not evaluated in relation to age (the seedlings were all the same age); the activity appears, instead dependent on the amount of roots and therefore the amount of compounds released.

P. purpurea with a large root system, planted alone, was able to completely eliminate *P. cinnamomi*. However, if *Q. suber* is present together with these seedlings, the inoculum potential is reduced but not eliminated since a host is present. Therefore, purple phlomis could be used as a non-host antagonistic plant. As *P. cinnamomi* has weak saprotrophic ability, the temporary removal of all known hosts or maintaining the area vegetation-free for several years, could lead to the complete elimination of the pathogen, although the more resistant structures, like chlamydospores and oospores may persist in soil up to six years (Mircetich & Zentmyer, 1967).

It would be worthwhile trying to mechanically eliminate all hosts from a plot (a more environmental friendly approach than the use of fumigants or herbicides) and planting mature *P. purpurea* seedling for at least 2 years. After that time, if *P. cinnamomi* is absent, susceptible hosts could be reintroduced and checked for infection establishment.

Host removal has been applied in orchards (Erwin & Ribeiro, 1996) and to forests using soil fumigants (Weste *et al.*, 1973). Crone *et al*. (2014) showed that host removal significantly reduced subsequent pathogen recovery, and 28 months after removal of all vegetation, by herbicide treatment, there were no *P. cinnamomi* recoveries. However when native plants regrew, some of which hosts for *P. cinnamomi*, this oomycete was recovered again (*ibid*.). Chlamydospores and oospores, the survival structures of *P. cinnamomi*, remain in the soil and when a host is present and the environmental conditions are favourable to the infection the life cycle begins. The inoculum increases and the oomycete can be recovered again.

Which compounds are exuded by *P. purpurea* roots and what may render this plant resistant to *P. cinnamomi* will be answered in the next chapters.

3.3.4. Conclusion

The main goal of this work was to determine the protective effect of *Phlomis purpurea* towards *Quercus suber* in a naturally infested area, having a non-infested area as a control. However, although initially *P. cinnamomi* was not found in the supposedly control area, this plant pathogen was recovered from soil collected during the sowing. This demonstrates that

this invasive pathogen is widespread at the Serra do Caldeirão - Algarve. Acorns from a top susceptible family, to *P. cinnamomi,* were sown in two areas of land each one with two plots. The winter was very dry and *P. purpurea* seedlings and acorns were watered bimonthly or monthly initially to enhance the establishment. The *Q. suber* emergence occurred during several months and even after one year of sowing new seeds emerged. In the plots without *P. purpurea* the emergence was significantly lower than in the plots in which acorns were planted together with *P. purpurea* showing that purple phlomis contributed to the emergence success. Purple phlomis seemed to enhance the survival of cork oak seedlings at area B. However, at area A, a bigger part of the emerged acorns together with *P. purpurea* compared with the plot without *P. purpurea* died and there were no significant differences between the two. The survival of *P. purpurea,* at the end of the trial, was higher in plot A2 than in plot B2, where we found signs of herbivory. Two methods were used to determine the soil inoculum potential. It was confirmed that the method using baits is the more effective to isolate this oomycete, mainly for two reasons: 1. the volume of soil analysed is 100 times bigger and 2. the release of zoospores from a single sporangia can infect several baits, increasing the chances of isolation. In this assay, *P. purpurea* significantly protect *Q. suber* from *P. cinnamomi* infection which confirms previous results (Neves *et al.*, 2014). However, *P. purpurea* did not reduced significantly the soil inoculum potential when planted together with *Q. suber* but seedlings with well-developed root system, planted alone, were able to completely eliminate *P. cinnamomi*.

The appendices can be found at **https://meocloud.pt/link/a1bcf83d-1669-4410-b338-b1c890ac3774/Appendices_Chapter%203.docx/** (see CD in attachment).

3.4. Acknowledgments

We would like to acknowledge José Albuquerque, Miguel Vieira, Pedro, Carla and the forest firefighters from “Associação dos produtores florestais da Serra do Caldeirão” for their help, essentially providing water for the seedlings during the field trial and for the discussions about cork oak decline.

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Chapter 4

Anti-Phytophthora cinnamomi activity of *Phlomis purpurea* root extract (PRE)

Part of this Chapter corresponds to part of the publication:

Neves, D.; Caetano, P.; Oliveira, J.; Maia, C.; Horta, M.; Sousa, N.; Salgado, M.; Dionísio, L., Magan, N., .Cravador, A. (2014). *Anti-Phytophthora cinnamomi* activity *of Phlomis purpurea* plant and root extracts. European Journal of Plant Pathology, 138:835–846.

The introduction of the article has been omitted, as a general introduction was presented in chapter 1.

4.1. Abstract

The effect of *Phlomis purpurea* crude ethanol root extract (PRE) was evaluated *in vitro* on *Phytophthora cinnamomi* life cycle structures. PRE was shown to inhibit mycelial growth, sporangial production, zoospore release, zoospore germination, chlamydospore production and chlamydospore germination. At 10 $mgml^{-1}$ inhibition of the pathogen structures was 85-100 % (Neves *et al.*, 2014).

In order to study the effectiveness of *P. purpurea* root extracts to control *Quercus suber* and *Q. ilex* subsp. *rotundifolia* root infection by *P. cinnamomi*, *in vivo* assays were set up. Two-week-old *Q. suber* seedlings were subjected to four different conditions, namely Mineral Salt Solution (MSS), 10 $mgml^{-1}$ *P. purpurea* root extract in MSS, 10 $mgml^{-1}$ extract with *P. cinnamomi* zoospores in MSS and zoospores in MSS only. The roots were detached from the seeds, placed on selective media and checked for the presence of *P. cinnamomi*. The viability of zoospores was also checked. *P. purpurea* root extracts at 10 $mgml^{-1}$ significantly (P <0.001) inhibited *P. cinnamomi* zoospore infection of *Q. suber* roots, whereas PRE at 5 $mgml^{-1}$ was shown to significantly protect *Q. ilex* subsp. *rotundifolia* against infection by this pathogen and at 10 $mgml^{-1}$ no *Q. ilex* subsp. *rotundifolia* roots were infected.

In addition, to evaluate whether the reduction of infection was due to the direct activity of PRE on the zoospores or to the induction of plant defences, another experiment was conducted, eliciting the plant roots for 24 h with PRE, before challenging with zoospores. PRE was able to elicit a defence response on *Q. suber* roots as revealed by the significant reduction extent of infection in roots.

4.2. Material and Methods

4.2.1. Plant material

Phlomis purpurea plants (*ca* 300) were collected in *Q. suber* stands across the Algarve (Concelhos Alcoutim, Aljezur and Olhão, freguesia de Moncarapacho), southern Portugal. They were stored at 21 °C for 1 week until processed. A voucher specimen from Moncarapacho was deposited in the herbarium of the Algarve University (accession number 13485).

Tap roots, 6–8 cm long, from 2-week-old *Q. suber* and *Q. ilex* subsp. *rotundifolia* (hereinafter *Q. ilex*) seedlings, all distinct genotypes and obtained from seedlings grown in vermiculite at 22 °C under natural photoperiod, were used in the *in planta* experiments.

4.2.2. Root extracts preparation

Fine roots from *ca* 100 plants of *P. purpurea* (50 g) were washed and chopped with a grinding-master (Moulinex® 1,2,3) and shaken for 7 days in 350 ml 70 % aqueous ethanol. The ethanol was then evaporated in a rotary evaporator (Heidolph 94200, Bioblock Scientific I, Schwabach, Germany) under reduced pressure at 35 °C. The extract was concentrated in a speedvac (Savant, SC 110A, Holbrook, USA) to constant weight, yielding approximately 5 g of a brownish sticky suspension which was dissolved in water and filtered through a 0.45 µm nitrocellulose filter (Millipore®). This procedure was repeated three times in order to obtain a final weight of 15 g necessary to perform all the experiments. *P. purpurea* crude root extract (PRE) was obtained with an average yield of 10 % (WPRE/Wroots).

4.2.3. *Phytophthora cinnamomi* isolates

Pure stock cultures of the *P. cinnamomi* isolates: PA37 and PA45, both mating type A2, isolated in the Algarve region, i.e., from *Quercus suber* roots at Lagos and from soil associated with declining *Q. suber* stands at S. Brás deAlportel were tested for pathogenicity. Isolation and culture maintenance took place on V8 Juice agar medium as described in detail in Chapter 2, section 2.2.2.

4.2.4. Inoculum preparation

Zoospores, of both PA45 and PA 37 strains (Chapter 2, section 2.2.2), were produced according to the procedure of (Byrt & Grant, 1979)**.** Briefly, five small plugs from the edge of *P. cinnamomi* colonies growing on V8A (Chapter 2, section 2.2.2), in Petri dishes were transferred to Miracloth membranes (Calbiochem-Novabiochem, Alexandria, Australia) overlying agar medium. After 28 days of growth at 24.5 °C, each oomycete isolate and Miracloth support was transferred to 100 ml 5 % V8-broth (V8B) and the culture shaken overnight (16 h) at 90 rpm. The nutrient medium was then replaced with a mineral salt solution (MSS) consisting of 0.01 M $Ca(NO_3)_2$. $4H_2O$, 0.005 M KNO_3 and 0.004 M $MgSO_4$. 7H2O dissolved in 1 l of distilled water, autoclaved and subsequently supplemented with 1 ml 0.1 M $C_{10}H_{12}N_2NaFeO_8$ solution, previously sterilised through a 0.22 µm filter (Millipore®). The culture was then shaken for a further 24 h. Sporangia were induced to release zoospores by incubating the Miracloth covered with MSS in Petri dishes (3 replicates per isolate) at 4 °C for 20 min. Then, the Petri dishes were exposed to fluorescent light (Philips TLD 30 W/54), at room temperature for 2–3 h.

The density of zoospores was determined by transferring the suspension to a 50 ml Falcon tube and allowing it to settle for 5 minutes. One millilitre of zoospore suspension was then transferred to a 1.5 ml Eppendorf tube and shaken for 60 s to induce zoospore encystment and consequent immobilisation to allow counting. Counting was performed using a Fuchs-Rosenthal haemocytometer slide (Hausser Scientific Company, Horsham, PA).

With this method, 10^4–10^5 zoospores ml^{-1} were routinely produced. A zoospore suspension (200 µl) was plated on a Petri dish containing NARPH medium (Chapter 2, section 2.2.2) and incubated at 24.5 °C to check their viability.

4.2.5. *In planta* inhibitory action of *P. purpurea* root extract (PRE) in *Quercus suber* and *Quercus ilex* subsp. *rotundifolia* infection by *Phytophthora cinnamomi* zoospores

Zoospores were produced as described above. Their concentration was determined using a haemocytometer and the suspension diluted with SDW to *ca* 10^4 zoospores ml^{-1}.

Quercus spp were subjected to the treatments below and left for 48–72 h or until they showed necrosis.

4.2.5.1. Direct effect of *Phlomis purpurea* root extract (PRE) on *Quercus suber* (Experiment 4.1)

Two-week-old *Q. suber* roots, obtained from germinated seeds, were subjected to four treatments (Figure 4.1):

(1) Immersion in mineral salt solution (MSS) (negative control);

(2) Immersion in MSS with 10 $mgml^{-1}$ PRE (negative control);

(3) Immersion in MSS with 10 $mgml^{-1}$ PRE and *P. cinnamomi* zoospores;

(4) Immersion in MSS with zoospores (positive control).

Figure 4.1. Two-week-old *Quercus suber* subjected to four treatments. From left to right, in triplicate, roots were immersed in mineral salt solution (MSS), MSS with 10 $mgml^{-1}$ PRE (negative control), MSS with 10 $mgml^{-1}$ PRE and *P. cinnamomi* zoospores and MSS with zoospores for 48 to 72 h or until roots showed necrosis.

Three *Q. suber* were used per treatment. Each germinated seed was considered one experimental unit. The experiment was repeated four times.

4.2.5.2. Direct effect of *Phlomis purpurea* root extract (PRE) on *Quercus ilex* subsp. *rotundifolia* infection (Experiment 4.2)

Two-week-old *Q. ilex* roots, obtained from seedlings, were subjected to eight different treatments:

(1) Immersion in mineral salt solution (MSS) (negative control);

(2) Immersion in MSS with 1 $mgml^{-1}$ PRE (negative control);

(3) Immersion in MSS with 5 $mgml^{-1}$ PRE (negative control);

(4) Immersion in MSS with 10 $mgml^{-1}$ PRE (negative control);

(5) Immersion in MSS with 1 $mgml^{-1}$ PRE and *P. cinnamomi* zoospores;

(6) Immersion in MSS with 5 $mgml^{-1}$ PRE and *P. cinnamomi* zoospores;

(7) Immersion in MSS with 10 $mgml^{-1}$ PRE and *P. cinnamomi* zoospores;

(8) Immersion in MSS with zoospores (positive control).

Five holm oak plants per treatment were used. Each germinated seed was considered one experimental unit. Three concentrations of PRE (1.0; 5.0 and 10 $mgml^{-1}$) were assayed. The experiment was repeated once.

4.2.5.3. Evaluation of *Phlomis purpurea* root extract (PRE) as an elicitor (Experiment 4.3)

Two-week-old *Q. suber* roots germinated from seeds were divided into two groups Group 1 was submitted to a pre-treatment with MSS and group 2 to 10 mgml^{-1} PRE in MSS both for 24 h. The aqueous solutions were discarded. Roots from group 1 were sub-divided into two groups: group 3 that was re-immersed in MSS (negative control) and group 4 that was immersed in a zoospore suspension in MSS (positive control). Roots from group 2 were sub-divided in two groups: group 5 that was kept in PRE in MSS and group 6 that was immersed in a suspension of zoospores in MSS (Figure 4.2).

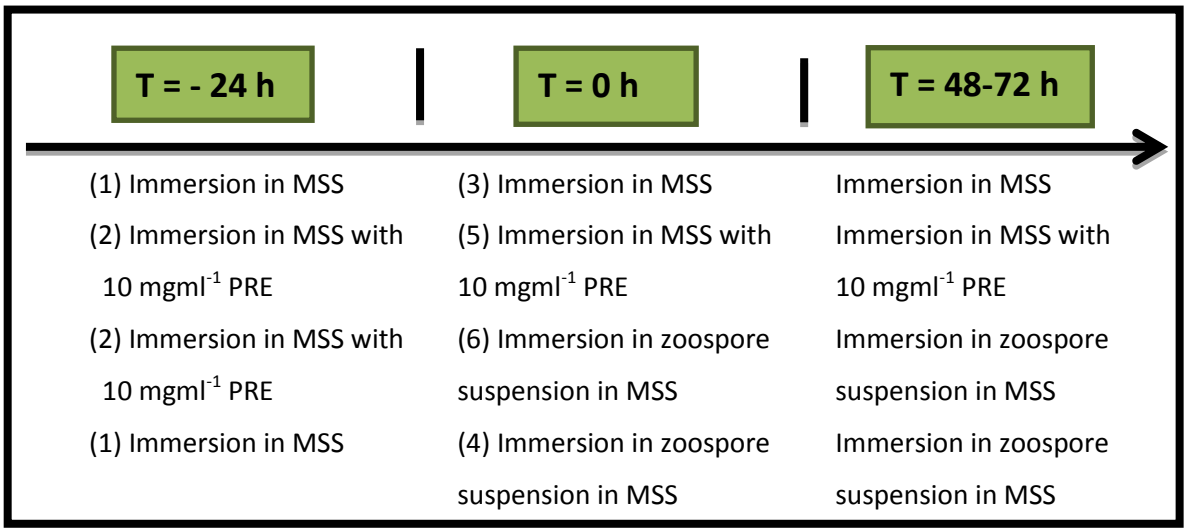

Figure 4.2. Two-week-old *Quercus suber* subjected to different treatments. Roots were either immersed in mineral salt solution (MSS) (group 1) or in MSS with 10 mgml^{-1} PRE (group 2). After 24 h, all solutions were discarded. Roots from group 1 were sub-divided into two groups: group 3 that was re-immersed in MSS (negative control) and group 4 that was immersed in a zoospore suspension in MSS (positive control). Roots from group 2 were sub-divided in two groups: group 5 that was kept in PRE in MSS and group 6 that was immersed in a group 5 that was kept in PRE in MSS and group 6 was immersed in a suspension of zoospores in MSS. Roots from groups 3-6 were immersed for 48 to 72 h or until roots showed necrosis.

Three plants per treatment were used. Each plant was considered one experimental unit. The experiment was repeated three times.

4.2.5.4. Re-isolation of *Phytophthora cinnamomi* from roots

Infection of root tissue by *P. cinnamomi* was assessed by re-isolation of the pathogen according to Sánchez et al. (2002): 1.0 cm long root segments from each of the inoculated and control germinated seeds were placed on Petri dishes with NARPH medium.

4.2.5.5. Calculation of the inhibitory effect of PRE by assessment of infected root tissue

The percentage of *P. cinnamomi* infected roots was calculated as follows:

% *P. cinnamomi* infected roots = 100 × (X/Y),

where: X = number of root segments with *P. cinnamomi* colonies and Y = total number of root segments in the Petri dishes.

4.2.6. Statistics

Data were analysed by analysis of variance (One-Way ANOVA), followed by Fisher's protected least significant difference (LSD) test or independent t-test if data was normally distributed and homogeneity of variance was confirmed using Levene's test (Levene, 1960). Otherwise, the nonparametric Kruskal-Wallis test or Mann-Whitney test (Steel & Torrie, 1985) was run with the software package "PASW statistics 18" (IBM software, 2009 SPSS Inc. Hong Kong). Statistical comparisons were made between the different conditions within the same plant species. The independent variables were the plant species under the treatments mentioned above. The dependent variables were the percentage of *P. cinnamomi* re-isolated from roots.

4.3. Results and discussion

4.3.1. Inoculum preparation

Zoospores were produced according to the procedure of Byrt and Grant (1979) which, briefly, is based on a period of nutritional deficiency, stirring, exposure to light and a decreased in growth temperature in liquid medium. Probably, in a natural environment, these are the same factors that stimulate sporulation: the lack of nutrients in a necrotized host tissue leads to intense sporulation and an attempt to infect new healthy tissues. The exposure to light to a soil pathogen also stimulates sporulation as a survival instinct and the presence of water during the cold season allows the dispersal of zoospores.

The number of zoospores produced, generally, varied between 10^4 and 10^5 zoospores ml^{-1}, the same as stated by the authors, and *ca* 10^4 zoospores ml^{-1} were used for infection. However, it must be stressed that the counting of cells which have their own mobility has an inherent error: when pipetting 1 ml, before shaking to induce zoospore

encystment and consequent immobilisation to allow counting, zoospores are swimming freely in an aqueous environment and not homogeneously distributed. This error was estimated to be between 10 and 20 % (Byrt & Grant, 1979).

4.3.2. *In planta* inhibitory action of *P. purpurea* root extract (PRE) in *Quercus suber* and *Quercus ilex* subsp. *rotundifolia* infection by *Phytophthora cinnamomi* zoospores

Zoospores are the motile infective structures of *P. cinnamomi*, which are, generally, attracted to roots by chemo and electro taxis, where they attach and invade the plant (reviewed by Hardham 2005). To simulate the natural infection process zoospores were produced and the plants inoculated to produce infection. However it is crucial that those structures are viable when the roots are infested. The viability was checked and after 48 h, the Petri dishes (three per repetition) were always completely full of germinated zoospores.

4.3.2.1. Direct effect of *Phlomis purpurea* root extract (PRE) on *Quercus suber* infection (Experiment 4.1)

Phlomis purpurea root extract (PRE) at 10 $mgml^{-1}$ was added to the zoospore suspension to evaluate the activity of PRE on *Q. suber* infection. It was previously shown that PRE at 10 $mgml^{-1}$ inhibited zoospore germination, *in vitro* (Neves *et al.*, 2014).

Forty eight hours post inoculation (hpi), the inoculated *Q. suber* roots without PRE started to show necrosis (not shown) which increased up to 72 hpi (Figure 4.4 H) while the inoculated roots containing PRE did not show any necrosis (Figure 4.4 G).

PRE was shown to protect *Q. suber* seedlings from infection by *P. cinnamomi* by reducing the number of infected root fragments. Thus, PRE at 10 $mgml^{-1}$ significantly inhibited (P<0.0001)[36] the infection of *Q. suber* roots by *P. cinnamomi* zoospores (Figure 4.3, 4.4). This presupposes the direct toxic effect of PRE towards zoospores. *Phytophthora cinnamomi* was always isolated from all roots immersed in a suspension of zoospores in MSS when PRE was not added, confirming that zoospores were able to successfully infect the host. On the other hand, the pathogen was never isolated from the negative controls (roots not challenged with the pathogen) meaning that there was no cross contamination.

[36] Appendix III.I.

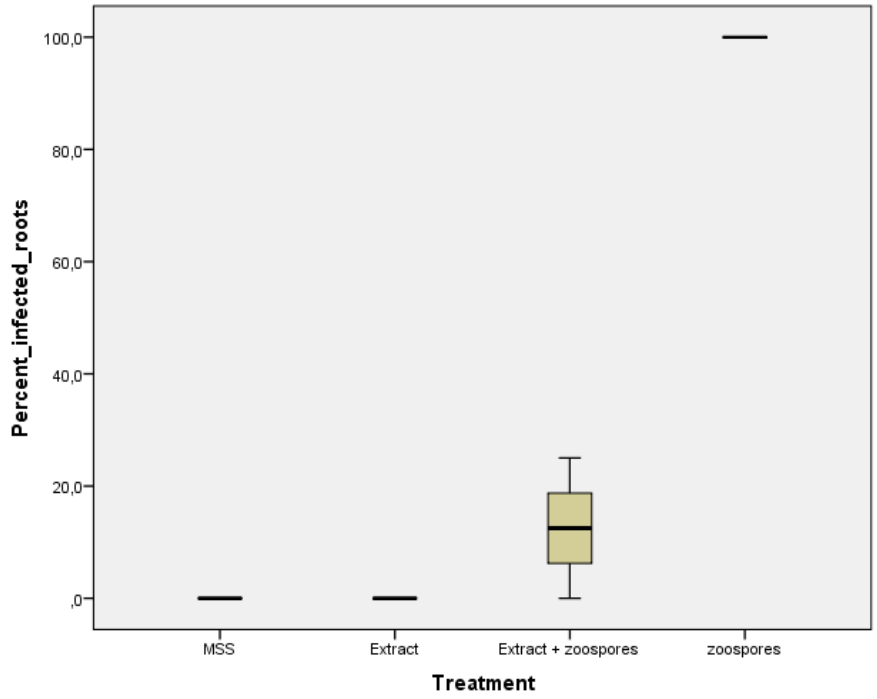

Figure 4.3. Percentage of *Quercus suber* roots infected by *Phytophthora cinnamomi* zoospores. MSS = Mineral Salt Solution; Extract = MSS with 10 mgml^{-1} *Phlomis purpurea* root extract (PRE); Extract + zoospores = MSS with 10 mgml^{-1} PRE and *P. cinnamomi* zoospores; zoospores = MSS with zoospores. MSS and Extract are the negative controls.

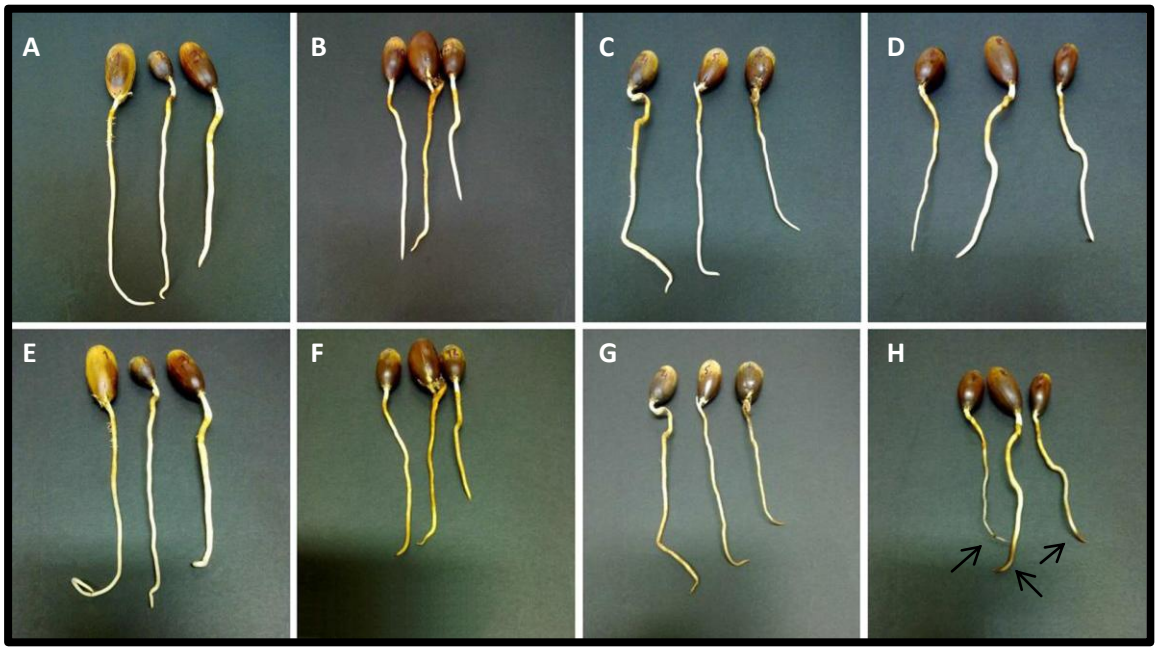

Figure 4.4. Effect of *Phlomis purpurea* root extract (PRE) on the infection of 2-week-old germinated *Quercus suber* roots by *Phytophthora cinnamomi* zoospores. *Q. suber* roots exposed to **A**=MSS (Mineral Salt Solution); **B**=MSS with 10 mgml^{-1} PRE; **C**=MSS with 10 mgml^{-1} PRE and *P. cinnamomi* zoospores; **D=**MSS with zoospores; **E**=MSS; **F**=MSS with 10 mgml^{-1}PRE; **G =**MSS with 10mgml^{-1} PRE and *P. cinnamomi* zoospores; **H**=MSS with zoospores (arrows point to necrosis). **A–D**: 0 h, **E–H**: 72 h.

4.3.2.2. Direct effect of *Phlomis purpurea* root extract (PRE) on *Quercus ilex* subsp. *rotundifolia* infection (Experiment 4.2)

The methodology was the same as in Experiment 4.1. However different concentrations of PRE were used in *Q. ilex***.** The treatments were: (1) immersion in mineral salt solution (MSS) (negative control); (2) immersion in MSS with 1 $mgml^{-1}$ PRE (negative control); (3) immersion in MSS with 5 $mgml^{-1}$ PRE (negative control); (4) immersion in MSS with 10 $mgml^{-1}$ PRE (negative control); (5) immersion in MSS with 1 $mgml^{-1}$ PRE and *P. cinnamomi* zoospores; (6) immersion in MSS with 5 $mgml^{-1}$ PRE and *P. cinnamomi* zoospores; (7) immersion in MSS with 10 $mgml^{-1}$ PRE and *P. cinnamomi* zoospores and (8) immersion in MSS with zoospores (positive control).

Phlomis purpurea root extract at 10 $mgml^{-1}$ (Treatment 7) was able to prevent infection of *Q. ilex* roots. PRE at 5 $mgml^{-1}$ (Treatment 6) was able to reduce the percentage of infected roots up to 92.0 %. *Phytophthora cinnamomi* was always isolated from all roots immersed in a suspension of only zoospores (Treatment 8) (Figure 4.4). On the other hand, the pathogen was never isolated from the negative controls (Treatments 1 to 4). PRE at 1 $mgml^{-1}$ (Treatment 5) did not differ from treatment 8[37]. However, these results show (Figure 4.5) as in 4.3.2.1 (Figure 4.2) a direct toxic effect of PRE to the pathogen.

[37] Appendix III.II.

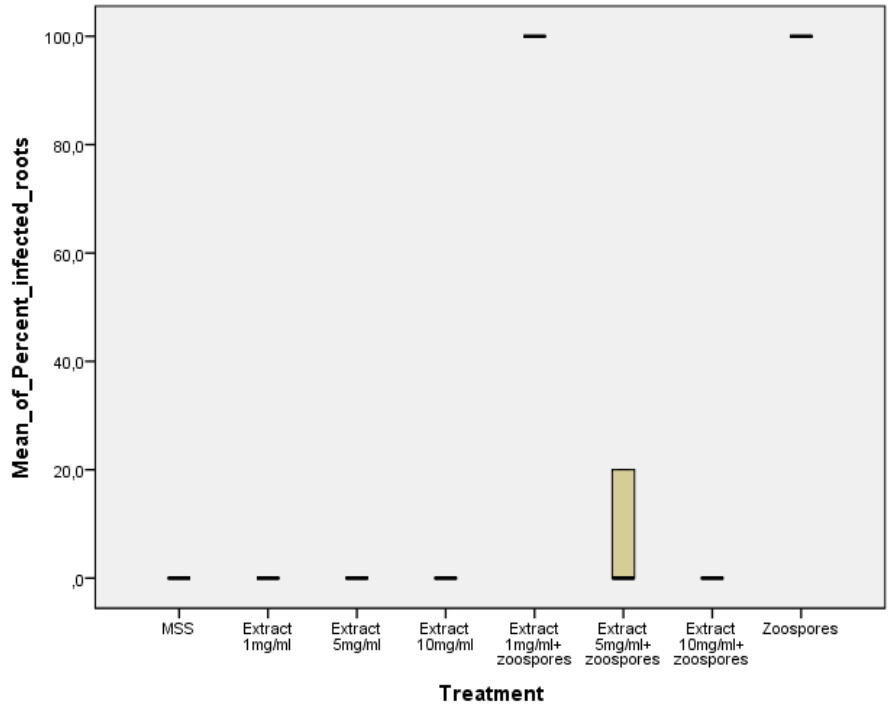

Figure 4.5. Mean of percentage of *Quercus ilex* subsp. *rotundifolia* roots infected by *Phytophthora cinnamomi* zoospores. MSS = Mineral Salt Solution; Extract 1 mg/ml = MSS with 1 $mgml^{-1}$ *Phlomis purpurea* root extract (PRE); Extract 5 mg/ml = MSS with 5 $mgml^{-1}$ PRE; Extract 10 mg/ml = MSS with 10 $mgml^{-1}$ PRE; Extract 1 mg/ml+zoospores = MSS with 1 $mgml^{-1}$ *Phlomis purpurea* root extract (PRE) with zoospores; Extract 5 mg/ml+zoospores = MSS with 5 $mgml^{-1}$ PRE with zoospores; Extract 10 mg/ml+zoospores = MSS with 10 $mgml^{-1}$ PRE with zoospores; Zoospores = *P. cinnamomi* zoospores. MSS and Extract 1, 5 and 10 mg/ml are the negative controls.

The reduction of the level of colonisation of tissues could be due to a direct effect of PRE on zoospores (Neves *et al.*, 2014). Several plant extracts have been shown to have a direct effect on *Phytophthora* spp. propagules (Kim *et al.*, 2003; Molina-Torres *et al.*, 2004; Curtis *et al.*, 2004; Stephan *et al.*, 2005). *In vitro* studies of inhibition of *P. cinnamomi* have been conducted using crude root extracts from avocado trees, showing inhibition of mycelial growth of the pathogen by 50–100 % (Sánchez-Pérez *et al.*, 2009).

Pathogens, including *P. cinnamomi*, secrete an arsenal of effector proteins, defined by Kamoun (2003) as "molecules that alter host cell structure and function, thereby facilitating infection (virulence factors or toxins) and/or triggering defence responses (avirulence factors or elicitors)" that modulate plant innate immunity (Kamoun, 2006). As in an "arms-race model" plants also trigger a rapid resistance response called the hypersensitive response (HR) that blocks pathogen growth by depriving biotrophic, in this case bio-necrotrophic, pathogens of food and by creating a highly oxidising environment that damages proteins and cell structures

leading to cell death, visualised as necrosis (Figure 4.4 H). Simultaneously, plants activate a complex series of responses, including local and systemic induced resistances. Depending on how fast activation of these responses is produced they can vary from effective to completely unsuccessful. It is known that plant extracts can also induce defence mechanisms against *Phytophthora* spp. in plants (Walters *et al.*, 2005; Moushib *et al.*, 2013). PRE either exerts a direct effect on zoospores and the infection of the host is prevented and/or induces a defence mechanism on the host so that the outcome is positive for the host.

For that reason Experiment 4.3 was conducted.

4.3.2.3. Evaluation of *Phlomis purpurea* root extract (PRE) as an elicitor in *Quercus suber* (Experiment 4.3)

Two-week old *Q. suber* roots were subjected to contact with PRE for 24 h before challenge with *P. cinnamomi*. Afterwards, PRE was discarded and roots immediately challenged with zoospores. Roots in mineral salt solution (MSS) and MSS plus 10 $mgml^{-1}$ PRE (PRE) were the negative controls. *Q. suber* roots were also immersed in MSS with zoospores as a positive control. Forty eight hours after inoculation, the *Q. suber* inoculated roots were evaluated for necrosis.

Prior contact of *Quercus suber* roots with PRE at 10 $mgml^{-1}$ for 24 h significantly inhibited (P=0.024) the infection of roots by *P. cinnamomi* zoospores (Figure 4.6) by reducing the number of root fragments infected (Figure 4.7). The oomycete caused necrosis on the *Q. suber* roots, as in Experiment 4.1 and was always isolated from all roots immersed in a suspension of zoospores in MSS. The pathogen was never isolated from the negative controls (roots not challenged with the pathogen). We can conclude that PRE induced resistance in *Q. suber* seedlings against *P. cinnamomi* infection. This resistance can be broad spectrum and long lasting but usually does not confer complete protection against the invaders, with many resistance elicitors providing between 20 and 85 % disease control (Walters *et al.*, 2005). The application of sugar beet extract (SBE) to potato leaves twice in 48 h led to a significant decrease in lesion size (approximately 20 %) caused by *P. infestans* (Moushib *et al.*, 2013).

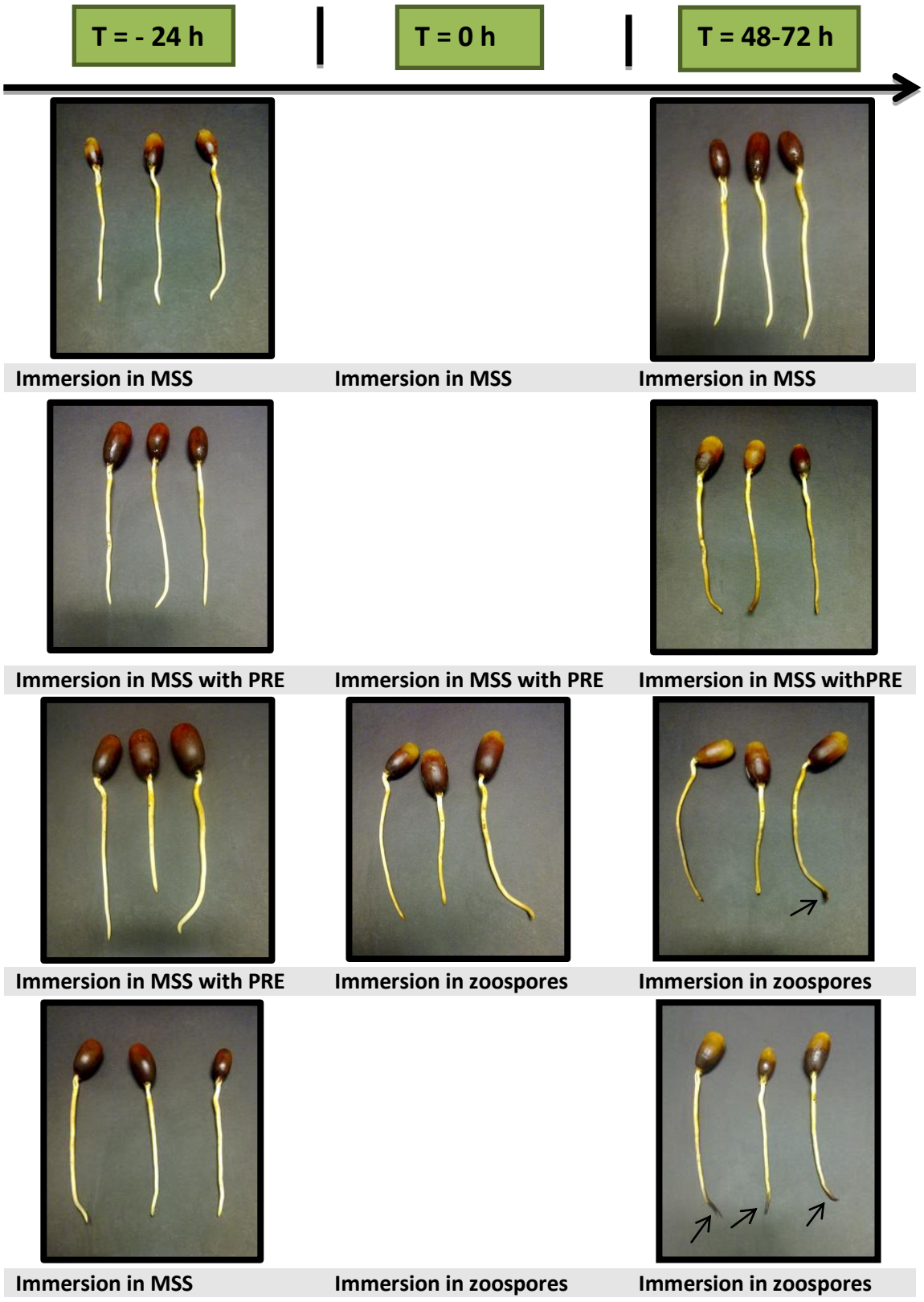

Figure 4.6. Two-week-old *Quercus suber* roots submitted to four treatments. Roots were either immersed in mineral salt solution (MSS) or in MSS with 10 $mgml^{-1}$ PRE (T=-24 h). After 24 h, all solutions were discarded. A group of roots that were in MSS continued in MSS and another group was immersed in a zoospore suspension. A group of roots that were immersed in MSS with 10 $mgml^{-1}$ PRE continued in PRE and another group were immersed in zoospores.

Roots were immersed for 48 h or until roots showed necrosis (T= 48-72 h). Arrows point to necrosis.

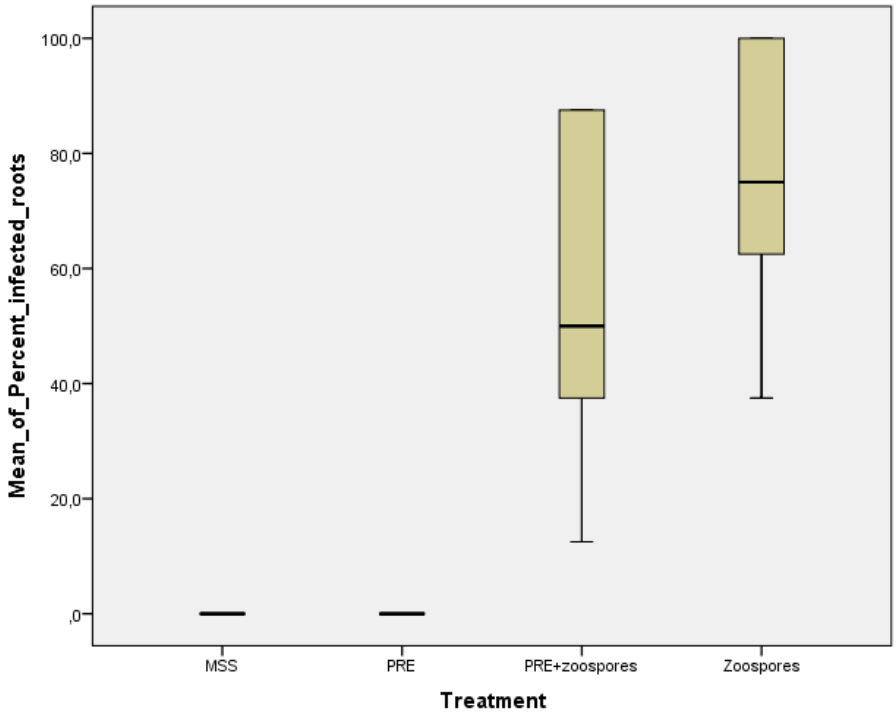

Figure 4.7. Mean of percentage of *Quercus suber* roots infected by *Phytophthora cinnamomi* zoospores. MSS = Mineral Salt Solution; PRE = MSS with 10 mgml^{-1} *Phlomis purpurea* root extract (PRE). PRE + zoospores = MSS with 10 mgml^{-1} PRE for 24 h and discarded, and infested with zoospores for 48 to 72 h; Zoospores = MSS with zoospores. MSS and PRE were the negative controls.

PRE significantly reduced the level of tissue colonisation from 73.6±22.9 to 52.8±30.5, in about 20 %[38,39] (Figure 4.7). However the optimal time interval between inducer treatment and challenge inoculation for maximum resistance to build up was not determined. Godard et al. (2009) have demonstrated the ability of two plant extracts, namely *Rheum palmatum* root extract (RPRE) and *Frangula alnus* bark extract (FABE), widely used in herbal medicine as laxatives due to their anthraquinone content, to protect *Vitis vinefera* from the oomycete *Plasmopara viticola*. These extracts are toxic to the pathogen, i.e. they reduce infection, and induce defence reactions, like PRE, with a maximum resistance determined 48 h after elicitation. Treatment with RPRE and FABE led to an accumulation of resveratrol, viniferins, and pterostilbene. RPRE induced intense production of phytoalexin in the absence of infection and reinforced the synthesis of defence compounds during a concomitant infection with *P. viticola*. Contrarily, FABE and emodin, an anthraquinone present in RPRE and FABE, induced

[38] Appendix III.III.
[39] Appendix III.IV.

very low concentrations of phytoalexins in the absence of infection, but the synthesis of phytoalexins was greatly stimulated during infection with *P. viticola*, as described for a priming effect, i.e., they stimulate defence mechanisms when an infection occurs (*Ibid.*).

Moushib *et al*. (2013) demonstrated that induced defence responses in potato plants were activated against *Phytophthora infestans*, after sugar beet extract (SBE) treatment. SBE triggered the so called pathogenesis-related (PR)-proteins (PR-1 and PR-2) induction suggesting that the protection conferred by SBE could be via induced resistance.

Several genes potentially involved in *Q. suber* response to infection by *P. cinnamomi* were identified, as their expression increased 24 h post-infection. These genes code for a cinnamyl alcohol dehydrogenase 1 (QsCAD1), a nucleotide binding and leucine-rich repeat domains (NB-LRR) resistance protein (QsRPc), a protein disulphide isomerase (QsPDI) and a cationic peroxidase (QsPox1) (Coelho *et al.*, 2006, 2011). Recently, a set of new genes potentially involved in the defence response of *Q. suber* were found; they encode a cinnamyl alcohol dehydrogenase 2 (QsCAD2), a thaumatin-like protein (QsTLP), a chitinase (QsCHI) and a 1,3-beta glucanase (QsGLU) (Ebadzad, 2014).

This oomycete can cause a slow decline or a sudden death in *Q. suber* plantlets and trees. In tolerant phenotypes, it is proposed that effector molecules released by *P. cinnamomi* during early infection, can be reduced and inactivated by QsCAD1. In the case of the sudden death, it is proposed that trees might contain inactive QsCAD1 proteins (Coelho *et al.*, 2006).

Phlomis purpurea root extract might have induced the expression of these genes, or even other PR genes, and enhanced the partial resistance against the pathogen. It would be interesting to address this issue. Different time courses for elicitation should also be studied to find which phytoalexins are produced.

The protection against oomycetes by plant extracts has been scarcely reported (Jaulneau *et al.*, 2011). However, plants can also be a source of elicitors (Devaiah *et al.*, 2009; Godard *et al.*, 2009; Jaulneau *et al.*, 2011; Moushib *et al.*, 2013). There are some studies showing that plant extracts (Deacon & Mitchell, 1985; Bowers *et al.*, 2004; Boughalleb *et al.*, 2009; Ambang *et al.*, 2010; Khoa *et al.*, 2011) and substances extracted from plants (Deacon & Mitchell, 1985; Curtis *et al.*, 2004; Bajpai *et al.*, 2008) protect plants against *Phytophthora* spp infection.

It was before demonstrated *in vitro,* that PRE is toxic to the pathogen (Neves *et al.*, 2014). The present work confirms that PRE partially protects *Q. suber* and *Q. ilex*, *in vivo* (Experiments 4.1 and 4.2) and possibly induces defence response against *P. cinnamomi* (Experiment 4.3).

The appendices can be found at **https://meocloud.pt/link/75be6d45-169f-4fac-8af6-13cba3729b73/Appendices_Chapter%204.docx/** (see CD in attachment).

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Chapter 5

Is *Phlomis purpurea* a host for *Phytophthora cinnamomi*?

The introduction has been omitted, as a general introduction was presented in Chapter 1.

5.1. Abstract

Phytophthora cinnamomi was not isolated from *Phlomis purpurea* roots in the pathogenicity assays in the greenhouse (Chapter 2) or in the field (Chapter 3). One of the goals of this work was to confirm the resistance of *P. purpurea* to *P. cinnamomi* under controlled conditions by histological techniques. *Quercus suber,* a susceptible host (Moreira-Marcelino, 2001; Caetano, 2007; Horta *et al.*, 2010) was used as a comparison.

Phlomis purpurea sections infested with zoospores and mycelia at 72hpi did not show any infection symptoms and no histological changes were seen either. Scarce *P. cinnamomi* hyphae were seen on the epidermal surface but the oomycete did not enter the roots. *P. purpurea* roots were shown to have a constitutive strengthened epidermis, with lignin and tannins, and an exodermis, composed of cellulose and suberin that can act, *per se*, as a physical barrier for the penetration of *P. cinnamomi*.

Quercus suber (a susceptible species) roots infested with both mycelia and zoospores, started to show infection symptoms at 16-24 hpi and showed extensive necrosis 72 hpi. At 48 hpi tissue damage and cell wall distortion occurred and hyphae proliferated both inter and intra-cellularly through the epidermis, cortical parenchyma and vascular cylinder. However roots elicited for 24 h with PRE, before challenging with the pathogen, showed epidermic and hypodermic irregular cells with walls thickened by deposition of lignin and tannins and, 48 hpi with mycelia and zoospores, hyphae was mainly restricted to the intercellular spaces of the epidermis, with small incursions into the cortex but did not reach the vascular cylinder. Moreover, *Q. suber* roots exposed simultaneously to PRE and zoospores for 48 h showed no macroscopic lesions on the roots and also no penetration of the tissues were visible under the microscope. This was probably due to the direct antagonistic activity of PRE on zoospores as shown *in vitro* (Neves *et al*. 2014).

5.2. Materials and Methods

5.2.1. Growth and maintenance of experimental plants

Phlomis purpurea and *Quercus suber* seeds were disinfected with 10 % sodium hypochlorite for 20 min and rinsed twice with sterile distilled water (SDW). Seeds were placed on a Petri dish involved in wet absorbent paper at 4 °C for 1 week and at then at 22 °C until the roots were *ca* 20 mm long. Every week the seeds were transferred to new wet absorbent paper to minimise contamination.

Experimental seedlings were grown using two different methods.

Method 1: Seedlings were transferred to 250 ml cups filled with washed sterile sand, covered with a clear cup and sealed with Parafilm® perforated with a needle to allow air exchange (Figure 5.1).

Figure 5.1. *Phlomis purpurea* seedlings in washed sterile sand. **(a)** Lateral view. **(b)** Top view. **(c)** Top view plantlets.

Method 2: Seedlings were transferred to tubes of vermiculite 30 mm in diameter (ø) and 250 - 300 mm long in plastic pots (Figure 5.2). Plantlets were kept in a plant growth cabinet (Clima Plus V, 16000 EHVP), with controlled temperature and humidity with a 16 h/8 h photoperiod at 23°C and 20°C, respectively and 80 % humidity. Seedlings were watered 2 to 3 times per week.

Method 3: *Phlomis purpurea* and *Q. suber* seedlings germinated in wet absorbent paper in 90 mm ø Petri dishes at 22 °C in the dark.

Figure 5.2. *Phlomis purpurea* seedlings in vermiculite. **(a)** Top view. **(b)** Close-up view.

5.2.2. *Phytophthora cinnamomi* growth and maintenance and zoospore production

The *Phytophthora cinnamomi* isolates PA37 and PA45, growth and maintenance are described in chapter 2, section 2.2.2.

Zoospores were produced according to the procedure of Byrt & Grant (1979) (see Chapter 4, section 4.2.4.).

With this method, 10^4–10^5 zoospores ml^{-1} were routinely produced. A zoospore suspension (200 µl) was plated onto V8A and incubated at 24.5 °C to check their viability.

5.2.3. Infestation procedures for *Phlomis purpurea* and *Quercus suber*

Seedlings were carefully removed from the vermiculite tubes, by cutting the tubes longitudinally, to avoid root damage (Figure 5.3).

Figure 5.3. *Phlomis purpurea* seedlings. **(a)** Growing inside the tubes with vermiculite. **(b)** Removal of the seedlings avoiding root damage.

The roots of 5 and 20-week-old *P. purpurea* seedlings and of *ca* 2-week-old *Q. suber* seedlings were inoculated with *P. cinnamomi* mycelia and zoospores.

Three different methods were used to inoculate the seedlings:

Method A: for the infestation with mycelia, a 0.5 x 0.5 cm agar plug containing actively growing mycelium was placed in contact with the main root surface at a distance of *ca* 5-10 mm from the root tip (elongation zone). When the objective was to determine the effect of elicitation with the *Phlomis purpurea* root extract (PRE), *Q. suber* roots were pre-treated with 10 $mgml^{-1}$ for 24 h and PRE discarded, prior to infestation with mycelia. The seedlings were laid on a tray and were wrapped in wet absorbent paper to keep a moist environment and incubated at 22 °C in the dark. Four seedlings for each species and condition were used. The experiment was performed once;

Method B: for the infestation with zoospores, the root tissue was infested with *ca* 50 ml of zoospore suspension in a 100 ml glass tube. Whenever the objective was to determine the effect of elicitation by the *Phlomis purpurea* root extract (PRE), *Q. suber* roots were pre-treated with 10 $mgml^{-1}$ PRE for 24 h and the PRE discarded, prior to infestation with

the zoospores. The spore concentration for root infestation was 8.5 x 10^4 zoospores ml^{-1}. Seedlings were incubated at 22 °C in the dark. Four seedlings for each species and condition were used. The experiment was performed once.

For both methods, a time course of 72 h for *P. purpurea* and 48 h for *Q. suber* was conducted to examine the infection process. Control and infested *P. purpurea* roots were cut in *ca* 5 mm long fragments at 0, 12, 24, 48 and 72 hours post infestation (hpi) with *P. cinnamomi* mycelia and zoospores. Similarly, *Q. suber* roots, and *Q. suber* roots pre-treated with PRE, were cut in *ca* 5 mm long fragments 48 hpi with *P. cinnamomi* mycelia and zoospores. Root segments were placed in formaldehyde, glacial acetic acid, ethanol and water (FAA) in 2.0 ml tubes with screw lids. For seedlings infected with mycelia, the segments from each root were numbered sequentially according to their proximity to the point of infestation, with segment 1 being closest to the point of infestation. Segments with the same number were then grouped together in a tube. The roots were studied by light microscopy after fixation with FAA. A preliminary assay was performed with *P. purpurea* seedlings challenged with *P. cinnamomi* zoospores at 0, 24 and 48 h to check whether fixation was successful.

Method C: *P. purpurea* and *Q. suber* seedlings *ca* 2 weeks old and 1 week old, respectively, and germinated in wet absorbent paper were infested with 20 µl zoospore suspension (1.0 x 10^5 zoospores ml^{-1}) on each root in the elongation zone and incubated at 22 °C in the dark in 90 mm ø Petri dishes. A time course of 120 h for *P. purpurea* and *Q. suber* was conducted to examine the infection process. Infested *P. purpurea* roots at 120 hpi were cut in *ca* 5 mm long fragments. *Q. suber* roots at 120 hpi were completely necrosed and crumbled as they were cut, so they were not used. *Phlomis purpurea* control and infested roots were washed in distilled water and fixed with Karnovsky (5 % glutharaldehyde + 5 % paraformaldehyde in phosphate buffered saline (PBS), pH 7.2 for 12 h at 4 °C (Lherminier *et al.*, 2003). The samples were washed 3 times with PBS for 10 min each. Samples were postfixed with 2 % OsO_4 in 0.02M PBS, pH 7.2 for 2 hours in a glass container. Samples were washed 3 times with PBS for 10 min each and kept at 4°C before being embedded in resin. Four seedlings for each species and condition were used. The experiment was performed once.

5.2.4. Histochemical staining of roots

Samples from method A and B (Table 5.1) were washed in 0.01 M PBS, pH 7.4, for 15 min and then dehydrated at room temperature in an increasing series of ethanol concentrations, starting at 70 % and increasing to 96 % and 100 % for no less than 20–30 min for each step.

The roots of 5-week old and 20-week-old seedlings of *P. purpurea* were divided into two groups, young and old, respectively, and a mixture of both were used at each time point for the preparation of paraffin and resin blocks.

Samples from method C were imbedded in resin.

Table 5.1. Sample treatments, time points and histological preparation of roots for methods A and B.

Sample number	Sample[1]	Treatment[2]	Time points (hpi)/ Tubes	Histology (P/R)/ Tubes[3]	Histologic cuts[4] (F/P/R)
0*	*Q. suber* +PRE	Control (MSS)	0; 48	P, R	P, R
1**	*Q. suber* +PRE	Control (PRE)	48	P, R	P, R
2	*Q. suber* +PRE	Zoospores	48	P, R	F, P, R
3	*Q. suber* +PRE	Mycelia	48 /(1, 2, 3, 4)	P /(1, 2, 3, 4) R /(1, 2, 3, 4)	P
4	*Q. suber* +PRE	Control (agar)	48 /(1, 2, 3, 4)	R	
5	*Q. suber*	Mycelia	48 /(1, 2, 3, 4)	R /(1, 2, 3, 4) P	F, P, R
6	*Q. suber*	Control (agar)	48	P, R	P
7*	*Q. suber*	Control (MSS)	48	P, R	R
8**	*Q. suber*	Control (PRE)	48	R	R
9	*Q. suber*	PRE + zoospores	48	P, R	F, P, R
10	*Q. suber*	Zoospores	48	P, R	F, P, R
11	*P. purpurea*	Control (MSS)	0; 6; 24; 48; 72	P(6; 72) R (0; 6; 24; 48; 72)	P R (72)
12	*P. purpurea*	Zoospores	6	P, R	P
13	*P. purpurea*	Zoospores	24	P, R	P
14	*P. purpurea*	Zoospores	48	R	R
15	*P. purpurea*	Zoospores	72	P, R	F, P, R
16	*P. purpurea*	Control (agar)	0; 6; 24; 48; 72	P(6 hpi), R	P
17	*P. purpurea*	Mycelia	6 /(1, 2, 3, 4)	P, R	P
18	*P. purpurea*	Mycelia	24 /(1, 2, 3, 4)	P, R	P, R
19	*P. purpurea*	Mycelia	48 /(1, 2, 3, 4)	R	R
20	*P. purpurea*	Mycelia	72 /(1, 2, 3, 4)	P, R	F, P, R

[1]PRE=Phlomis root extract; [2]MSS=Mineral salt solution; [3]P=Paraffin, R=Resin; [4]F=Freezing, P=Paraffin, R=Resin, *Treatment of sample 0 is the same as treatment of sample 7 and only one was analysed, ** Treatment of sample 1 is the same as treatment of sample 8 and only one was analysed.

5.2.4.1 Preparation of roots in paraffin

Four roots of each sample were treated twice with isoamyl acetate for 30 min, with xylene saturated with paraffin at 60 °C for 5 min and with pure paraffin for 20 min before being embedded, vertically and horizontally, in liquid paraffin (60 °C) in a block. Histological cuts in paraffin (12-14 microns) were performed using a microtome apparatus (Microm–HM

340 E). A series of 6-8 sections were placed on glass slides previously covered with Mayer's albumin to allow adhesion of the cuts to the slides. A total of 10 slides per sample were made.

Direct Red staining

This dye is commonly used to stain collagen in animal tissues. It is very hydrophilic and stains also reticulin fibers and mucins. In plants, it is not usually employed but nevertheless, we decided to try it. In some samples it gave good staining, especially with epifluorescence, as it specifically binds to walls with deposits of suberin and/or lignin.

It is known that Direct Red stains the cell walls of fungi and *Phytophthora spp* (F. García, personal communication). Samples 5, 10, 15 and 20 were selected for direct red staining.

Safranin and fast green staining

Safranin, also called basic red 2, is a "basic dye" meaning that is will stain acidic features of tissue (nuclei and lignified secondary cell walls that surround mature xylem cells in vascular bundles) brilliant red.

Fast green or fast green FCF is an acidic dye that stains the basic components of tissue (such as the cytoplasm and cellulosic primary cell walls) in brilliant green colour.

Before staining, deparaffination was conducted, and the slides dried on a heating plate set to 58 °C for 10-15 min to remove any water from the sections, soften the paraffin, attach the tissue to the slide and aid in the spreading out of sections to remove folds. The sections were deparaffinised using the following series: xylene, 5 min, to another container with xylene 5 min, 100 % ethanol 2 min, 95 % ethanol 1 min, 90 % ethanol 1 min and 70 % ethanol 1 min.

Deparaffination was conducted on the following samples: 0, 1, 2, 3 (1, 3, 4), 4, 5 (1, 2, 3, 4), 7, 8, 9, 10, 11 (0, 6, 72), 12, 13, 14, 15, 16, 17, 19 and 20.

The following samples were selected for safranin and fast green staining: 2, 3, 5, 9, 10, 15 and 20.

The staining with safranin was performed for *ca* 5 min. To wash out the excess stain, running water was gently poured on the slides for 5 min. For differentiation, the slide was covered with 1 % acid-alcohol (1 % concentrated HCl in 70 % etanol) for 10 s and finally washing with running water.

The fast green staining was conducted for 10 s and a clearing solution was added for 10 s to enhance transparency. The slide was then covered with xylene + 4 drops 100 % ethanol for 10 s to remove residual water and finally xylene was added for 1 min to dehydrate.

5.2.4.2. Preparation of roots in resin

Three roots of each sample were infiltrated in a mixture of propylene oxide: embedding agent[40] (Spurr)(2:1, v/v), vortexed, and allowed to stand for 2-3 h. The mixture was replaced with a 1:1 propylene oxide: embedding agent overnight and then replaced with a 1:3 propylene oxide: embedding agent for 2-3 h. The mixture was drained and fresh embedding agent (100 % Spurr) added and allowed to stand overnight. The samples with pure resin were placed in propylene flat embedding molds (EMS 70905) and oriented appropriately to cut transversely or longitudinally. The molds were inserted into box cocoon BE8 (EMS 64300) and were closed to prevent access of oxygen to the samples. Curing took 16-24 h at 60 °C. Histological sections of 2 microns were made using an ultramicrotome (Ultratome Nova LKB Bromma) with a diamond knife (DIATOME Histo 45°).

Toluidine blue staining for lignin and tannin detection

A series of 16-20 sections were placed on glass slides, dried at 65 °C and dyed 15-20 s with toluidine blue (TB). The samples were washed with water and dried again. TB is a metachromatic stain which serves as a histochemical stain. It reacts with different cell wall components to produce a variety of colours which can be used to identify a variety of cell and tissue types. The colours generated can provide information on the nature of the cell and its walls. TB is a cationic dye that binds to negatively charged groups. An aqueous solution of this dye is blue, but different colours are generated when the dye binds with different anionic groups in the cell. For example, a pinkish purple colour will appear when the dye reacts with carboxylated polysaccharides such as pectic acid; green, greenish blue or bright blue with polyphenolic substances such as lignin and tannins; and purplish or greenish blue with nucleic acids (Yeung, 1998).

5.2.4.3. Preparation of roots by freezing

A few samples [5 (3, 4), 10, 15 and 20] were frozen and 30-35 microns transverse sections made with a microtome (LEICA CM1325). The tissues were placed on a glass slide and the excess water removed.

[40]Embedding agent (such as resin or paraffin wax) which is then hardened. This is achieved by cooling in the case of paraffin wax and heating (curing) in the case of the epoxy resins. In this case is Spurr was used.

Detection of autofluorescence

Autofluorescence (AF) was visualised under blue-violet and ultraviolet lights. One of the primary contributors of plant autofluorescence is chlorophyll, but lignins, carotenes, and xanthophylls also produce a significant level of fluorescence emission (Olympus corporation, 2009). It is also produced in response to attempted hyphal penetration (F. García, personal communication).

Aniline blue for callose detection

A series of 16-20 transverse sections were laid down in glass slides and stained with aniline blue according to the method of Jensen (1962) and Eschrich and Currier (1964) to detect callose. Briefly, transverse sections were cleared of soluble pigments in 90 % ethanol in water for 24h. Sections were then transferred into a solution of 0.5 % (w/v) aniline blue buffered in 0.15M di-potassium-hydrogen orthophosphate (K_2HPO_4) pH 9.0 and left during 30 min at room temperature. Transverse sections were rinsed twice and covered with Fluormount (an antifade solution) before being mounted onto glass slides. Callose depositions are seen as bright light blue fluorescence under ultraviolet epi-fluorescence lights (365 nm; Olympus cube U-MWU2). Callose depositions are seen as bright light blue fluorescence under ultraviolet epi-fluorescence lights.

Calcofluor white M2R staining

Calcofluor-white (CFW) is a special fluorescent stain that binds to beta 1-3 and beta 1-4 polysaccharides, such as those found in cellulose and chitin, and when excited with ultraviolet radiation, will fluoresce with an intense bluish/white colour.

In plants, it is used for staining cell walls. Samples were stained with the fluorochrome dye CFW at 0.1 % for 15-20 s, the excess was removed with absorbent paper and a drop of 10 % KOH added for 10 s. Again, the excess was removed with absorbent paper and a drop of citifluor antifade added. The tissues were observed under UV light by epifluorescence.

To observe the preparations, an Olympus Provis AX-70 light field with an epifluorescence system Olympus U-ULS 100 HG was used. For epifluorescence observations, a cube U-MWBV (excitation filter: 400-440 nm, barrier filter: 475 nm) and a cube U-MWU (420 nm barrier filter 330-385 nm excitation filter) were used. The pictures were taken with an Infinity 2CCD digital camera (Lumenera Corp., Ottawa, Canada) and treated with image acquisition software Infinity Analyze Camera Software v6.4.1 (Lumenera Corp.).

Root samples (5, 10, 15 and 20) were also observed in the low temperature scanning electron microscope (LTSEM). The samples were washed with abundant distilled water and frozen with liquid nitrogen. Then, samples were inserted into a cryo-observation system, where ice sublimated in the surface for 15 min and were coated with gold for 30 s. To observe the samples, microscope JEOL JSM 5410 model LTSEM Service UPV microscopy was used and images were captured with a digital camera Altra 20 and treated with imaging software GetIt analysis1.

5.3. Results and discussion

5.3.1. Growth and maintenance of experimental seedlings

Phlomis purpurea and *Quercus suber* seedlings were prepared to inoculate the roots, do the histochemical staining and observe the tissues.

Method 1, using washed sterile sand was developed to grow the seedlings in aseptic conditions, but most seedlings failed to grow, were difficult to take out from the containers without breaking the fine roots and were contaminated with fungi. Aseptic plant material is the ideal for research proposes. Nevertheless, when it is not possible, alternatives must be implemented. An alternative procedure (Method 2) was implemented with a non-sterile, inert substrate (vermiculite) with which it was easy to remove the roots and also the plantlets by cutting the plastic tube. Vermiculite is a traditional substrate that generally replaces soil to improve access to roots for research purposes. An alternative method could have been a soil-free plant growth system (SPS) (Gunning & Cahill, 2009) to ease the access to the root system with no interference from soil adhering to the roots allowing tissues to be collected undamaged. However this hydroponically-like growth can influence the root suberization. Portions of hydroponically growing maize roots elevated above the solution into an enclosed humid chamber, like SPS, suberized both endodermis and exodermis while the submerged portions of the root system and that of the controls remained largely unsuberized (Enstone & Peterson, 1998).

Another method (3) consisted in germinating and growing *P. purpurea* and *Q. suber* in a soil free media, i.e. in wet absorbent paper in 90 mm ø Petri dishes. The main radicle of *P. purpurea* plants generally stopped growing and secondary roots grew immediately behind.

5.3.2. Effect of *Phytophthora* cinnamomi infestation on *Phlomis purpurea* and *Quercus suber*

To determine whether infestation procedures influenced the interaction of *P. purpurea* and *Q. suber* with *P. cinnamomi*, three root infestation methods were examined: (1) root infestation with mycelia in agar (2) root dipping in a zoospore suspension and (3) direct infestation of roots growing on filter paper, with zoospores. All three infestation methods produced the same results with no macroscopic lesions or symptoms displayed in root tissue for *P. purpurea* and the presence of necrotized tissue for *Q. suber* (except for most of PRE elicited seedlings, described hereinafter). At 72 hpi, control and infested *P. purpurea* did not show any necrosis on the fine roots (Figure 5.4). A similar study was conducted on the model plant *Arabidopsis thaliana* Col-0, a resistant plant to *P. cinnamomi* infection (Rookes *et al.*, 2008). This oomycete did not produced macroscopic lesions or symptoms in the roots and leaves after 21 days post infestation (*Ibid.*). In a study by Cahill *et al*. (1993) all resistant plants displayed macroscopic, small (up to 5 mm) lesions after infestation with *P. cinnamomi* zoospores. For instance *Zea mays* (maize) displayed yellow lesions at 24 hpi and *Triticum aestivum* (wheat) orange-brown lesions at 8 hpi (*Ibid.*). Moreover, *Zea mays* at 120 -168 hpi displayed lesions reaching a maximum of 1.3 cm (Allardyce, 2011). In our work no macroscopic lesions were seen on *P. purpurea* at 120 hpi (Figure 5.5). At the macroscopic level *P. purpurea* reacted as *A. thaliana* Col-0 roots. This plant infested with zoospores did not show HR-like cell death, as contact with a germ tube or appressorium-like structure alone did not induce cell death (Rookes *et al.*, 2008).

Using Method 2, one could argue saying that zoospores are negatively geotropic (Erwin and Ribeiro, 1996); thus, the highest densities of zoospores should be at the surface and therefore would not be able to infect *P. purpurea* roots at the elongation zone. However this assumption collapses when the roots of *Q. suber*, using the same method are infected showing macroscopic necrosis.

In *Z. mays*, at 72 hpi, multiple lateral roots were formed around the lesion (Allardyce 2011). This ability is probably a response to increase the uptake of water and nutrients to face the loss of necrotized tissue. Contrariwise, there was no evidence of lateral root formation at any time point in *P. purpurea* control and infested roots (Figure 5.5).

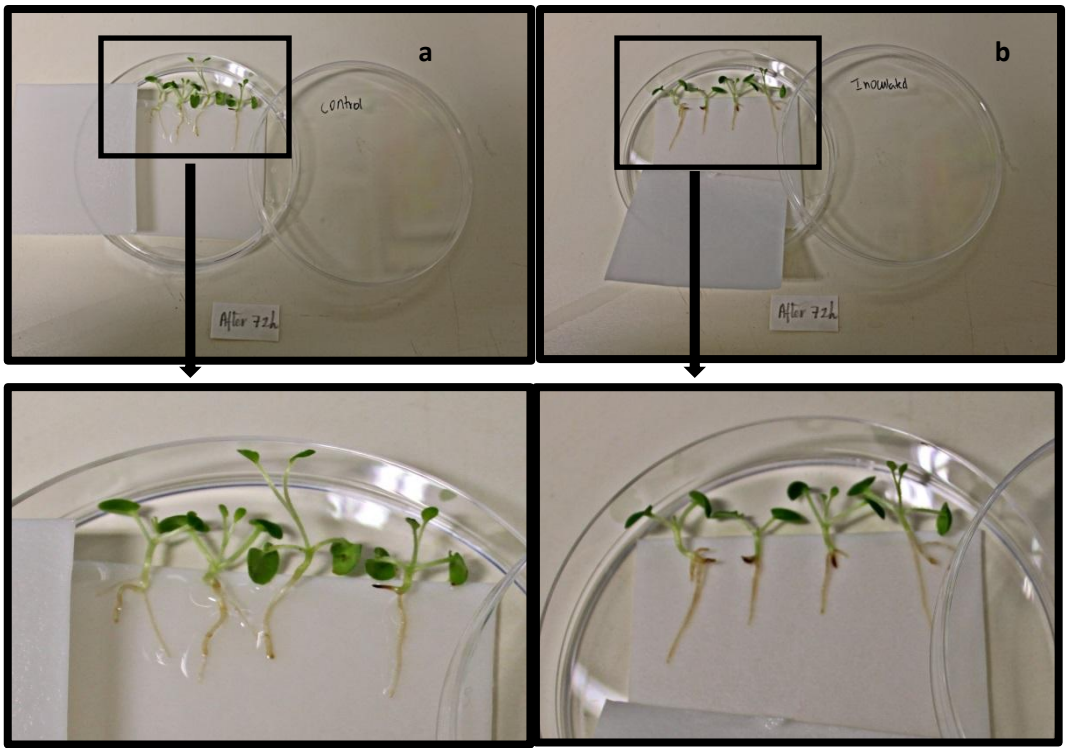

Figure 5.4. *Phlomis purpurea* seedlings **(a)** control **(b)** infested with *Phytophthora cinnamomi* zoospores exhibit no disease symptoms at 72 hpi, not even restricted lesions at the points of infestation.

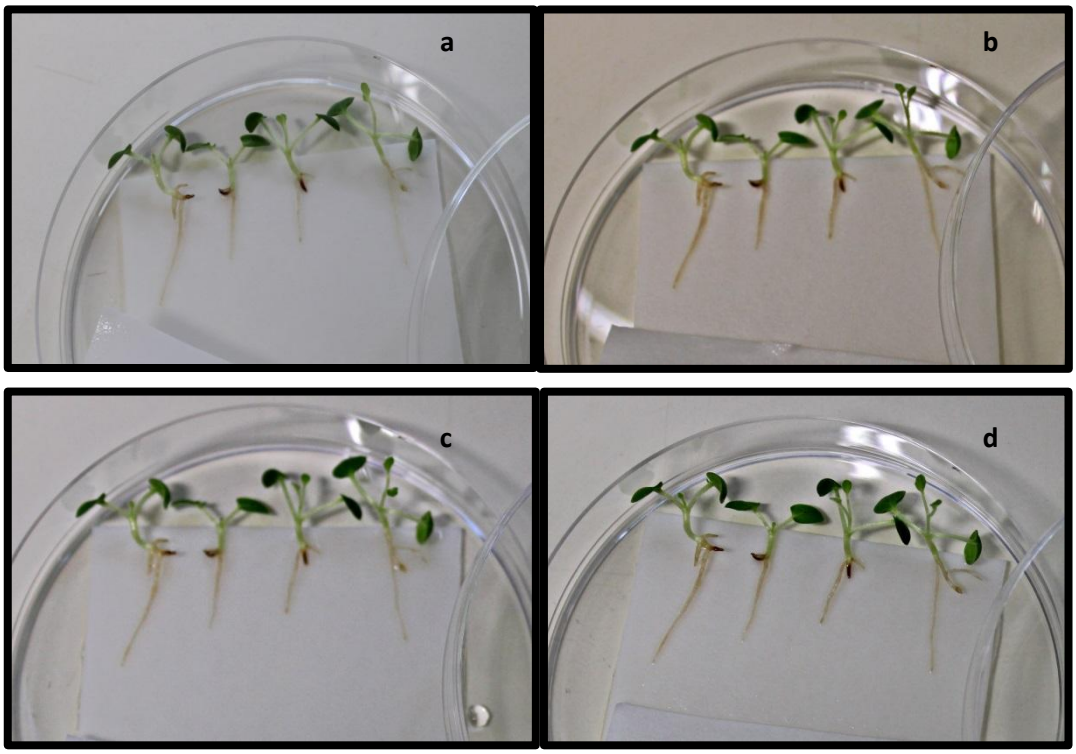

Figure 5.5. *Phlomis purpurea* seedlings **(a)** 66 hpi **(b)** 72 hpi **(c)** 96 hpi **(d)** 120 hpi infested with *Phytophthora cinnamomi* zoospores, without lateral root formation around the infestation point.

In contrast, *Q. suber* (a susceptible species) started to show symptoms at 16 hpi and at 72 hpi, showed extensive root necrosis (Figure 5.6b) contrasting with the whitish colour and turgid appearance of non-infested controls (Figure 5.6a). Similar studies have shown that *Q. suber* infested roots appeared necrotic at the infestation point (Maia *et al.*, 2008; Horta *et al.*, 2010; Medeira *et al.*, 2012).

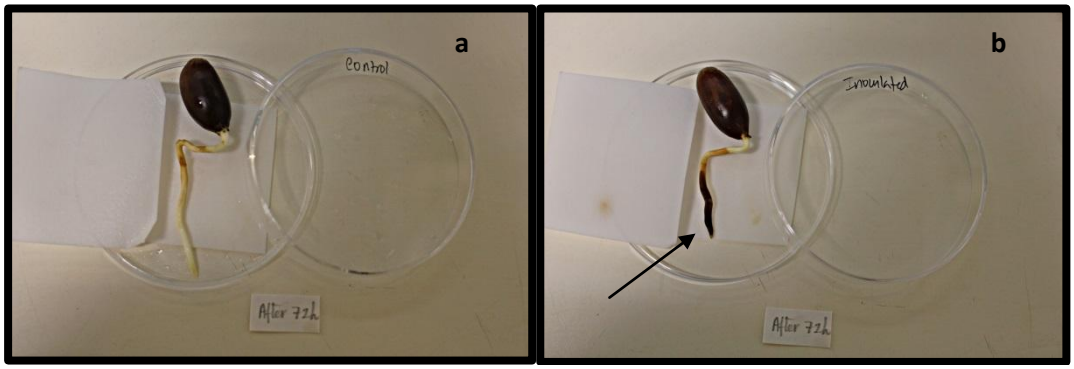

Figure 5.6. *Quercus suber* seedlings **(a)** control; **(b)** at 72 hpi with *Phytophthora cinnamomi* zoospores exhibit a spreading dark root lesion (white arrow headed).

When *Q. suber* roots were elicited with PRE for 24 h prior to infestation with *P. cinnamomi* zoospores, they showed only a little macroscopic necrosis on one root when compared with non- elicited roots (see chapter 4, Figure 4.6), suggesting PRE elicits a defence response. It was also shown in chapter 4, that PRE inhibited *P. cinnamomi* infection of *Q. suber* radicles, possibly by having a direct inhibitory effect on zoospores (see Figure 4.4), as PRE was shown to inhibit zoospore germination *in vitro* (Neves *et al.*, 2014).

5.3.3. Histochemical analysis of roots

Roots were prepared in paraffin, resin and were also frozen.

Histological sections of roots in paraffin were stained with direct red, as this staining specifically binds to walls with deposits of suberin and/or lignin. Safranin and fast green stains were also used to stain acidic features of tissue (nuclei and lignified secondary cell walls that surround mature xylem cells in vascular bundles) brilliant red and the basic components of tissue (such as the cytoplasm and cellulosic primary cell walls) a brilliant green colour, respectively.

Histological sections of roots in resin were stained with toluidine blue (TB), which is used as a metachromatic dye to differentiate various modifications in the cell walls or the presence of other compounds although its success depends heavily on the pH at which the

staining is performed. TB staining produces a bright blue when it binds to polyphenolic substances such as lignin and tannins. Cells which are lignified should present a thicker and lighter bright blue colour than those with only primary walls that should present a darker blue colour in the walls. With proper pH, suberin should appear blue-red but if not simply appear bluer.

Histological sections of frozen roots were stained with aniline blue for the detection of callose depositions as they are seen as bright light blue fluorescence under ultraviolet epi-fluorescence lights and with calcofluor-white (CFW) that binds to beta 1-3 and beta 1-4 polysaccharides, such as those found in cellulose and chitin, and when excited with ultraviolet radiation, will fluoresce with an intense bluish/white colour.

5.3.3.1. Histochemical analysis of *Phlomis purpurea* infested with *Phytophthora cinnamomi*

Lignin, tannin, suberin and cutin production

Lignin, tannin, suberin or cutin were detected constitutively in the epidermis and vascular tissue of *P. purpurea* (Figure 5.7 a) and within *P. cinnamomi* infested root tissue at all time points (Figure 5.7 b and c). This is well visible through the bright blue colour produced by toluidine blue, showing a thickened epidermis in control and infested roots (Figure 5.7).

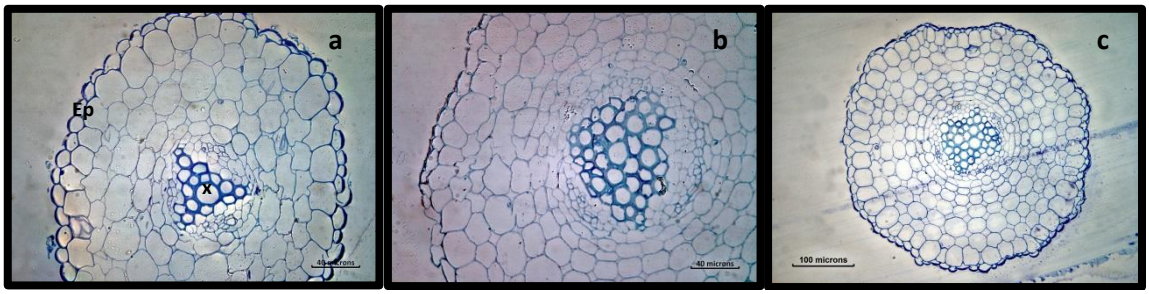

Figure 5.7. Light microscopy observation of transverse sections of *Phlomis purpurea* roots. Lignin and tannin presence within epidermis (Ep) and vascular tissue (x) of *P. purpurea* roots (bright blue colour produced by toluidine blue staining) in: **(a)** control root tissue, scale bar = 40 µm; **(b)** following inoculation with *P. cinnamomi* zoospores at 6 hpi, scale bar = 40 µm; **(c)** following inoculation with *P. cinnamomi* zoospores at 24 hpi, scale bar = 100 µm.

In the non-host *Z. mays* on the contrary, no lignin could be detected within control root tissue (Allardyce, 2011), suggesting *Z. mays* does not produce lignin constitutively and hence penetration by *P. cinnamomi* can occur whereas in *P. purpurea*, probably due to the character of the lignified epidermis, *P. cinnamomi* is not at all able to penetrate it. A clonal line of *Eucalyptus marginata* derived from resistant seedlings obtained through selection within a

susceptible family (RS) had the highest constitutive levels of lignin which appears to have contributed to the resistance observed in that line (Cahill *et al.*, 1993). Moreover, these authors state a relationship between resistance to *P. cinnamomi* in selected clonal lines of *E. marginata* and the constitutive and induced levels of phenylalanine-ammonia-lyase (PAL), lignin and phenolic compounds. On another hand, a decrease in the concentration of either lignin or phenolic compounds after inoculation and/or lower constitutive levels of those compounds in seedlings was correlated with susceptibility (*ibid*.). PAL is one of the enzymes involved in the phenylpropanoid pathway responsible for the synthesis of lignin or phenolic compounds.

Direct red was used for staining cell walls of *P. cinnamomi* but no hyphae were found inside the root tissues of *P. purpurea*. Instead it revealed that *P. purpurea* roots have a complete constitutive exodermis (monolayer with Casparian bands) composed of cellulose and suberin (Figure 5.8). Suberin lamellae are deposited onto the inner surface of exodermal and endodermal cell walls. The suberin lamellae, wich is a complex biopolyester comprised of both poly(phenolic) and poly(aliphatic) domains (Bernards, 2002), protect against pathogen invasion and possibly root drying during times of stress (Enstone *et al.*, 2003; Pollard *et al.*, 2008). In *Z. mays*, *Fusarium culmorum* penetrated the epidermis at many sites but did not breach the exodermis in which all cells possessed both Casparian bands and suberin lamellae, providing effective barriers to the growth of *F. culmorum* (Kamula *et al.*, 1994).

Preformed root suberin was found to be implicated in partial resistance of soybean to *P. sojae* (Thomas *et al.*, 2007; Ranathunge *et al.*, 2008). Also, rapid production and deposition of suberin in the exodermal layer might be implicated in resistance of *Rubus idaeus* to *P. rubi* (Valenzuela-Estrada *et al.*, 2012). During periods of drought the epidermis often dies and the exodermis becomes the outermost protective layer of the root (Enstone *et al.*, 2003). The production of highly lignified and suberized periderm is assumed to be a generalised host response to wounding and fungal invasion by enhancing defence against compressive forces, and preventing the diffusion of toxins and enzymes from fungi into host cells and the translocation of water and nutrients from host cells to fungi, as suggested in other host-pathogen interactions (Vance *et al.*, 1980; Hawkins & Boudet, 2003). Within cell walls, lignin is deposited to form an interpenetrating network with hemicelluloses. This matrix surrounds the cellulose fibrils and forms a physically impenetrable barrier to polysaccharidases, produced during microbial attack, that cannot reach cellulose and hemicelluloses in fully lignified tissues (Vance *et al.*, 1980).

The presence of an exodermis can be a very relevant factor in the resistance of *P. purpurea* to *P. cinnamomi* infection. In fact plants with multiple exodermis like *Phragmites australis* and *Typha latifolia* were shown not to be infected by *Phytophthora gallica* (Jung & Nechwatal, 2008) and *P. cinnamomi, P. citrophthora, P. cryptogea, P. nicotianae, P. palmivora* (Ridge, 2013), respectively.

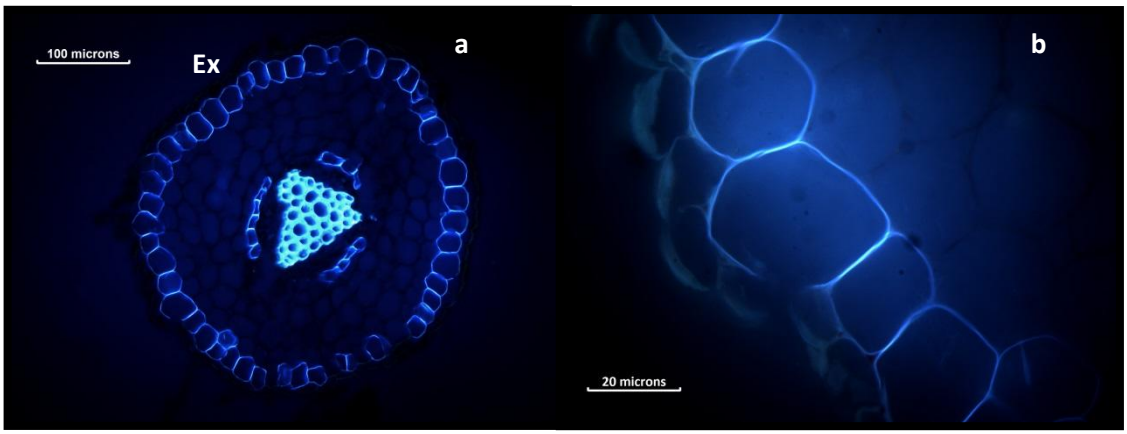

Figure 5.8. Light microscopy cross-sections of *Phlomis purpurea* control root after 72h in mineral salt solution. **(a)** Transverse section in paraffin stained with direct red showing a constitutive "reinforcement" of the exodermis (Ex), scale bar = 100 µm; **(b)** Close up view , scale bar = 20 µm.

As no signs of *P. cinnamomi* were seen in the *P. purpurea* roots stained paraffin and resin sections, it was decided to make thicker sections, possible by freezing the root samples.

Calcofluor white (CFW) staining of infested samples with zoospores at 72h revealed *P. cinnamomi* hyphae around but not entering the epidermis (Figure 5.9 A and B).

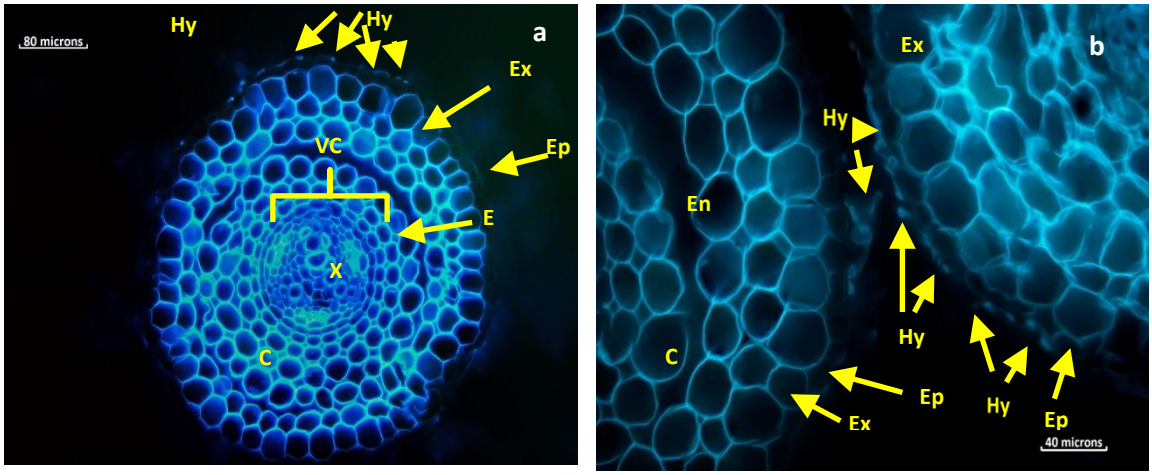

Figure 5.9. Transverse sections obtained by freezing and stained with calcofluor. Epifluorescence under UV light. Section of *Phlomis purpurea* roots inoculated with *P. cinnamomi* zoospores at 72 hpi, scale bar = 80 µm **(a)** and scale bar = 40 µm **(b).** Vascular cylinder (Vc), X=xylem; En=endodermis; C=cortex; Ex=exodermis; Ep=epidermis; Hy=hyphae were observed on the surface of the root (arrows)

Phlomis purpurea sections infested with zoospores at 72hpi, stained with aniline blue, showed deposition of callose tissue (Figure 5.10 – arrow headed) and *P. cinnamomi* hyphae close to the epidermis (Hy). In one, and just one out of hundreds of sections (<1 %), hyphae were seen entering the epidermis (Hy*) but never progressing further (Figure 5.10).

In fact the epidermis, in the field, often dies and is degraded by microorganisms and under lab conditions this layer can also die, then the exodermis becomes the outermost part of the root (Enstone *et al.*, 2003). Without the protective epidermis the exodermis become important in preventing pathogen entry into the root, and the phenolic compounds of suberin are thought to be a key aspect in this regard (Bernards, 2002).

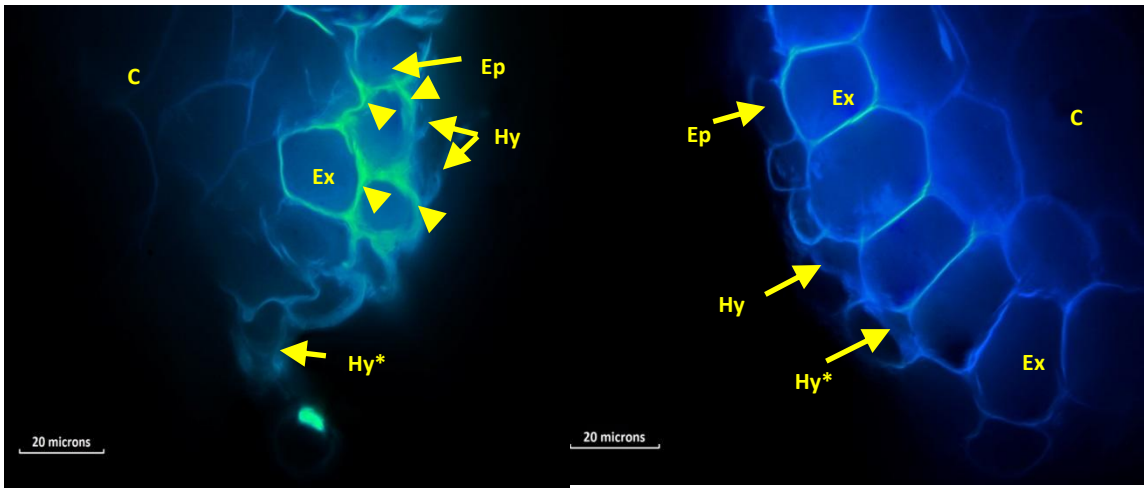

Figure 5.10. Transverse sections obtained by freezing and stained with aniline blue. Epifluorescence under UV light. Sections of *Phlomis purpurea* roots inoculated with *Phytophthora cinnamomi* zoospores at 72 hpi, scale bar = 20 µm. C=cortex; Ex=exodermis; Ep=epidermis; Hy=hyphae on the surface of the root; Hy*=hyphae inside the epidermis. Callose deposits (arrows).

The observations of infested *Phlomis purpurea* by low temperature scanning electron microscope (LTSEM) could show crushed *P. cinnamomi* hyphae on the root epidermis, but the entry of this pathogen was not observed (Figure 5.11).

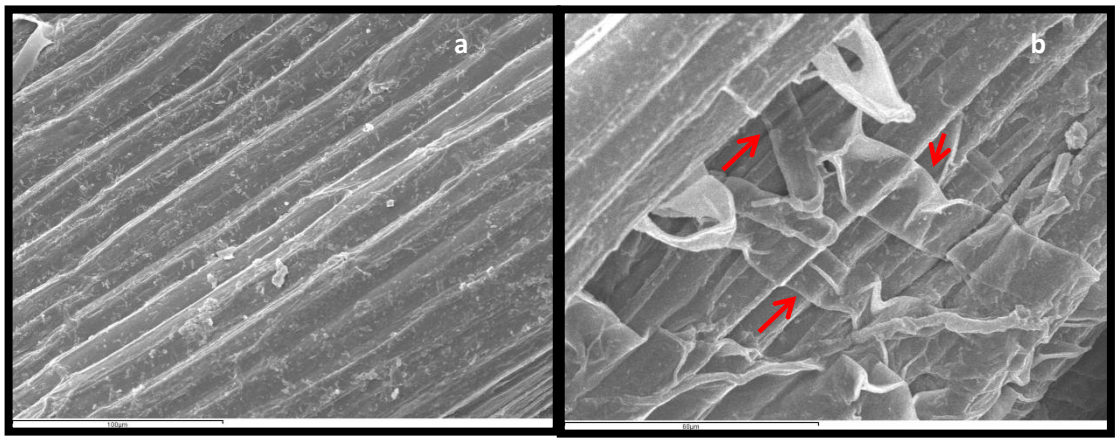

Figure 5.11. Low temperature scanning electron microscope of *Phlomis purpurea* **(a)** Control root after 72h, scale bar = 100 µm; **(b)** At 72hpi showing crushed hyphae, scale bar = 60 µm.

Given this, the constitutive presence in *P. purpurea* roots of a strengthened epidermis, with lignin and tannins, and an exodermis, composed of cellulose and suberin can act, *per se*, as a physical barrier for the penetration of *P. cinnamomi*.

5.3.3.2. Histochemical analysis of *Quercus suber* elicited with *Phlomis purpurea* root extract (PRE)

To determine the effect of elicitation with PRE, *Q. suber* roots were pre-treated with 10 $mgml^{-1}$ PRE for 24 h and PRE was discarded, prior to inoculation with the zoospores. *Q. suber* roots elicited with PRE show epidermic and hypodermic irregular cells with walls thickened by deposition of lignin, tannin and suberin or cutin. PRE seems to elicit a defence response by inducing an increase of lignin and tannin deposition (Figure 5.12b). After inoculation the shape of the cells is much more irregular and the depositions of lignin and suberin, stained by toluidine blue increase, especially in tangential internal cell walls (Figure 5.12c). These occurrences probably prevented the incursion of *P. cinnamomi*, as there was only a restricted lesion on the roots (Chapter 4, Figure 4.6) and no evidence of the pathogen inside the host tissues (Figure 5.12).

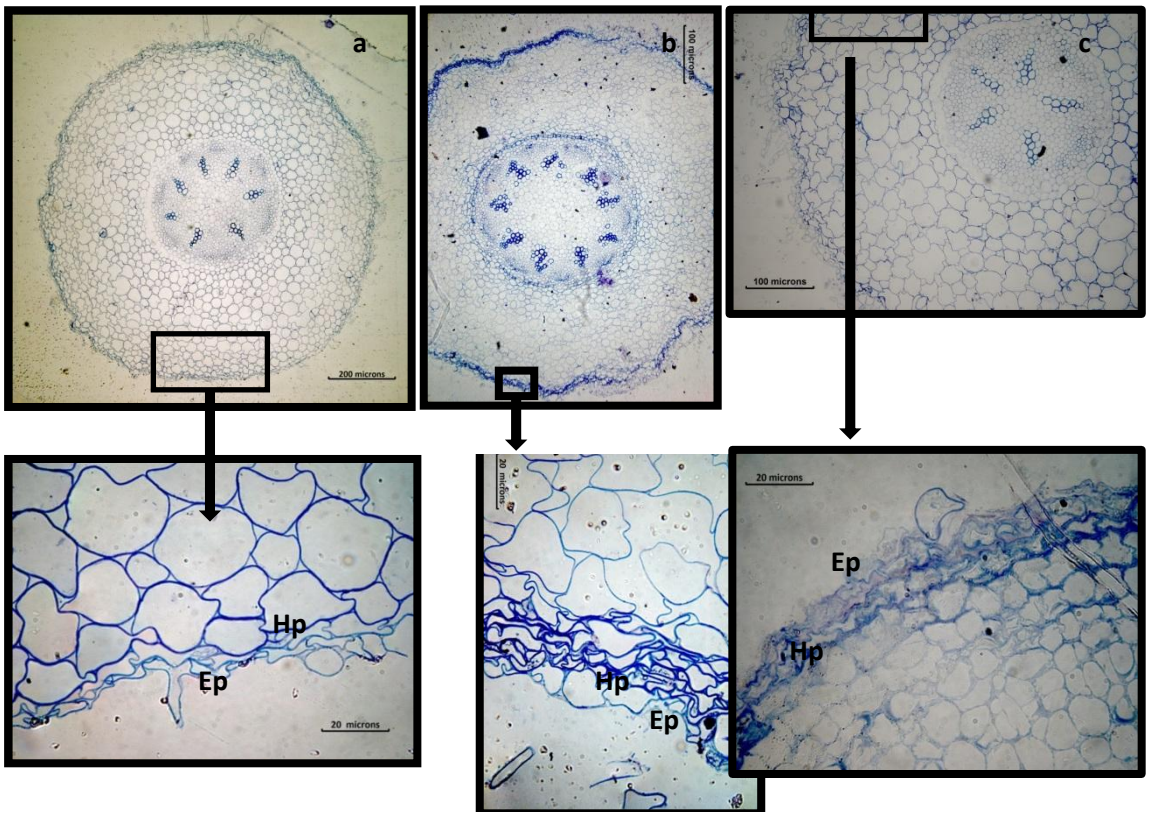

Figure 5.12. Light microscopy transverse sections of *Quercus suber* roots elicited with *Phlomis purpurea* root extract (PRE). Presence of lignin and tannin in *Q. suber* roots within epidermis and vascular tissue (bright blue colour produced by toluidine blue staining) in: **(a)** control root tissue, at time 0 h (sample 0), scale bar = 200 µm; close-up view showing no constitutive accumulation of lignin and tannin in the epidermis (Ep), scale bar = 20 µm; **(b)** *Q. suber* root tissue after elicitation with PRE (sample 1), showing an accumulation of lignin and tannin in the epidermis and suberin in the hypodermis (Hp), scale bar = 100 µm; close-up view, scale bar = 20 µm; **(c)** *Q. suber* root tissue following infestation with *P. cinnamomi* zoospores at 48 hpi (sample 2), showing a reinforcement of the epidermis (Ep) and hypodermis (Hp), scale bar = 100 µm; close-up view showing accumulation lignin and tannin in the epidermis and suberin in the hypodermis, non-invaded cortex and vascular cylinder, scale bar = 20 µm.

Autofluorescence (AF) was observed under ultraviolet light, as bright blue colour, at 48 hpi with mycelia, within the epidermal tissue but more intense in the vascular cylinder (VC). There is minimal AF in the epidermis, probably associated with attempted hyphal penetration (Figure 5.13). There is no exodermis present in *Q. suber* roots, which is in agreement with other observations (Horta *et al.*, 2010; Medeira *et al.*, 2012). Discrete areas of AF was also visualized 24 hpi in *Zea mays*, and intense AF, directly associated with attempted hyphal penetration was visible throughout the endodermis and stelle at 72 hpi (Allardyce, 2011).

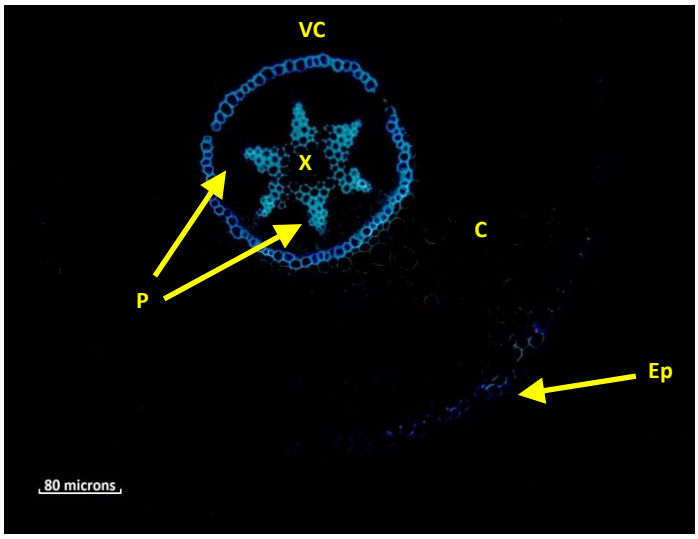

Figure 5.13. Autofluorescence visualised under ultraviolet light in *Quercus suber* roots elicited with *Phlomis purpurea* root extract following 48 h inoculation with *Phytophthora cinnamomi* mycelia. Transverse section, unstained, showing minimal epidermal but intense vascular cylinder (VC) autofluorescence. Frozen cut: X=xylem; P=phloem; C=cortex; Ep=epidermis; scale bar = 80 µm.

To have more chances to visualize *P. cinnamomi*, roots were frozen and thicker sections, than resin and paraffin, were prepared. Callose production in root tissue was detected at 48 hpi with *P. cinnamomi* mycelia, as bright light-blue colour produced by aniline blue staining. Intense callose deposition in the phloem (P) and minimal depositions in the epidermis (Ep) were observed (Figures 5. 15 and 5.16, yellow arrow-headed). However, in root tissues at 48 hpi with *P. cinnamomi* zoospores, some deposition of callose was seen in the epidermis but only a few in the phloem.

Callose depositions in the phloem are a natural process especially during winter when plants stop their photosynthetic activity. It also occurs during infectious processes that reach the phloem. If the hyphae had managed to reach the vascular cylinder, then the amount of phloem callose deposited in the sieve plates would be very high, but this was not the case since no hyphae were detected close to the vascular cylinder (F. Garcia, personal communication).

One of the effects of the PRE could also be increasing callose deposits in the phloem to prevent hyphae reaching this structure and use it for infective process.

Callose deposition in the cell walls is a defense mechanism of cells against stress of any kind. The presence of callose in the outer walls of the epidermis, in this case, is related to the infective process. The minimal depositions in the epidermis (Ep) in roots infested with *P. cinnamomi* mycelia (Figures 5.14 and 5.15) compared with the more intense callose deposition in roots infested with *P. cinnamomi* zoospores is due to the fact that zoospores cause more damage than mycelia. This is comprehensible as zoospores are known as the main infective structures.

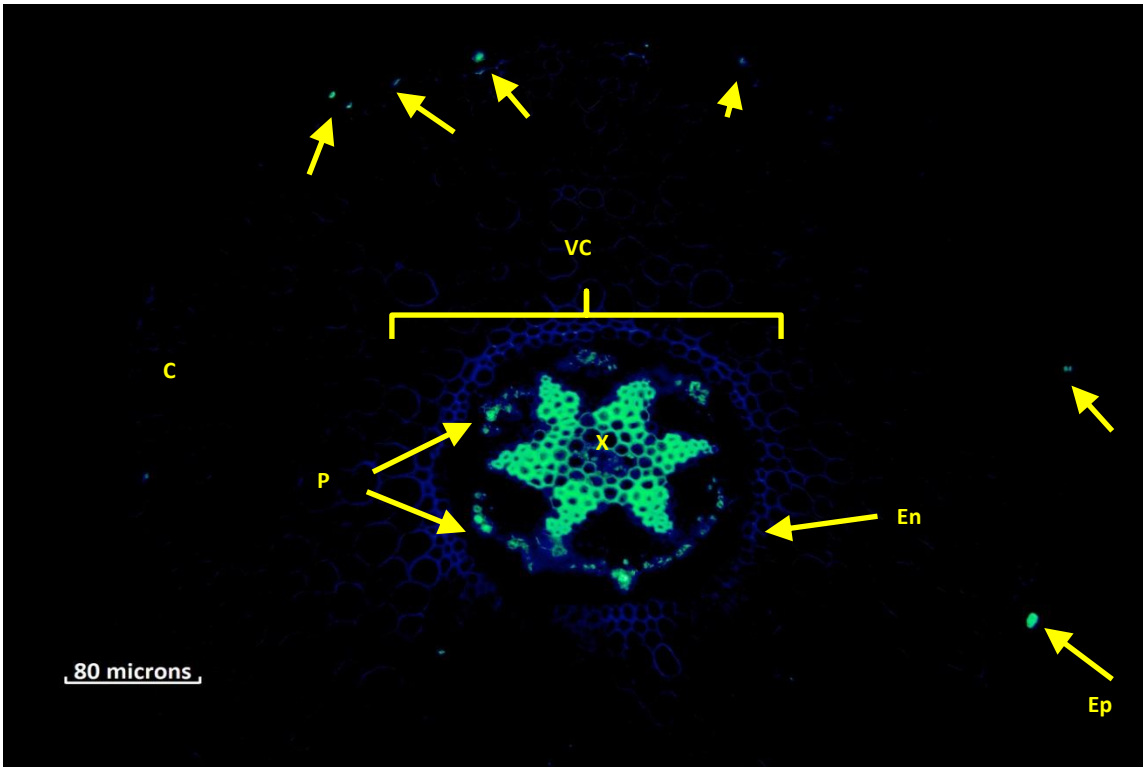

Figure 5.14. Light microscopy transverse sections under ultraviolet light (bright light-blue colour produced by aniline blue staining), in *Quercus suber* roots elicited with *Phlomis purpurea* root extract following 48 h inoculation with *P. cinnamomi* mycelia showing intense callose deposition in the phloem (P) (yellow arrowheads) and minimal depositions in the epidermis (Ep) (yellow arrowheads). Frozen cut: VC=vascular cylinder, X=xylem; En=endodermis; C=cortex; scale bar = 80 µm.

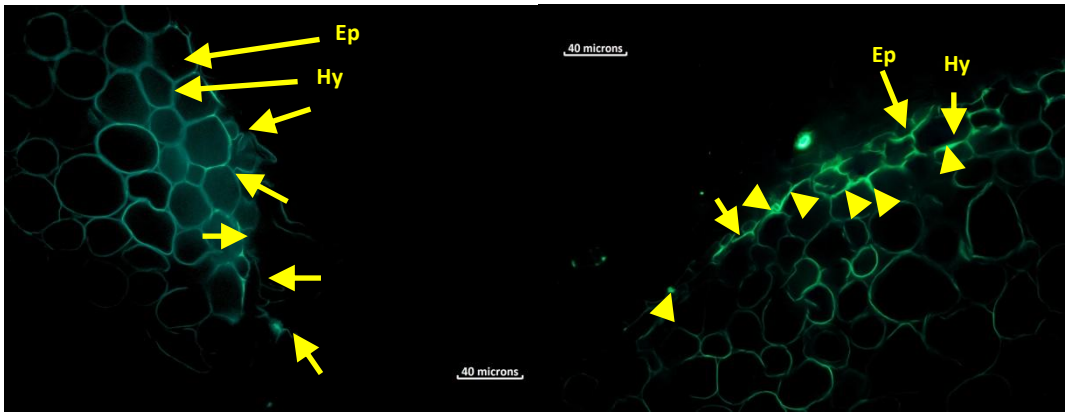

Figure 5.15. Light microscopy transverse sections under ultraviolet light (bright light-blue colour produced by aniline blue staining), in *Quercus suber* roots elicited with *Phlomis purpurea* root extract following 48 h infestation with *P. cinnamomi* mycelia, with intense callose deposition (yellow arrowheads). Frozen cut. En=endodermis; Hy=hypodermis; scale bar = 40 µm.

Phytophthora cinnamomi, in PRE elicited samples, was mainly restricted to the intercellular spaces of the epidermis, with small incursions into the cortex but did not reach the vascular cylinder. Somehow, PRE was able to induce a response by *Q. suber* roots, with a similar outcome to the eliciated roots with cryptogein and capsicein in which the pathogen penetrated and colonized the cortex within 48 hpi but did not colonized the vascular cylinder (Medeira *et al.*, 2012).

5.3.3.3. Histochemical analysis of *Quercus suber* infested with *Phytophthora cinnamomi* zoospores plus PRE

Quercus suber roots were exposed simultaneously to PRE and zoospores (see Table 5.1, sample 9) for 48 h with no macroscopic lesions being produced on the roots (see Chapter 4, Figure 4.4 G) and also no penetration of the tissues were visible under the microscope.

5.3.3.4. Histochemical analysis of *Quercus suber* roots infested with *Phytophthora cinnamomi* mycelia and zoospores

The *Q. suber* roots infested with *P. cinnamomi* mycelia at 48 hpi, presented callose production as shown by bright light-blue colour produced by aniline blue staining. Intense callose deposition in the phloem (P) sieve plates and minimal depositions in the epidermis (Ep) was observed (Figure 5.16). Conversely, in *Zea mays*, inoculated with zoospores at 24-48 hpi, the majority of callose was deposited in the epidermal and cortical tissues of the roots and at

72 hpi there was intense deposition in the vascular tissue, particularly across sieve plates (Allardyce, 2011). Usually, there is callose constitutivelly in the sieve plates of the phloem, but when a big deposition occurs, this can be caused by stress, including a biotic stress as is the case of the inoculation of *P. cinnamomi* (F. Garcia, personal communication).

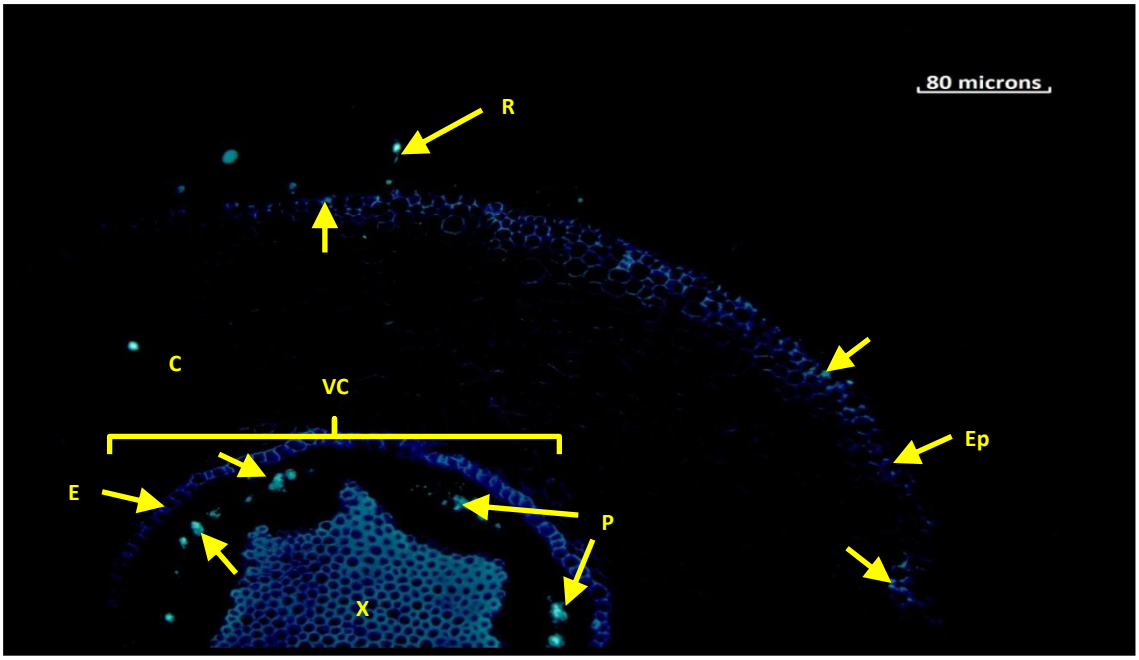

Figure 5.16. Light microscopy transverse sections under ultraviolet light (bright light-blue colour produced by aniline blue staining) in *Quercus suber* roots 48 hpi with *P. cinnamomi* mycelia, showing intense callose deposition in the phloem (P) sieve plates (yellow arrowheads) and light depositions in the epidermis (Ep) (yellow arrowheads). Frozen cut: VC =vascular cylinder, X=xylem; En=endodermis; C=cortex; scale bar = 80 µm.

The results obtained from *Q. suber* roots infested with *P. cinnamomi*, both mycelia and zoospores, at 48 hpi, shows tissue damage with cell wall distortion (Figure 5.17), red arrow-headed). Cell wall distortion and disruption accompanied infection was observed in the roots of all 13 plant studied, ranging from susceptible to resistant (Cahill *et al.*, 1989).

Frozen cuts stained with calcofluor showed that in 48h this oomycete was able to colonize *Q. suber,* with hyphae proliferating both inter and intra-cellularly through the epidermis, cortical parenchyma and vascular cylinder (Figure 5.18). These results observations by Maia *et al*. (2008), Horta *et al*. (2010) and Medeira *et al*. (2012).

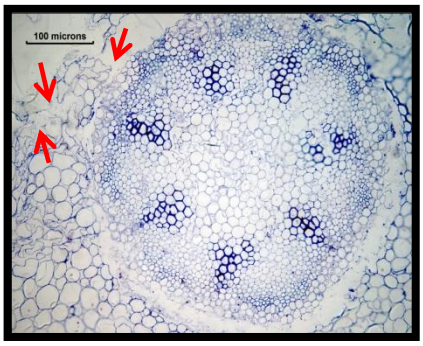

Figure 5.17. Light microscopy of transverse sections of *Quercus suber* roots, 48 hpi, stained with toluidine blue, showing tissue damage at the cortex (arrow-headed), scale bar = 100 µm.

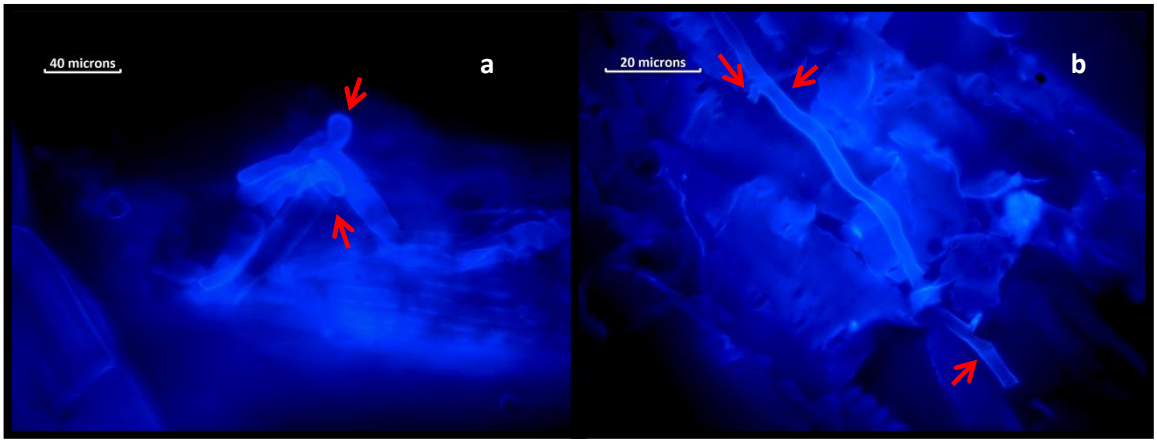

Figure 5.18. Longitudinal section of *Quercus suber* roots at 48 hpi with *P. cinnamomi* obtained by freezing and treating with calcofluor. Emitted fluorescence under UV light. **(a)** inoculation with mycelia, scale bar = 40 µm. **(b)** inoculation with zoospores, scale bar = 20 µm. Hyphae were observed in the cortex (arrows).

The observations of infested *Quercus suber* by LTSEM showed *P. cinnamomi* hyphae and zoospores entering the root epidermis (Figure 5.19).

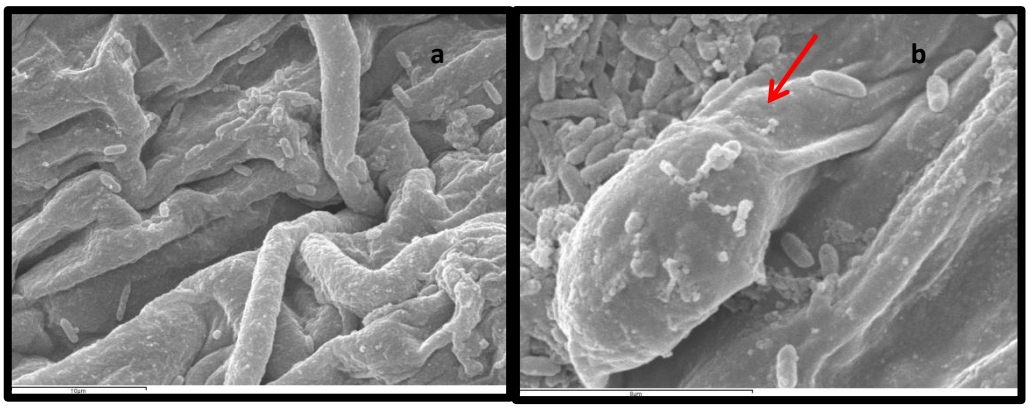

Figure 5.19. Low temperature scanning electron microscopy of *Quercus suber* roots at 48 hpi with *P. cinnamomi* zoospores: **(a)** showing hyphal penetration; scale bar = 10 µm; **(b)** showing a penetrating zoospore (red arrow), scale bar = 8 µm.

From the anatomy of *Q. suber*, shown in the figures above, there are no constitutive morphological or structural defence mechanisms, namely an exodermis that can act as a physical barrier. In that way the roots are more prone to attack of invaders and have to rely on biochemical defence mechanisms.

5.4. Conclusions

These results, arising from microscopic observations of roots of *Phlomis purpurea* control seedlings and infested with *P. cinnamomi* zoospores and mycelia, suggest that a preformed barrier made of constitutive cell wall fortification in the form of suberin, lignin, tannins and cellulose may be the most important feature to avoid pathogen invasion. It is widely accepted that suberin establishes boundaries within tissues, serves as a barrier against free water and ion passage, and works as a shield against pathogen attacks. The exudation of secondary metabolites and/or antimicrobial peptides (see Chapter 6) could also explain the avoidance of this oomycete by the plant, preventing efficiently, together with physical barriers, the invasion. It should be stressed that *P. cinnamomi* could never be isolated either from *P. purpurea* seedlings directly inoculated with zoospores and mycelia or from *P. purpurea* inoculated with *P. cinnamomi* in the greenhouse assays and in the field (see chapters 2 and 3). On the other hand even in resistant Arabidopsis Col-0 plants, infestation with *P. cinnamomi* resulted in pathogen encystment, germination and hyphal growth and the pathogen could be re-isolated from the roots (Rookes *et al.*, 2008).

Phlomis purpurea is therefore proposed to be a non-host, type 1 resistant plant to *P. cinnamomi*. Type I non-host resistant, relies on passive or preformed defences (Osbourn, 1996, 2001; Heath, 2000; Mysore & Ryu, 2004; Nürnberger & Lipka, 2005; Fan & Doerner, 2012), including physical barriers such as cytoskeleton and/or chemical strategies such as secondary metabolites and antimicrobial proteins (Broekaert *et al.*, 1995).

Studies on chemotaxis of *P. cinnamomi* zoospores towards the roots of both resistant and susceptible plant species have demonstrated that in all species examined, the roots both attracted and were penetrated by zoospores (Hinch & Weste, 1979). For instance, both disease tolerant *Eucalyptus st johnii* and susceptible *E. obliqua* roots, were penetrated by *P. cinnamomi* zoospores (Tippet *et al.*, 1976). Tyler (2002) state that recognition of the host plant by soilborne *Phytophthora* pathogens is essential for root infection. It would be of interest to see how *P. purpurea* avoids penetration of *P. cinnamomi* zoospores through real time videomicroscopy as done by Jones *et al*. (1991).

Quercus suber, although producing cork, and its young roots being suberized, the first signs of suberization, according to Machado *et al*. (2013) were observed in the endodermis and as the root matures, acquiring a brown coloration, the formation of three to four layers of suberized phellem cells takes place as the result of periclinal divisions of meristematic cells (phellogen). Generally, at earlier stages of root development, rhizodermis, with its cell walls containing a diffuse nonlamellar suberin, is the first line of defense because it is in direct contact with the environment. Later on, endodermis, with its Casparian strip, takes the role as the last barrier against pathogen invasion due to suberization (Huitema *et al.*, 2004). However, *Q. suber* root cap and endodermic passage cells do not show suberin deposition, and *P. cinnamomi* finds its way in through the only root zone that shows no suberization.

Unlike *P. purpurea*, there is no exodermis present in *Q. suber* which could provide a physical barrier against the entrance of microorganisms, so leaving those roots unprotected.

5.5. Acknowledgments

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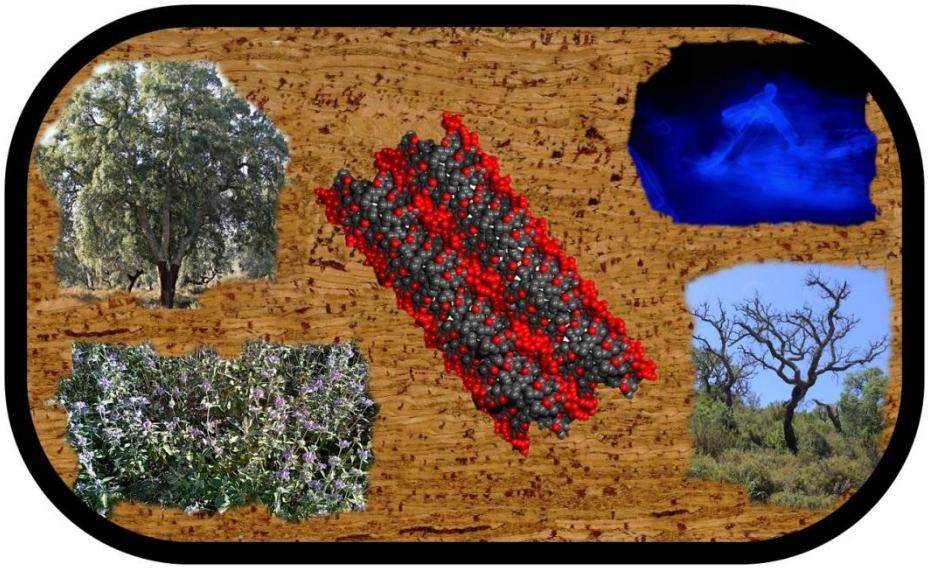
Part II

Determination of metabolites in *Phlomis purpurea* roots challenged and not challenged with *Phytophthora cinnamomi*.



Chapter 6

Introduction and literature review

6.1. Plant secondary metabolites

Plants, unlike animals, cannot run away from predators or stress environments. In that way, plants developed strategies to defend themselves. For that propose, plants produce a vast array of over 100,000 secondary metabolites, which are low-molecular-mass (< 1,500 to 2,000 Da) natural products (Dixon, 2001; Bednarek, 2012) that are specific to a plant species (Arbona *et al.*, 2013). Secondary metabolites, although not secondary in importance, are distinct from the components of intermediary (primary) metabolism in that they are generally nonessential for the basic metabolic processes of the plant (Dixon, 2001).

Pathogen defence in plants, upon recognition of pathogen attack, can be grouped into three types of antimicrobial secondary metabolites: preformed phytoanticipins, inducible phytoalexins and infection-triggered breakdown products of preformed or induced metabolites (Bednarek *et al.*, 2005). Phytoanticipins and phytoalexins (VanEtten *et al.*, 1994) can be subdivided into a variety of biosynthetically distinct classes, such as terpenoids, aliphatic acid derivatives, phenolics and phenylpropanoids (Dixon, 2001; Vogt, 2010), whereas their breakdown products, as well as toxic breakdown products from other precursors, are chemically highly diverse (Bednarek *et al.*, 2005). Phytoanticipins are a part of the genetically inherited resistance mechanisms that make plants constitutively resistant to the majority of pathogenic organisms and pests present in the environment. Phytoalexins are included, together with cell wall reinforcement, accumulation of reactive oxygen species (ROS), hypersensitive response (HR) and transcriptional activation of defence-related genes, in a second line of defence which is activated in the immediate vicinity of the infected or wounded sites in an attempt to prevent the spread of the pathogen throughout the plant. A third line of defence is associated with the phytohormone salicylic acid (SA), in which local attack by a necrotizing pathogen induces broad-spectrum resistance throughout the plant system-systemic acquired resistance (SAR), against an array of pathogens (Grant & Lamb, 2006; Ibáñez *et al.*, 2010).

The roots of plants have the ability to synthesize a remarkable diversity of secondary metabolites, and to adjust their metabolic activities in response to biotic and abiotic stress (Bais *et al.*, 2004). This includes the ability to exude a complex array of metabolites into the rhizosphere, potentially affecting the inter-relationships between plants and beneficial or harmful soil-borne organisms (Bais *et al.*, 2004; Haichar *et al.*, 2008; Badri & Vivanco, 2009). These metabolites can often be separated into two classes: low-molecular weight compounds, which include, oxygen, water, inorganic acids, organic acids, amino acids, sugars, phenolics and an array of secondary metabolites which are generally species specific (Arbona *et al.*, 2013), and high-molecular weight compounds like mucilage and proteins.

The classes of compounds, including some compounds secreted by roots and involved in plant defence are listed in Table 6.1.

Table 6.1. Classes of compounds released in plant root exudates, their molecular weight and formula. Adapted from (Narasimhan *et al.*, 2003; Badri & Vivanco, 2009).

Class of compounds	Single components	Molecular weight (MW) $gmol^{-1}$	Molecular formula (MF)
Carbohydrates	Arabinose, Ribose, Pentose, Xylose	150.129900	$C_5H_{10}O_5$
	Glucose, Galactose, Fructose	180.155880	$C_6H_{12}O_6$
	Sucrose	342.296480	$C_{12}H_{22}O_{11}$
	Rhamnose	164.156480	$C_6H_{12}O_5$
	Raffinose	504.437080	$C_{18}H_{32}O_{16}$
	Mannitol	182.171760	$C_6H_{14}O_6$
Amino acids	Proteinogenic amino acids (20)	–	–
	L-hydroxyproline	131.12986	$C_5H_9NO_3$
	Homoserine	119.119160	$C_4H_9NO_3$
	Mugineic acid	320.295800	$C_{12}H_{20}N_2O_8$
	Aminobutyric acid	103.11976	$C_4H_9NO_2$
Organic acids	Acetic acid	60.051960	$C_2H_4O_2$
	Succinic acid	118.088040	$C_4H_6O_4$
	L-aspartic acid	133.102680	$C_4H_7NO_4$
	Malic acid	134.087440	$C_4H_6O_5$
	L-glutamic acid	147.129260	$C_5H_9NO_4$
	Salicylic acid, *p*-hydroxybenzoic acid	138.120740	$C_7H_6O_3$
	Shikimic acid	174.151300	$C_7H_{10}O_5$
	Isocitric acid	192.123520	$C_6H_8O_7$
	Sinapic acid	224.20998	$C_{11}H_{12}O_5$

Class of compounds	Single components	Molecular weight (MW) $gmol^{-1}$	Molecular formula (MF)
Cont. **Organic acids**	Lactic acid	90.077940	$C_3H_6O_3$
	Caffeic acid[b]	180.157420	$C_9H_8O_4$
	Gallic acid	170.119540	$C_7H_6O_5$
	Tartaric acid	150.086840	$C_4H_6O_6$
	Ferulic acid[b]	194.184000	$C_{10}H_{10}O_4$
	Protocatachuic acid	154.120140	$C_7H_6O_4$
	p-coumaric acid[b]	164.158020	$C_9H_8O_3$
	Oxalic acid	90.034880	$C_2H_2O_4$
	Citric acid	192.123520	$C_6H_8O_7$
	Piscidic acid	256.20878	$C_{11}H_{12}O_7$
	Chorismic acid	226.182800	$C_{10}H_{10}O_6$
Flavonols	Naringenin	272.252780	$C_{15}H_{12}O_5$
	kaempferol	286.236300	$C_{15}H_{10}O_6$
	Quercitin, Isoquercitin	302.235700	$C_{15}H_{10}O_7$
	myricetin	318.235100	$C_{15}H_{10}O_8$
	naringin	580.534580	$C_{27}H_{32}O_{14}$
	rutin	610.517500	$C_{27}H_{30}O_{16}$
	Genistein[b]	270.236900	$C_{15}H_{10}O_5$
	strigolactone	234.29092	$C_{14}H_{18}O_3$
Lignins	Catechol	110.110640	$C_6H_6O_2$
	benzoic acid	122.121340	$C_7H_6O_2$
	nicotinic acid	123.109400	$C_6H_5NO_2$
	phloroglucinol	126.110040	$C_6H_6O_3$
	cinnamic acid[b]	148.158620	$C_9H_8O_2$
	gallic acid	170.119540	$C_7H_6O_5$
	ferulic acid	194.184000	$C_{10}H_{10}O_4$
	syringic acid[b]	198.1727	$C_9H_{10}O_5$
	sinapoyl aldehyde	208.21058	$C_{11}H_{12}O_4$
	chlorogenic acid	354.30872	$C_{16}H_{18}O_9$
	Vanillic acid[b]	152.147320	$C_8H_8O_3$

Class of compounds	Single components	Molecular weight (MW) $gmol^{-1}$	Molecular formula (MF)
Cont. **Lignins**	sinapyl alcohol	210.22646	$C_{11}H_{14}O_4$
	quinic acid	192.166580	$C_7H_{12}O_6$
	pyroglutamic acid	129.113980	$C_5H_7NO_3$
Coumarins	Umbelliferone	162.142140	$C_9H_6O_3$
Aurones	Benzyl aurones synapates	-	-
	sinapoyl choline	-	-
Glucosinolates	Cyclobrassinone(nine)	234.3405	$C_{11}H_{10}N_2S_2$
	desuphoguconapin	-	-
	desulphoprogoitrin	-	-
	desulphonapoleiferin	-	-
	desulphoglucoalyssin	-	-
Anthocyanins	Cyanidin	322.697240	$C_{15}H_{11}ClO_6$
	delphinidin	303.243640	$C_{15}H_{11}O_7^+$
	pelargonidin	271.244840	$C_{15}H_{11}O_5^+$
Indole compounds	Indole-3-acetic acid	175.18396	$C_{10}H_9NO_2$
	brassitin	220.29078	$C_{11}H_{12}N_2OS$
	sinalexin	-	-
	brassilexin	174.22234	$C_9H_6N_2S$
	methyl indole carboxylate	175.183960	$C_{10}H_9NO_2$
	Camalexin[b]	200,25962	$C_{11}H_8N_2S$
Fatty acids	Linoleic acid	280.445480	$C_{18}H_{32}O_2$
	oleic acid	282.461360	$C_{18}H_{34}O_2$
	palmitic acid	256.424080	$C_{16}H_{32}O_2$
	stearic acid	284.477240	$C_{18}H_{36}O_2$
Sterols	Campestrol	400.680120	$C_{28}H_{48}O$
	sitosterol	414.706700	$C_{29}H_{50}O$
	stigmasterol	412.690820	$C_{29}H_{48}O$
Allomones	Jugulone (juglone)[b]	174.152840	$C_{10}H_6O_3$
	Sorgoleone[b]	357.463260	$C_{22}H_{29}O_4^-$

Class of compounds	Single components	Molecular weight (MW) $gmol^{-1}$	Molecular formula (MF)
Cyclic hydroxamic acids	DIMBOA	211.171460	$C_9H_9NO_5$
	DIBOA	181.145480	$C_8H_7NO_4$
Proteins and enzymes	PR proteins	-	-
	lectins	-	-
	Proteases[b]		
	acid phosphatases	-	-
	peroxidases	-	-
	hydrolases	-	-
	lipase	-	-
Others	hederagenin	472.699720	$C_{30}H_{48}O_4$
	gypsogenin	470.683840	$C_{30}H_{46}O_4$
	Trifolirhizin	446.404080	$C_{22}H_{22}O_{10}$
	Maackiain	284.263480	$C_{16}H_{12}O_5$
	DL-Glutamic acid	147.12926	$C_5H_9NO_4$
	Salicylglutamic acid	267.23472	$C_{12}H_{13}NO_6$
	DL-Aspartic acid	133.10268	$C_4H_7NO_4$
	Arginyl-glycyl-aspartic acid	346.339680	$C_{12}H_{22}N_6O_6$
	Catechin gallate[b]	442.372320	$C_{22}H_{18}O_{10}$
	Barosmin, diosmin	608.544680	$C_{28}H_{32}O_{15}$
	Tamoxifen[b]	371.514560	$C_{26}H_{29}NO$
	Silymarin[b]	482.436180	$C_{25}H_{22}O_{10}$
	Xanthohumol	354.396380	$C_{21}H_{22}O_5$
	Resveratrol[b]	228.243280	$C_{14}H_{12}O_3$
	Digitoxigenin	374.513660	$C_{23}H_{34}O_4$
	Epigallocatechin gallate (EGCG)[b]	458.371720	$C_{22}H_{18}O_{11}$
	Hamamelitannin	484.3644	$C_{20}H_{20}O_{14}$
	Aesculitannin C[b]	862.74066	$C_{45}H_{34}O_{18}$
	Procyanidin	594.519640	$C_{30}H_{26}O_{13}$
	Rosmarinic acid[b]	360.31484	$C_{18}H_{16}O_8$

[a]Source: Pubchem http://www.ncbi.nlm.nih.gov/pccompound. [b]Involved in plant defence

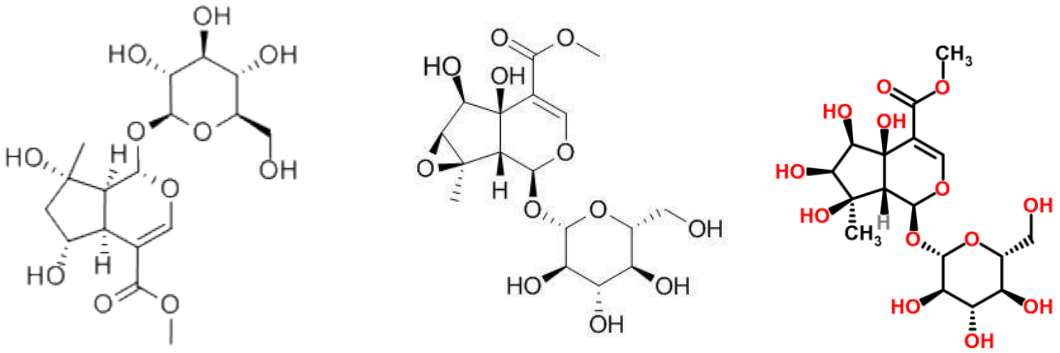
Metabolites reflect the integration of gene expression, protein interaction and other different regulatory processes. Therefore, metabolomics is the most transversal technology and can be applied to different organisms with little or no modifications (Arbona *et al.*, 2013).

6.2. Plant extracts with anti-*Phytophthora* spp activity

Plant extracts are mixtures of compounds that may have antimicrobial properties, including anti-oomycete activity as research, mainly on *Phytophthora infestans* (see Chapter 1, section 1.6, Table 1.1) shows. Other examples include the inhibition action of crude extracts of *Thevetia peruviana* seeds against *P. megakarya*, one of the causal agents of black pod disease of *Theobroma cacao* (Ambang *et al.*, 2010); of *Xanthium strumarium* leaf, fruit and inflorescence from Iran against mycelial growth of *P. drechsleri* (Bahraminejad *et al.*, 2012) or substances isolated from plant extracts such as nyasol isolated from *Anemarrhena asphodeloides* rhizomes (Park et al. 2003) with anti-*P. capsici* activity (Andrews, 2001) and rosmarinic acid, from the roots of *Ocimum basilicum* with anti- *P. drechsleri*, *P. megasperma* and *P. parasitica* (Bais *et al.*, 2002).

6.3. Compounds isolated from plants of the genus *Phlomis*

Flavonoids are the major phytoconstituents isolated from the genus *Phlomis* (Amor *et al.*, 2009; Li *et al.*, 2010). Apigenin, chryseriol, kaempferol, luteolin, naringenin and their glycosides are abundant in this genus (ibid). The majority of flavonoids, isolated from the *Phlomis* spp, are flavones, flavonols and usually 7- or 3- glycosylated flavonoids (ibid). Flavonoids exhibit a wide range of bioactivities including antioxidant, cardiovascular, anti-inflammatory, antibacterial, antiviral, immune-stimulating, anti-allergic and estrogenic activities (Reviwed by Rice-Evans *et al.*, 1996). Other class of compounds such as iridoids have been isolated from the *Phlomis* species, among them the shanzhiside methyl ester, reported in several *Phlomis* spp., for instance, in the aerial parts of *P. rigida* (Takeda *et al.*, 2000), the roots of *P. umbrosa* (Liu *et al.*, 2007) and also the roots of *P. younghusbandii* (Li *et al.*, 2011b) (Figure 6.1). From *P. younghusbandii* roots other five iridoids were found: sesamoside, 7,8-dehydropenstemoside, penstemoside, 8-*O*-acetylshanzhiside methyl ester and phlomiol. Phenylethanoid glycosides were also found, for example, acteoside (verbascoside), alyssonoside and isoacteoside (ibid). Iridoids exhibit a wide range of bioactivities including cardiovascular, anti-hepatotoxic, anti-inflammatory, immunomodulator choleretic, hypoglycemic, analgesic, anti-mutagenic, antispasmodic, antitumor, antiviral, and anti-microbial activities (reviewed by Tundis *et al*. 2008).



A. shanzhiside methyl ester **B.** Sesamoside **C.** Phlomiol

Figure 6.1. Iridoids isolated from *Phlomis umbrosa* (A) and *P. younghusbandii* roots (B, C).

Other secondary metabolites include compounds with phenylethylalcohol glycoside structures, caffeic acid derivatives, such as verbascoside and forsythoside. Verbascoside was present in 13 *Phlomis* spp (reviewed by Amor et al. 2009), including in roots of *P. umbrosa* (Liu *et al.*, 2007) and of *P. younghusbandii* (Li *et al.*, 2011b). Forsythoside was reported in the aerial parts of 11 *Phlomis* spp. (Amor *et al.*, 2009). Up to 2010, 151 compounds were isolated from the genus *Phlomis*, including terpenoids, flavonoids, phenylethanoid glycosides, lignans, essential oils, etc., with diverse biological activities including anti-mutagenic, antioxidant, anti-inflammatory, antiprotozoal, antibacterial and antifungal activities (Li *et al.*, 2010).

From the air-dried aerial parts of *P. purpurea,* several flavonoids were isolated: apigenin ($C_{15}H_{10}O_5$), apigenin-7-glucoside ($C_{21}H_{20}O_{10}$), apigenin-7-coumarouylglucoside, chrysoeriol ($C_{16}H_{12}O_6$), chrysoeriol-7-glucoside ($C_{22}H_{22}O_{11}$), chrysoeriol-7-*p*-coumarouylglucoside, isorhamnetin-3-glucoside ($C_{22}H_{22}O_{12}$), isorhamnetin-3-*p*-coumarouylglucoside, kaempferol-3-glucoside (also known as astragalin) ($C_{21}H_{20}O_{11}$), kaempferol-3-*p*–coumarouylglucoside, luteolin-7-glucoside ($C_{21}H_{20}O_{11}$) and *p*-coumaric acid ($C_9H_8O_3$) (Tomas-barberan *et al.*, 1992). Flavonoid's basic structural feature is the 2-phenyl-benzo[α]pyrane, which consists of two benzene rings linked through a heterocyclic pyrane ring (Figure 6.2) (Cushnie & Lamb, 2005).

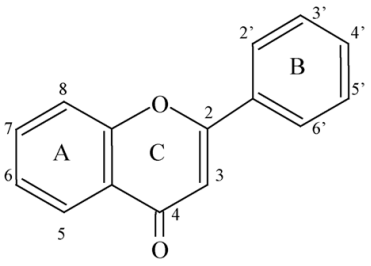


Figure 6.2. The 2-phenyl-benzo[α]pyrane, the basic structural feature of flavonoid compounds which consists of two benzene rings (A and B) linked through a heterocyclic pyrane ring (C) with positions numbered.

Flavonoids can be classified according to their biosynthetic origin. Some classes, for example chalcones, flavanones, flavan-3-ols and flavan-3, 4-diols, are intermediates in biosynthesis as well as end products that can accumulate in plant tissues. Other classes are only known as end products of biosynthesis, for example anthocyanidins, proanthocyanidins, flavones and flavonols (Cushnie & Lamb, 2005). Flavonoids are a widely distributed group of polyphenolic compounds with well-known antioxidant activity. Their properties have been found to include anticancer, antiviral, antiinflammatory activities, effects on capillary fragility, and an ability to inhibit human platelet aggregation (Benavente-Garcia *et al.*, 1997; Harborne & Williams, 2000). Flavonoid compounds are also, known to have antimicrobial properties, including fungitoxic such as the isoflavanone kievitone and the isoflavan vestitol (Harborne, 1999). For instance, kievitone was reported to strongly inhibit the germination of *Phytophthora vignae* f. sp. *adzukicola* encysted zoospores (Harada & Kondo, 2009).

Phlomis purpurea crude root extract was reported to inhibit *P. cinnamomi* mycelial growth (Neves, 2007), sporangial production, zoospore release and germination as well as chlamydospore production and viability (Neves *et al.*, 2014) but until the present work no compounds responsible for that activity were known.

6.4. From the plant to the identification of the active principle(s)

Several methods for plant extraction are used (see Chapter 1, Table 1.1). The extract is filtrated or centrifuged and evaporated to the dryness, weighed and resuspended in a convenient solvent. Usually a bioassay is done to determine the activity of the crude extract on microorganism(s). Fractionation is then performed successively with organic solvents with increasing or decreasing polarities (Ambang *et al.*, 2010). The fractionation can be guided by thin layer chromatography (TLC) and analysed by HPLC. Bioassay guided fractionation must be performed to identify the active fraction(s). Isolation and purification of the compound(s) precede the identification using spectroscopic and spectrometric techniques and X-ray diffraction. A brief flow chart is represented on Figure 6.3.

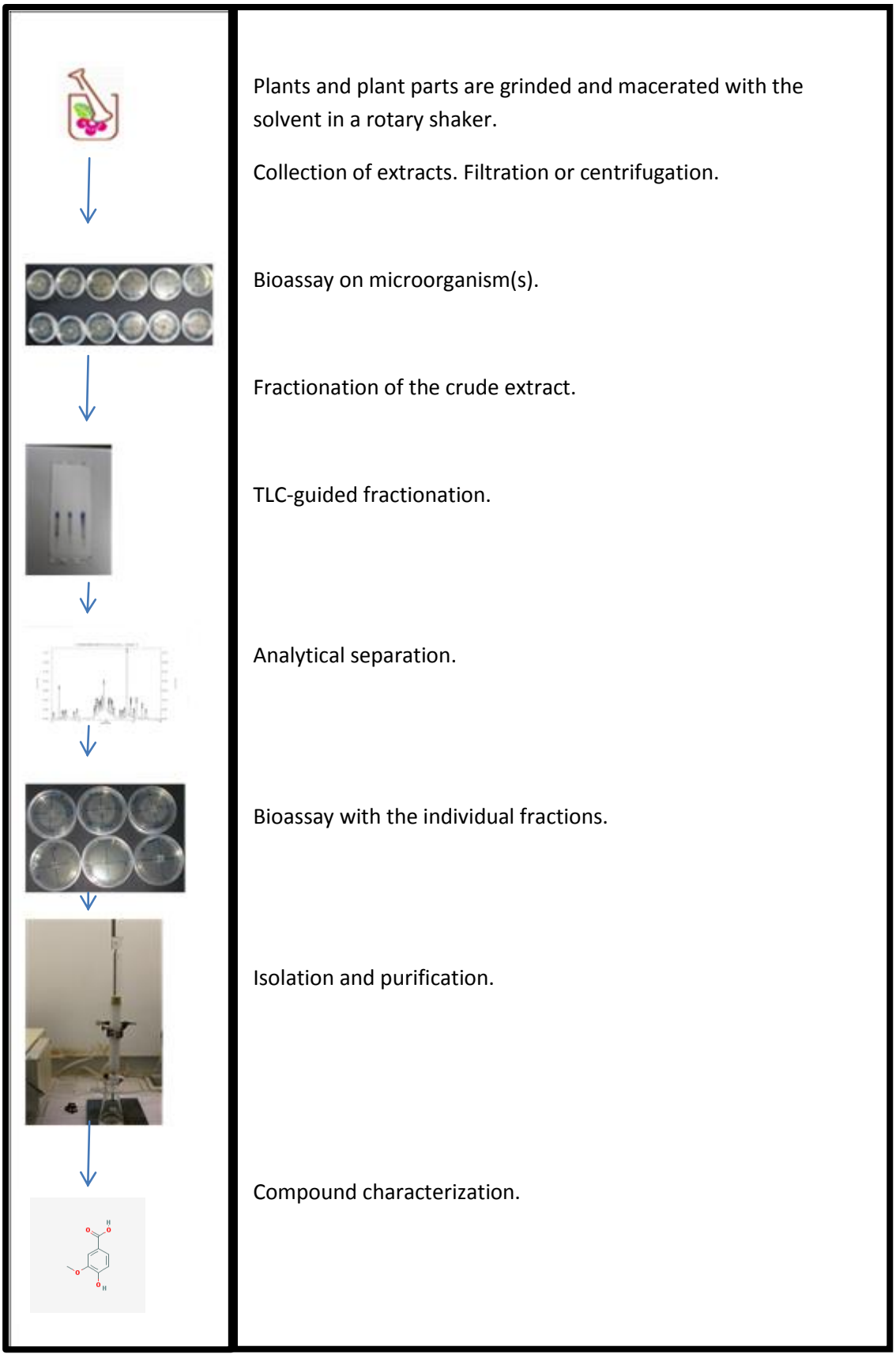


Figure 6.3. A flow chart representation of methods involved in extraction, fractionation, separation, isolation, purification and compound characterization from plant extracts. Bioassays are performed in intermediate steps to evaluate the extract activity.

6.5. Metabolite fingerprinting, metabolite profiling and metabolomics

It is convenient to distinguish between metabolite fingerprinting, metabolite profiling and metabolomics. Metabolic fingerprinting is the application of a broad analytic technology to obtain enough information (e.g. differences between two samples) to help to orientate a research project (Kopka *et al.*, 2004) without aiming to get quantitative data. Metabolite profiling is the measurement of hundreds or potentially thousands of pre-defined metabolites. Sample preparation and clean-up are directed to the chemical properties of these compounds. It requires a streamlined pipeline for extraction, separation and analysis, so that large numbers of metabolites can be measured in a robust and quantitative manner while in the presence of the extraordinarily complex mixture of chemicals that is found in cellular extracts (Sumner *et al.*, 2003; Kopka *et al.*, 2004). Metabolomics, is the complete form of metabolite analysis, i.e., the "comprehensive analysis of the whole metabolome under a given set of conditions" (Fiehn *et al.*, 2000) or the unbiased, comprehensive qualitative and quantitative measurement of all metabolites present in a given system (Sumner *et al.*, 2003; Kopka *et al.*, 2004; Bhalla *et al.*, 2005; Hall, 2006; Allwood *et al.*, 2008).

There is another approach: target analysis that has been applied for a long time and aims at determination and quantification of known metabolites using the best analytical technique that gives the best performance for the compounds of interest (Roessner & Bown, 2009).

Therefore, while transcriptomic and proteomic data do not tell the whole story, metabolic profiling can give an instantaneous picture of the physiology of that cell, tissue or organ. The combination of the three gives a holistic view of the complex interaction between gens and metabolites (Bino *et al.*, 2004; Bhalla *et al.*, 2005)

From the problem formulation until the data analysis, there are obligatory steps (Figure 6.4). All steps have to be carefully programmed.

Figure 6.4. Workflow in metabolite analysis.

6.5.1. Sample preparation

Some practical steps need to be kept in mind for the sample preparation. For instance, when plants are challenged with a pathogen in a time course, it must be essential that infective propagules are viable and that samples at set time-points are immediately freeze dried in liquid nitrogen to stop the enzymatic activity. Great care must be taken not to partially thaw tissues before extracting metabolites (Fiehn, 2001). For plant exudates, the same process applies. Exudates can be lyophilized which also prevents enzyme function.

Frozen samples, which must be separated by tissue type, can be ground using a ball mill in pre-chilled holders followed by the addition of organic solvents. The choice of extraction buffers has to be compatible with the final objectives of the analysis. Usually polar organic solvents, like alcohols, are directly added to homogenize frozen tissues for the extraction of polar components, often followed by non-polar solvents such as dichloromethane for gaining sufficient recovery of lipophilic metabolites. As a well-established alternative the extraction with cold methanol is able to cover polar and medium polar metabolites. For certain groups of compounds specific solvents or conditions (acidic environment) might be needed (Kopka *et al.*, 2004; Bhalla *et al.*, 2005). Given the chemical diversity of metabolites, it is virtually impossible to invent a method that allows the extraction of all metabolites present in a sample (Kopka *et al.*, 2004). Samples are dried via speed vacuum concentration, stored at -80 °C or directly analysed (Fiehn *et al.*, 2000).

It is worth to point the importance of the biological variance that arises from quantitative variations in metabolite levels between plants of the same species grown under identical conditions (Sumner *et al.*, 2003). Biological variations typically exceed analytical variations as stated by Roessner and colleagues (2000) who reported that the biological variability of tuber slices from wild type plants grown under identical conditions exceeded the analytical variability of GC/MS by a factor of ten. Sumner and coworkers suggested that the average biological variance for *Medicago truncatula* is approximately 50 % (unpublished data). A way to reduce biological variance and strengthen the differences is to pool the samples to reduce the individual variability (biological variance) (Sumner *et al.*, 2003). However, pooling samples can result in undesirable dilution of specific up or down-regulated metabolites (ibid). Another way is to use clones and hairy roots could serve that purpose. The amount of metabolites (alkaloids), per gram dry root material, were shown to be the same for both transformed roots and wild type *Hyoscyamus muticus* (Zolala *et al.*, 2007). Hairy root production by employing *Agrobacterium rhizogenes*-mediated transformation method has greatly facilitated the study of root-specific metabolism and contributed to our understanding of this remarkable plant organ. To induce the hairy root cultures the axenic explants are co-

cultivated with *A. rhizogenes*, generally for 1 to 2 days in a culture media. Then, the explants in co-culture are transferred to culture media supplemented with antibiotic to kill the bacteria. This process is repeated until no bacteria grow (2 to 4 weeks). The transformed plant cells are selected and root excised to grow on solid or directly to liquid media without antibiotics and sub-cultured every 15 days (Santos *et al.*, 1998, 2002, 2005). To allow the production of metabolites in larger scale bioreactors can be used (Stoger, 2012).

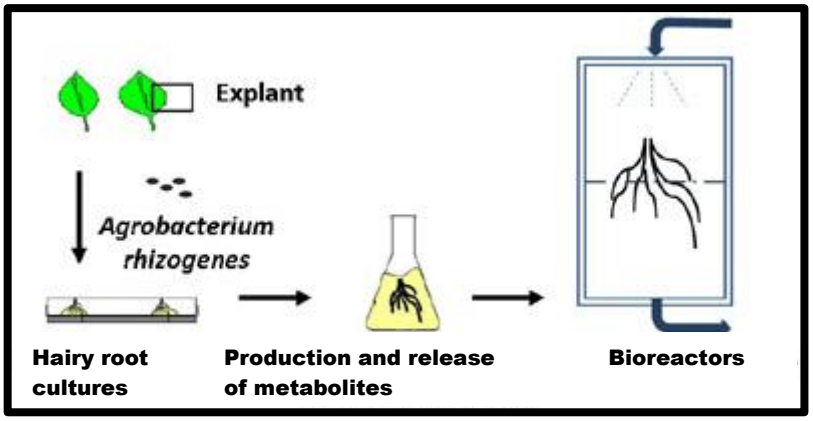


Figure 6.5. Induction and maintenance of hairy root cultures. Adapted from Stoger 2012.

Roots must now be accounted as the site of unique metabolic activities and, in many cases, as main contributors to the production of secondary metabolites in the whole plant (Flores *et al.*, 1999).

6.5.2. Analytical technologies to analyze metabolites

There are several technologies to analyze the extracted metabolites. Generally, the technology platform depends on the type of sample to be analyzed and on the type of information one would like to get.

Liquid chromatography coupled to mass spectrometry (LC-MS) is an analytical chemistry technique that combines the physical separation capabilities of liquid chromatography (HPLC) with the mass analysis capabilities of MS. LC-MS is a powerful technique that has a very high sensitivity and selectivity and allows the separation, general detection and potential identification of chemicals of particular masses, so it is useful in many applications including the analysis of natural-product extracts. In HPLC, the sample is forced by a liquid at high pressure (the mobile phase) through a column that is packed with a stationary phase. Metabolites are introduced into the MS by electrospray ionization (ESI). The analytes enter the MS as charged molecules. Ionization can occur via protonation ($+H^+$) or

deprotonation ($-H^+$). Another adducts can be formed. This can lead to single and multiple ions whose analysis helps in the determination of masses of particles, the elemental composition of a sample or molecule, and hence in the elucidation of the chemical structures of molecules. LC-MS has been one of the most significant chromatographic-spectrometric-coupled technologies of the 21st century (Allwood *et al.*, 2008; Li *et al.*, 2011a). However evaluation needs expert knowledge and the biggest challenge is to develop an automated procedure for evaluation and metabolite quantification from raw chromatograms and consequently to identify the huge numbers of unknown metabolites and analytes detected by these powerful analytic platforms (Kopka *et al.*, 2004).

Gas chromatography coupled to mass spectrometry (GC-MS), is a technique in which compounds are separated by GC and then transferred to the MS for further separation and detection. However, derivatization is necessary to render metabolites volatile. There are several chemicals that suite this propose but trimethylsilylation is comonly used as it complies best with the requirements of a non-biased metabolite profiling. The other major limitation of GC-MS for non-target analysis is that most peaks are still not identified. This is due to the high sensitivity and resolution of capillary GC-MS, but a frustration for the biologist (Kopka *et al.*, 2004; Koek *et al.*, 2011).

Both GC-MS and HPLC-MS provide good selectivity and sensitivity (Sumner *et al.*, 2003).

The primary advantage of HPLC-MS over gas chromatography-mass spectrometry (GC-MS) is that it is capable of analysing a much wider range of components such as compounds that are thermally labile, exhibit high polarity or have a high molecular mass, provided that they are ionizable by MS ionization source (Fernie *et al.*, 2004). Compounds are identified by matching their chromatographic retention times and mass-spectral fragmentation patterns to known and predicted information available in databases.

The main alternative to mass-spectrometry-based approaches for metabolic profiling is nuclear magnetic resonance (NMR). This is a very useful technique, since in-principle any chemical species that contains protons gives rise to signals (Krishnan *et al.*, 2005). This method offer the advantage that, because of the intensive use over the years, they have been subjected to intensive validation (Fernie *et al.*, 2004). NMR technologies have a lower sensitivity compared with mass-spectrometry methodologies. However, computational analyses and the chemometric software associated with NMR are highly developed (Kopka *et al.*, 2004). NMR is important for unequivocal determination of metabolite structure, which is one of the major handicap of metabolite profiling (ibid). NMR has rapid analysis times but has lower sensitivity thus allowing only the visualization of the more concentrated metabolites

(Sumner *et al.*, 2003). Furthermore, NMR is non-invasive, which can be a particularly important advantage in certain situations (e.g. small amount of sample).

In the selection of the most suitable technology a compromise must exist between speed, selectivity and sensitivity (Sumner *et al.*, 2003).

Other technologies can give supplementary information about a specific compound. Infrared spectroscopy (IR) exploits the fact that molecules absorb specific frequencies that are characteristic of their structure and can be a complementary tool to identify and study chemical compounds.

X-ray diffraction is a powerful technique to determine structural properties and to determine atomic arrangements of a compound.

6.5.3. Data analysis

Raw data is an eventual source of information and consequently, an eventual source of knowledge (Goodacre *et al*. 2004 citing Henry Nix, 1990). Due to the large amount of data, bioinformatics is used for the storage, retrieval and analysis of datasets (Sumner *et al.*, 2003).

Metabolomic data analysis consists of four essential areas:

1. Raw data processing (machine output) to allow comparison of different data sets. This can be achieved by bioinformatics and statistical tools to convert this data into information. This can be spectral alignment, baseline correction and noise reduction (Hall, 2006).

2. Data mining to select metabolites of interest. Several multivariate methods for classifying and modeling analytical data includes unsupervised methods such as principal component analysis (PCA) (Mendes, 2002). PCA is one of the oldest and most widely used multivariate techniques and constitute the first step in evaluating metabolomics data (Sumner *et al.*, 2003; Bhalla *et al.*, 2005). It works extremely well for detecting patterns, trends, and groups among samples (Bhalla *et al*. 2005). The concept behind PCA is to use orthogonal transformation to convert a set of observations (metabolite concentrations) of possibly correlated variables into a set of values of linearly uncorrelated variables (principal components). Other of the most udely used and simplest techniques are hierarchical clustering (HCA) or supervised approaches such as partial least squares (PLS) (Sumner *et al.*, 2003).

3. Data presentation in a simplistic and understandable format. The biggest challenge of metabolomics for bioinformatics might be the current lack of appropriate databases and data exchange formats (Sumner *et al.*, 2003). There is an urgent need for databases to identify metabolites and to describe the known chemistry. For pathway visualization, various tools have already been developped (e.g. KEGG, AraCyc, MetNet, BioPathAtMAPS, DOME, MetaCyc).

However, such tools depend on the previous identification of metabolites (Bino *et al.*, 2004; Hall, 2006).

4. Effective data storage and database construction (Hall, 2006). To share information is of major importance also in this field. This data could be used for future investigations, provided that it is then still possible to access all associated information concerning for instance, the species, type of material, growing conditions, sample preparation and extraction, etc. Input from research. Our knowledge will increase with the input from multiple research groups.

The two main bottlenecks in this field are the limited ability to identify the detected metabolites caused by the absence of reference standards (Hall, 2006).

The applications of metabolomics are wide, from biomarker discovery to therapy monitoring (Goodacre *et al.*, 2004) and are expanding.

The future challenge is the integration of Transcriptomics, Proteomics and Metabolomics within a single framework that will allow a better understanding of how plants respond to a changing environment.

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Chapter 7

Isolation and structural characterization of a novel nortriterpene with anti-*P. cinnamomi* activity

The introduction has been omitted, as a general introduction was presented in Chapter 6.

7.1. Abstract

The effect of *Phlomis purpurea* crude ethanol root extract (PRE) was evaluated *in vitro* on *Phytophthora cinnamomi* mycelial growth, sporangial production, zoospore release and germination as well as on chlamydospore production and viability (Neves *et al.*, 2014). The objective of this study was to identify the chemical substance(s) that is (are) responsible for the inhibition of *P. cinnamomi*. PRE was analysed by HPLC-ESI/MS/MS coupled to photodiode array detection and tandem mass spectrometry. HPLC fractions were recovered and their anti-*P. cinnamomi* activity determined. The fraction showing a major compound with m/z 513.3 ($[M+Na]^+$) significantly inhibited (100 %, $P \leq 0.001$) *P. cinnamomi* mycelial growth at 0.5 $mg.ml^{-1}$.

This compound was isolated from the PRE by preparative chromatography on silica gel with purity of *ca* 90 % and was further purified by recrystallization. It is a novel nortriterpene denominated phlomispurpentaolone, structurally characterized by 1D and 2D nuclear magnetic resonance (NMR), electrospray ionization mass spectrometry (ESI MS/MS), infrared spectroscopy (IR) and X-ray diffraction (XRD). At a concentration of 0.1 $mgml^{-1}$, it inhibited mycelial growth of *P. cinnamomi* by 75.7 %, 100 times more effectively than PRE.

7.2. Materials and Methods

7.2.1. Biological material

Phytophthora cinnamomi isolate

Phythophthora cinnamomi isolate PA37 was used in the bioassays (see Chapter 2, section 2.2.2).

Plant material

Phlomis purpurea roots were harvested from a field at S. Brás de Aportel in October 2010. A voucher specimen was deposited in the herbarium of the University of Algarve (accession number 13485).

Chemicals and reagents

Ethanol 96 % (Merck, Darmstadt, Germany), ascorbic acid (Sigma, St. Luis, USA) and HPLC-grade water (Milli-Q system, Millipore, Bedford, MA, USA) were used for extract preparation.

7.2.2. Extract preparation

The plant extraction protocol was adapted from Carini *et al.* (2001). Fine roots from *ca*. 100 plants of *P. purpurea* (50 g) were washed and chopped with a grinding-master (Moulinex® 1,2,3) and shaken for 7 days in 350 ml of 70 % aqueous ethanol containing 0.1 % ascorbic acid to prevent oxidation (Molina-Torres *et al.*, 2004). The insoluble residue was re-extracted twice with the same solvent and the extracts combined.

These conditions were optimized by Neves (2007). The ethanol was evaporated in a rotary evaporator R-114, in a Waterbath B-4180 (supplied by Buchi) at 60°C and with a pump Vac V500 at an operating pressure of 230 Vac (230psi). The extract was concentrated in a speedvac (Savant, SC 110A, Holbrook, USA) to constant weight, yielding approximately 5 g of a brownish sticky suspension which was dissolved in water and filtered through a 0.45 µm nitrocellulose filter (Millipore®). This procedure was repeated four times in order to obtain *ca* 20 g needed to perform all the experiments. The *Phlomis purpurea* crude root extract (PRE) was dissolved in sterile Milli-Q water.

7.2.3. HPLC analysis of PRE and fractionation

Chemicals and reagents

Acetonitrile, ethyl acetate, methanol and formic acid (HPLC grade, Merck, Darmstadt, Germany) and HPLC-grade water (Milli-Q system, Millipore, Bedford, MA, USA) were used for the mobile phase preparation. Hederagenin was purchased from Extrasynthése (Genay, France). Rutin, chlorogenic acid, caffeic acid, rosmarinic acid and vanillic acid were purchased from Sigma–Aldrich (Steinheim, Germany).

Apparatus and chromatographic conditions

HPLC analysis of PRE was performed using a liquid chromatograph (Merck-Hitachi, model 655A-11), a Rheodyne 7125 injection valve with a 50 µL loop, a 655A variable-wavelength UV detector (set at 254 nm) and a L-5000 cc controller. The samples were analysed using a LiChrospher 100® column RP-18 endcapped, 125 mm x 4 mm i.d. filled with particle substrate sized 5 µm in diameter (Merck KGaA, Germany). The mobile phase consisted of a gradient of acetonitrile:water in a dilution mode (Table 7.1). The chemicals were degassed in an ultrasonic bath. The flow rate was of 0.8 $mlmin^{-1}$. The HPLC system was operated at room temperature (23 ± 1°C). Gradient elution was performed using two solvents: acetonitrile and 0.1 % (v/v) formic acid in Milli-Q water with the linear gradient combination described in the Table 7.1.

Table 7.1. HPLC Solvent gradient (flow 0.8 $mlmin^{-1}$).

Time (min)	CH_3CN (%)	0,1 % $HCOOH/H_2O$ (%)
0	15	85
3	15	85
8	50	50
10	50	50
12	80	20
15	80	20
16	15	85

The main purpose of gradient elution is to move strongly retained components of the mixture faster, yet having the least retained component well resolved. Starting with a low content of the organic component in the eluent, the least retained components are allowed to be separated. Strongly retained components will sit on the adsorbent surface on the top of the column, or will move very slowly. These are hydrophobic compounds in the hydrophobic (non-polar) stationary phase here used. When the amount of organic component in the eluent (acetonitrile) starts to increase, the strongly retained components will move faster, because of the steady increase of the competition for the adsorption sites.

Recovery of *P. purpurea* fractions

To determine which PRE fraction had the anti-*P. cinnamomi* activity (see section 7.2.5), fractions were recovered as follows: PRE was centrifuged for 5 min at 16,110x*g* (Eppendorf tube, 5415 D), diluted by a factor of 10 with Milli-Q water and filtered through a 0.45 µm membrane (Durapore, Millex - HV). Fifty microliters were injected and fractions were collected every 3 min with a fraction collector (Pharmacia Biotech Frac-100 collector), in glass tubes previously heated at 400 °C for 6 h in an electric muffle furnace., Six fractions of 200 ml each were collected in about 200 runs. Fractions were evaporated to 1 ml in 100 ml round-bottom flasks in a rotary evaporator at 60 °C. They were then transferred to a 1.5 ml Eppendorf tube and evaporated to dryness in the speedvac at 45 °C. The tubes with the dry residue were transferred to a desiccator and further dried to constant weigh (Table 7.2). The residue of each fraction was dissolved in Milli-Q water and used in the bioassays to identify the active fraction(s). F5 was shown to inhibit *P. cinnamomi* mycelia growth (see results).

Table 7.2. Dry weight of HPLC fractions

Fractions	Weight (mg)
1	117.6
2	9.8
3	6.9
4	6.1
5	3.5
6	6.1

7.2.4. LC-MS analysis of the fractions

The PRE fractions were analysed by HPLC-PDA-ESI/MS/MS in positive mode. The LC (Agilent 1200) was linked to a mass spectrometer [Bruker, high-capacity ion trap (HCT ultra)], equipped with a diode-array detector (DAD) set at 254 nm and an electrospray ion source (ESI). The capillary voltage was set at -4000 V. Mass spectral data were acquired over m/z 50-1200 using Fullscan and MS^2 modes. Nitrogen was used as nebulizer gas (MS → 20 psi: 8 l/min, 300 °C; LC-MS→50 psi: 10 l/min: 365 °C).

The samples were analysed using a LiChrospher 100® column RP-18 endcapped, 2.1 mm i.d. (Merck KGaA, Germany). The mobile phase consisted of a gradient of acetonitrile:water in a dilution mode (Table 7.1). The flow rate was of 0.4 ml min^{-1}. The HPLC system was operated at room temperature (23 ± 1°C). Gradient elution was performed using two solvents: acetonitrile and 0.1 % (v/v) formic acid in Milli-Q water with the linear gradient combination described in the Table 7.1.

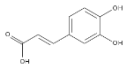
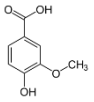
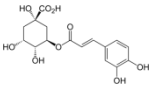
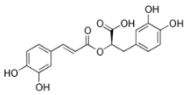
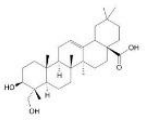
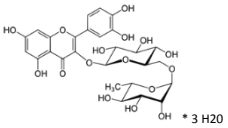
The intensity was measured in absorbance units (mAU).

7.2.5. Bioassays

The PRE, the PRE HPLC fractions and the pure compound isolated by the preparative chromatography (section 7.2.6) were evaluated for inhibitory activity on *P. cinnamomi* mycelia growth.

Commercially supplied natural compounds including phenolic acids, rutin and hederagenin were also evaluated, taking into account their activity on other *Phytophthora* spp (Bais *et al.*, 2002; Widmer & Laurent, 2006) and making the hypothesis that they might be present in the PRE. Moreover, combinations between these compounds were also tested to look for synergies. The compounds and combinations used are described in Table 7.3.

Table 7.3. Commercially supplied compounds and their combinations at 200 µM which were tested on *Phytophthora cinnamomi* mycelial growth.

Compound	Molecular formula	Molecular weight
Caffeic acid		M =180.16
Vanillic acid		M =168.15
Chlorogenic acid		M =354.31
Rosmarinic acid		M =360.31
Hederagenin		M =472.70
Rutin	 * 3 H20	M =610.52
Caffeic+Vanillic acid		
Caffeic+Chlorogenic acid		
Caffeic+Rosmarinic acid		
Vanillic+Chlorogenic acid		
Vanilic+Rosmarinic acid		
Chlorogenic+Rosmarinic acid		
Caffeic+Vanillic+Chlorogenic acid		
Caffeic+Vanillic+Rosmarinic acid		
Caffeic+Chlorogenic+Rosmarinic acid		
Vannilic+Chlorogenic+Rosmarinic acid		

The PRE at concentrations of 0.5 $mgml^{-1}$, 1.0 $mgml^{-1}$ and 5 $mgml^{-1}$, HPLC fractions, the pure compound isolated by preparative chromatography (section 7.2.6) and commercially supplied compounds at concentrations of 0.5 $mgml^{-1}$, 0.1 $mgml^{-1}$ (212 µM) and 200 µM respectively, were used incorporated into V8A culture medium. Six millilitres of culture media were poured into 50 mm Petri dishes and allowed to solidify. Control Petri dishes received the same amount of culture media and the corresponding amount of solvent [water 100 % or ethanol:water (10 %/90 %)]. A mycelial agar plug (5 mm in diameter) of isolate PA 37 was then

cut from the edge of an actively growing colony and placed on each Petri dish. Petri dishes were sealed with Parafilm®, put into plastic bags, grouped according to the treatment and incubated, in the dark, at 24.0 °C.

The length and width of the colonies were measured every day until colonies reached the edge of the dish (about 2-3 days). These assays were performed in triplicate. For PRE and each HPLC fraction, the experiment was performed once. The experiment was repeated three times for the pure compound isolated by preparative chromatography and twice for the commercially supplied compounds.

After 3 days (72 h), the percentage of inhibition by each substance and by the PRE was calculated after subtracting the diameter of each agar plug (5 mm) from the diameter of each colony, as follows:

Inhibition (%) =100 [(Ut-Tr)/Ut], where, Ut =mean value for untreated control and Tr =mean value for media supplemented with plant extracts (Mekuria, 2005).

7.2.6. Preparative separation of the active compound by column chromatography

All solvents used were PA grade or equivalent. Acetone was purchased from Sigma–Aldrich (Steinheim, Germany). Ethanol absolute, methanol and sea sand (0.25-0.3 mm) washed thin grain were obtained from Panreac (Barcelona, Spain). Ethyl acetate was purchased from Lab-Scan (Gliwice, Poland). Silicagel 60 (0,040-0,063 mm), 230-400 mesh ASTM was acquired from Merck (Darmstadt, Germany).

Evaporation of *Phlomis purpurea* crude root extract

Phlomis purpurea crude root extract (1,250 ml) was filtered to remove particles in suspension. The filtrate was evaporated in a rotary evaporator (Buchi, Rotavapor RII) operating with a pump at pressure 230 Vac (230psi) (Buchi, RV – 500) at 60 ^{o}C till dryness. The resulting residue was further dried in a desiccator for several days to constant weight (*ca* 6 g).

The residue was solubilised in absolute EtOH and analysed by thin layer chromatography (TLC, Silicagel 60 F254) using as eluent EtOAc:MeOH (97.5 %/2.5 %).

Liquid – liquid extraction

The crude *P. purpurea* extract (*ca* 20 g) in 100 ml EtOH/H_2O (v/v, 1/1) was extracted four times with 50 ml EtOAc. The organic and the aqueous phases were concentrated *ca* 5 ml to 10 ml.

The TLC analysis showed that the compound that eluted in HPLC F5, where the major biological activity was concentrated, was present in the organic phase (in each extract), and in

a minor amount in the aqueous phase (Figure 7.1). This was further confirmed by LC-MS (Figures 7.2, 7.3). The organic phase was evaporated to dryness. A third of the residue (4.8 g/3=1.6 g) was solubilised in about 20 ml EtOAc, mixed with 15 g silica (Silicagel 60 (0,040−0,063 mm), 230-400 mesh ASTM) and the EtOAc evaporated in the rotary evaporator.

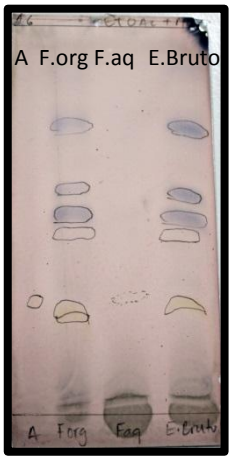


Figure 7.1. Thin layer chromatography (TLC). A – active compound from F5, "F.org" =organic phase, "F.aq" =aqueous phase, "E. Bruto"=PRE. Compound is arrow-headed. The revealing was under UV light and by spraying with anisaldehyde, followed by heating.

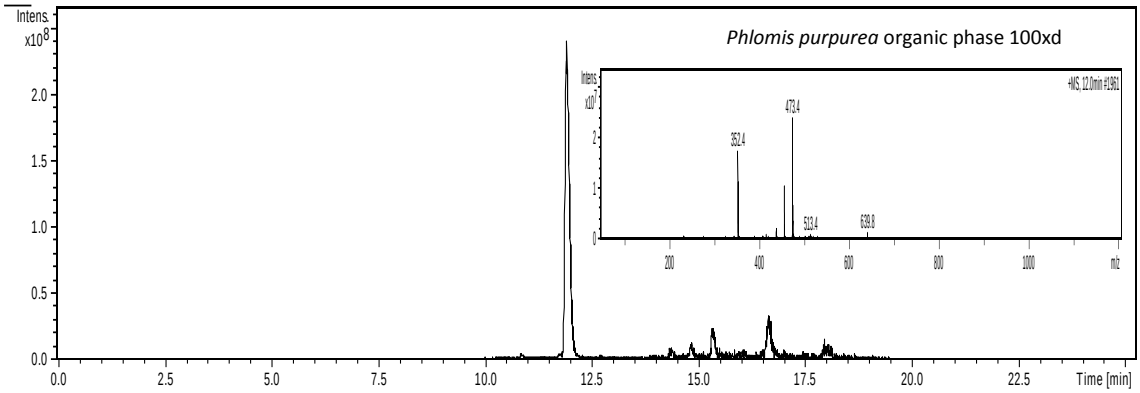


Figure 7.2. LC-MS of the organic phase of *Phlomis purpurea* extract diluted 100 times. For conditions see section 7.2.4. The intensity was measured in absorbance units (mAU).

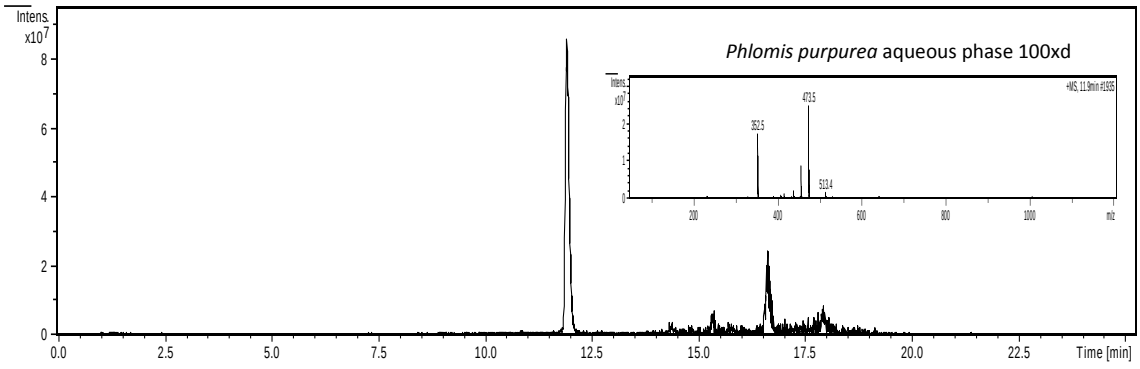


Figure 7.3. LC-MS of the aqueous phase of *Phlomis purpurea* extract diluted 100 times. For conditions see section 7.2.4. The intensity was measured in absorbance units (mAU).

Column chromatography separation

A 35 cm glass column with 3 cm diameter was packed with 70 g of silica to 3/4 of the column length and EtOAc added slowly to avoid clumps and air bubbles. A 2 cm layer of washed sea sand followed by the sample entrapped in silica were applied on top of column. The column was fed with EtOAc at a rate of 2 $mlmin^{-1}$ under pressure with a solvent delivery module (Shimadzu LC - 6A) (Figure 7.4). This elution system was chosen based on an optimization by Campos (2012).

Figure 7.4. Column chromatography separation apparatus.

Fifty millilitre fractions were collected, evaporated and analysed by (TLC). Six to 7 L of EtOAc was used. The active compound eluted from 4.5 to 6 L of EtOAc after which 1 % MeOH was added to accelerate the elution.

The fractions containing the pure active compound were combined and evaporated to dryness. The residue was resuspended in 10 ml EtOAc and submersed in an ultrasonic bath (P Selecta – Ultrasons H-D) until it completely solubilised. The resulting solution was transferred to a 25 ml Erlenmeyer flask that was immersed in a water bath (P Selecta – Precisterm) at 70 °C and shaken to reduce the amount to about 5 ml of solvent. A few drops of pentane were added to decrease the solubility of the compound in EtOAc and facilitate recrystallization. The solution was left at room temperature for 2 days and for another day at 4 °C at which point a precipitate was formed. This was filtered through a sintered glass filter nr. 3 and the precipitate washed with cold EtOAc.

The separation was repeated twice under the same conditions.

7.2.7. Spectroscopic and spectrometric analyses of the purified pure compound

The purified active compound was analysed by TLC: silica gel 60 F254 plates (AL TLC 20x20; Merck); revealing was under UV light and by spraying with anisaldehyde , followed by heating.

MS data were obtained on a Bruker, HCT ultra spectrometer (section 7.2.4).

IR spectra were recorded on an IR spectrophotometer (Bruker, Tensor II) using KBr pellets.

NMR spectra were recorded on a Bruker Avance 600 MHz (Bruker BioSpin Corporation, Billerica, MA) controlled by Topspin V3.1 and equipped with a 1.7 mm TCI Cryo probe. 1D spectra were recorded using an approximate sweep width of 8400 Hz and a total recycle time of approximately 7 s. The resulting time-averaged free induction decays were transformed using an exponential line broadening of 1.0 Hz to enhance signal to noise. The 2D data were recorded using the standard pulse sequences provided by Bruker. At minimum a 1K x 128 data matrix was acquired using a minimum of 2 scans and 16 dummy scans with a spectral width of 10000 Hz in the f2 dimension. The 2D data sets were zero-filled to at least 1k data point. Post-acquisition data processing was performed with either Topspin V3.1 or MestReNova V8.1.

Phlomispurpentaolone:

IR (KBr): 3382, 2958, 2929, 2860, 1730, 1461, 1382, 1282, 1272, 1122, 1072, 1039, 742 (Figure 7.13).

Positive ESI MS/MS: m/z: 513.3 $[M+Na]^+$ (Calcd for $C_{29}H_{46}O_6Na$: 513.3186); 491.3 ($[M+H]^+$, $C_{29}H_{46}O_6^+$; calc. 491.337265 (Figure 7.16).

The ^1H and ^{13}C NMR nuclear magnetic resonance (NMR) data will be published soon elsewhere[41].

7.2.8. X-Ray Crystallographic Analysis

Structural characterization was further elucidated by single-crystal X-ray diffraction, using a diffractometer MD2 (MAATEL); absorption correction Multi-scan; *T*min, *T*max 0.897, 0.992, respectively; number of measured, independent and observed [$I > 2\sigma(I)$] reflections were 32427, 9594, 8160, respectively; R_{int} 0.097; ϑmax (°) 27.7; $(\sin \vartheta/\lambda)_{max}$ ($Å^{-1}$) 0.476[42].

[41] We thank Dr. Gregory S. Walker from Pfizer Inc. Groton CT, for recording NMR spectra
[42] In Unidade de Diffraccion de RaiosX, RIAIDT, Santiago de Compostela

7.2.9. Statistical analysis

Analyses of the results were performed using the software package "PASW statistics 18" (IBM software, 2009 SPSS Inc. Hong Kong). The dependent variable was the colony diameter, with extracts, fractions, pure compound isolated by preparative chromatography and commercially supplied natural compounds concentrations and replicates as the independent variables. The mean values were compared with those from control plates or among them by Fisher's protected least significant difference (LSD) test at $P \leq 0.05$ (Steel and Torrie 1985) and by Independent Samples T-Test when less than 3 groups were present.

7.3. Results and discussion

7.3.1. Extract preparation

The *Phlomis purpurea* crude root extract (PRE) was prepared 1) to provide material to determine its inhibitory activity on *Phytophthora cinnamomi* mycelia growth, 2) to fractionate it by HPLC and determine which fraction had the highest inhibitory activity on *P. cinnamomi* mycelia growth and finally 3) to ascertain which compound(s) is (are) responsible for that activity.

From the liquid-solid extraction of *P. purpurea* roots (200 g) with EtOH/H_2O (70/30) *ca.* 20 g of PRE were obtained [yield (w_{PRE}/w_{roots}) =10 %]. Wójcik (2010) obtained yields ranging between 6.2 and 8.6 %, whereas Campos (2012) obtained a yield of 3.6 %. These differences might be due to the variation in water content of the roots that were collected at different times and in different locations.

7.3.2 Activity of *Phlomis purpurea* crude root extract (PRE) on *Phytophthora cinnamomi* mycelia growth

The activity of PRE on *P. cinnamomi* mycelial growth was evaluated by Neves (2007) and Neves *et al* (2014). However, as it was necessary to prepare new extracts, a new evaluation was carried out.

PRE significantly inhibited *P. cinnamomi* mycelia growth at 0.5, 1.0 and 5.0 $mgml^{-1}$ PRE by 53.2, 62.5 and 74.1 %, respectively (Figure 7.5). These results are comparable with those obtained by Neves (2007) and Neves *et al*. (2014). In Neves *et al*. (2014), V8A was also used as culture media, and the inhibition was found to be slightly higher (72.8 and 80.8 % inhibition at PRE 1.0 and 5.0 $mgml^{-1}$, respectively). In the work by Neves (2007), using another culture

media (potato dextrose agar - PDA), the percentages of inhibition were 78.3 % at 1.0 $mgml^{-1}$ PRE and 93.1 % at 5.0 $mgml^{-1}$ PRE. These differences might be due to a difference in the PRE composition, since plants were harvested at different places, with different biotic and abiotic conditions, as it is known that these factors can change the quantitative composition of plant extracts. The stage in the plant life cycle can also affect the chemical composition of the plant and hence of the plant extract.

Root extracts from other plant species have been evaluated for their activity against *P. cinnamomi.* For example, selected crude root extracts from *Persea americana* var. *drymifolia* (Mexican avocado) with potential resistance to *P. cinnamomi*, were able to inhibit its mycelial growth, *in vitro*. Seven accessions inhibited mycelial growth by more than 50 % (exact percentage not specified) and two accessions inhibited mycelial growth by 100 %, but the concentration is not mentioned by the authors (Sánchez-Pérez *et al.*, 2009).

In the next step PRE was fractionated by HPLC and the fraction with the best inhibitory activity on *P. cinnamomi* mycelial growth was determined.

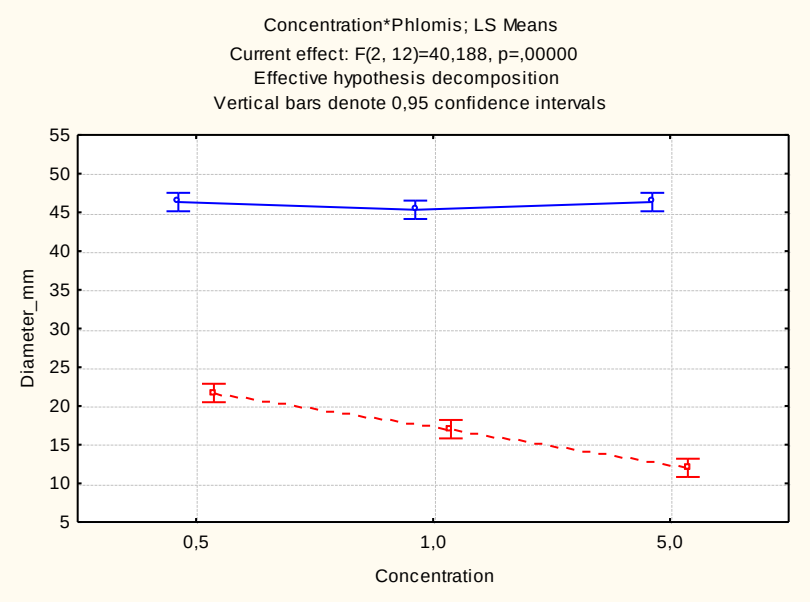


Figure 7.5. Diameter of colonies versus concentration of PRE *Phythophthora cinnamomi* mycelial growth, at day 3, in V8A supplemented with *Phlomis purpurea* crude root extract at 0.5, 1.0 and 5 $mg.ml^{-1}$ (red) and not supplemented (blue-control).

7.3.3. HPLC analysis and fractionation of *Phlomis purpurea* crude root extract (PRE)

HPLC analysis of *Phlomis purpurea* crude root extract (PRE) was performed and fractions were collected in order to isolate a fraction with anti-*P. cinnamomi* activity and finally, the compound responsible for that activity.

The PRE was analysed by HPLC using a column LiChrospher® 100 RP-18 endcapped which is suited for the chromatography of acidic, neutral and weakly basic compounds, substances frequently found in all analytical fields.

The chromatogram gave information about the number of components in the extract and about their polarity. As a reverse stationary phase was used, the compounds with lower retention times are more polar and with increasing of the time, the polarity decreases.

Six fractions were collected every 3 min (Figure 7.6), over ca 200 runs, evaporated and their activity on *P. cinnamomi* mycelial growth evaluated.

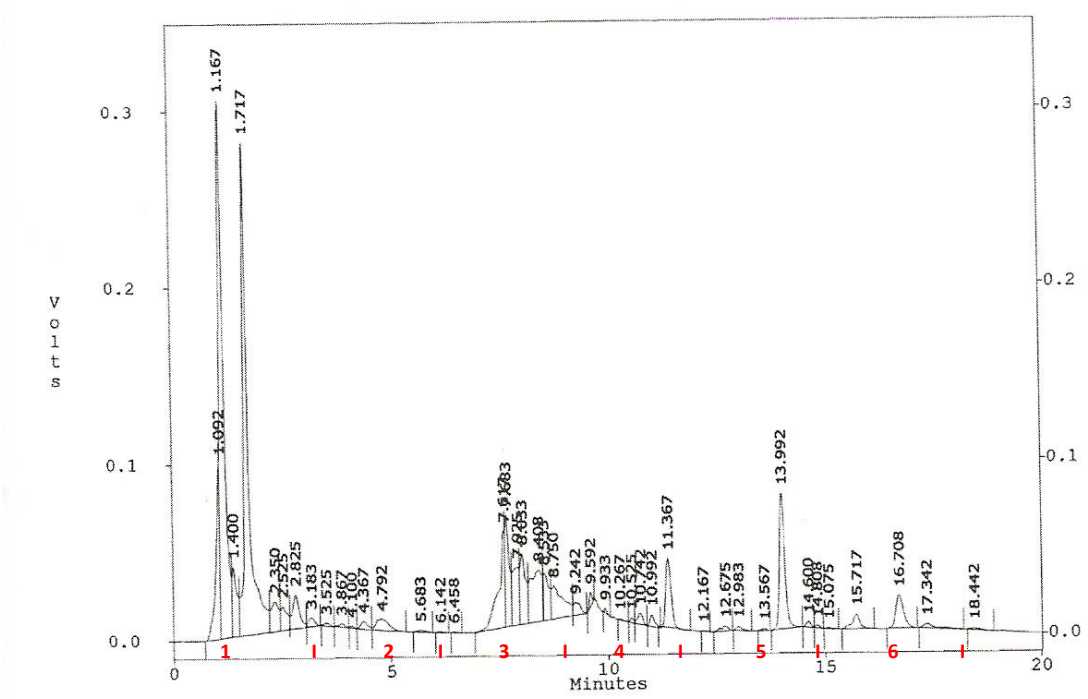


Figure 7.6. HPLC chromatogram of *Phlomis purpurea* crude root extract (PRE) eluted from LiChrospher® 100 RP-18 endcapped. Detection was at 254 nm. Gradient elution was performed using two solvents: acetonitrile and 0.1 % (v/v) formic acid in Milli-Q water in conditions described under section 7.2.3. In red are the numbers of fractions collected.

7.3.4. Activity of HPLC fractions of PRE on *Phytophthora cinnamomi* mycelial growth

All HPLC fractions of PRE were screened for their activity on *P. cinnamomi* mycelial growth. Fractions 3, 4, 5 and 6 (F3, F4, F5 and F6) significantly inhibited *P. cinnamomi* mycelial growth (PCMG) at 0.5 $mgml^{-1}$. Fractions 1 (F1) and 2 (F2) had no activity on PCMG (Figure 7.7).

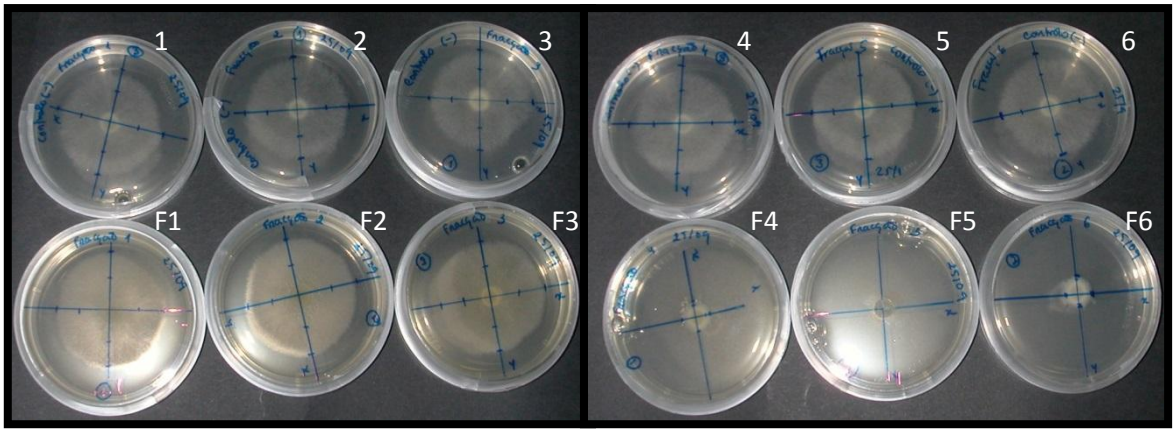


Figure 7.7. Activity of *Phlomis purpurea* root extract (PRE) HPLC fractions on *Phytophthora cinnamomi* mycelial growth, at day 3, in V8A supplemented with PRE. HPLC fractions tested at 0.5 mg.ml^{1} collected by HPLC. Petri dishes 1 to 6 are controls and F1 to F6 are HPLC collected fractions.

Fraction 5 completely inhibited the mycelial growth of the oomycete at 0.5 mgml^{-1} (100 % inhibition). However, F3, F4 and F6 were also active[43] (Table 7. 4), either because they also contained the active compound that is present in F5, or due to the presence of other product(s) that are also biologically active (Table 7. 5).

In the above cited work, root extracts of *Persea americana* accessions that completely inhibited *P. cinnamomi* mycelial growth were fractionated by preparative TLC. Fractions were recovered and bio assayed to identify the active fractions. A 100 % inhibition against mycelial growth of *P. cinnamomi* was obtained in two fractions (Sánchez-Pérez *et al.*, 2009). The preparative TLC fractionating procedure used in that work might have been a more expeditious way to recover PRE fractions than the time consuming procedure that we have chosen (we had no available preparative HPLC) but not so efficient in separating the complex PRE mixture.

[43] Appendix IV.I

Table 7.4. Colony growth of *Phytophthora cinnamomi* in presence of 0.5 mgml^{-1} PRE fractions.

Sample	Colony diameter* ± SD	Percentage of inhibition of *Phytophthora cinnamomi* (%)
Control F1 F1	45.7 ± 0.6 a 45.7 ± 0.6 a	0.0
Control F2 F2	45.7 ± 0.6 a 46.3 ± 0.6 a	0.0
Control F3 F3	46.0 ± 0.0 a 44.3 ± 0.6 b	4.0
Control F4 F4	46.3 ± 0.6 a 14.0 ± 2.0 b	69.6
Control F5 F5	46.3 ± 0.6 a 0.0 ± 0.0 b	100.0
Control F6 F6	46.3 ± 0.6 a 13.0 ± 1.0 b	71.7

*Statistical comparison was made between the control and respective fraction. The means of three replicates ± the Standard Deviation (SD) followed by the same letter are not significantly different following Fisher's protected LSD (Least Significant Difference) test (Steel and Torrie, 1985) with $P<0.05$.

7.3.5. ESI LC/MS analysis of the fractions

The six fractions collected every three minutes over 200 runs (Figure 7.6) were analysed by ESI LC/MS. The full-scan mass spectra of each fraction are presented in Figure 7.8.

The full-scan mass spectrum profile of F6 shows a high similarity with that of F5 supporting the hypothesis that the activity exhibited by F6 is due to the same compound present in both fractions. Fraction 4 has a completely different full-scan mass spectrum, so it is reasonable to expect that its biological activity is caused by a different substance(s).

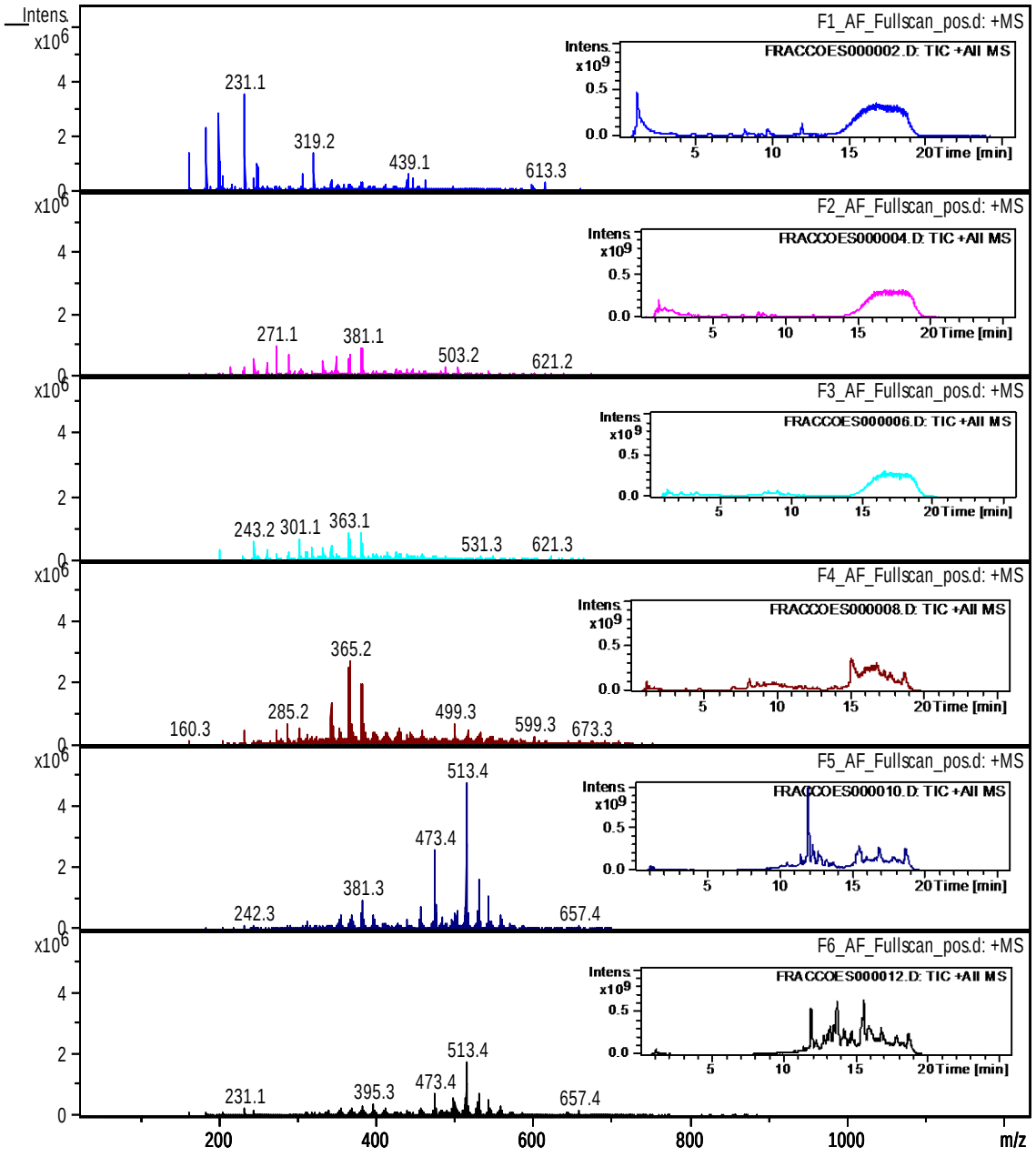


Figure 7.8. Full-scan ESI-MS for each collected fraction (F1 to F6). For conditions see section 7.2.4. The intensity was measured in absorbance units (mAU).

The full-scan mass spectrum profile of F6 shows a high similarity with that of F5 supporting the hypothesis that the activity exhibited by F6 is due to the same compound present in both fractions. Fraction 4 has a completely different full-scan mass spectrum, so it is reasonable to expect that its biological activity is caused by a different substance(s).

The mass spectrum of compound eluting at 14 min retention time (F5) in positive ion mode appears in the eluted at *ca* 12 min using LC-MS (see section 7.2.4). It is shown in Figure 7.9.

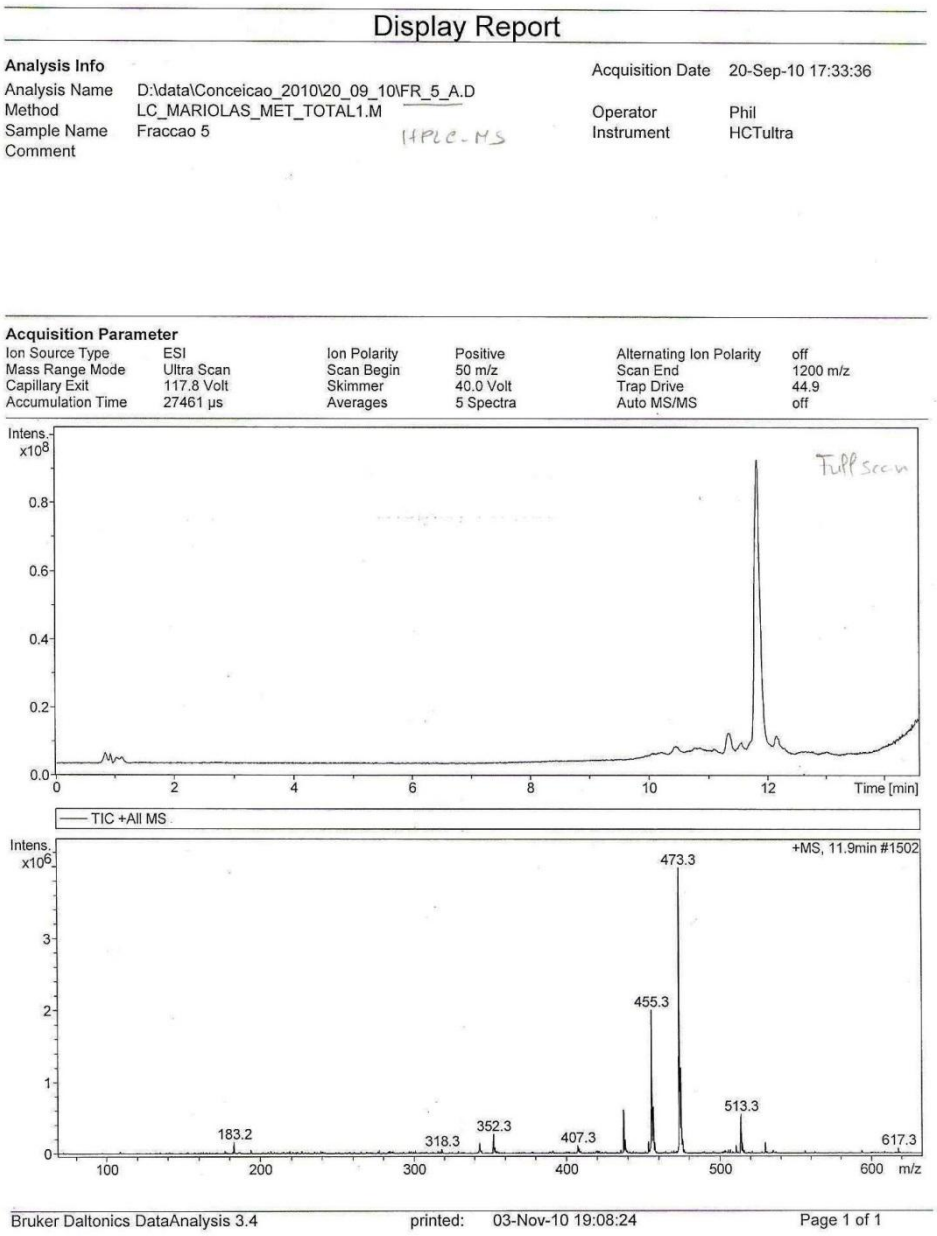


Figure 7.9. LC-MS of the peak with retention time 11.9 obtained in full-scan mode. For conditions see section 7.2.4. The intensity was measured in absorbance units (mAU).

This compound responsible for the inhibition of *P. cinnamomi* mycelial growth appears to have both hydrophobic and hydrophilic characteristics because it elutes at an intermediate retention time. It interacts with the non-polar phase which confers hydrophobicity but also has hydrophilic characteristics because it is soluble in water.

7.3.6. Activity of commercially supplied natural compounds on *Phytophthora cinnamomi* mycelial growth

It was first hypothesised a $[M+H]^+$ =473.4 for the above compound. This m/z value for the ($M+H^+$) molecular ion is compatible with the molecular formula of hederagenin, an aglycone derived from β-amyrin, a frequently occurring saponin of which *P. purpurea* is rich (Madl *et al.*, 2006). Therefore hederagenin and other natural compounds including phenolic acids and rutin were bio-assayed, based on their inhibitory activity against other *Phytophthora* spp (Bais *et al.*, 2002; Widmer & Laurent, 2006) and/or on their presence in PRE. Three natural compounds were identified in PRE: chlorogenic acid with retention time 1.6 min detected with m/z 355; caffeic acid with retention time 2.0 min and m/z 181 and vanillic acid with retention time 2.1 min, observed at m/z 169, all in positive ion mode (Cravador *et al.*, 2010).

The results of bioassays with these compounds obtained from commercial supply and their possible synergies based on the hypothesis that they may be present in the PRE (hederagenin, rosmarinic acid and rutin) and on the knowledge that they are present (caffeic, chlorogenic and vanillic acids) (Cravador *et al.*, 2010) are shown in Table 7.5.[44]

Phenolic acids, including rosmarinic acid (RA), an ester of caffeic acid (CA), rutin and hederagenin were tested at 200 µM. Vanillic acid (VA) and its combination with CA showed the highest percentage of *P. cinnamomi* inhibition, with 33.3 and 36.7 %, respectively. Vanillic acid present in F1, could have contributed to the anti-*P. cinnamomi* activity of PRE. This fraction (F1) did not show any bioactivity against this oomycete, probably because VA is present in trace amounts and therefore not enough to cause inhibition of *P. cinnamomi* mycelial growth. The inhibition of *P. cinnamomi* mycelial growth by VA, in our work, was higher than the 13.1 % inhibition of *Phytophthora capsici* mycelial growth observed with 248 µM VA by Candela *et al* (1995).

Caffeic acid inhibited *P. cinnamomi* mycelial growth by 10.6 % but its ester, RA, had no inhibitory effect. CA and RA, isolated from medicinal plants, were shown to inhibit zoospore germination of *P. capsici*, *P. megakarya* and *P. palmivora in vitro*, at concentrations of 3 and 6 $mgml^{-1}$ respectively, corresponding to 16,650 µM (*ca* 83 times more than the concentration used in the present work) of both CA and RA (Widmer & Laurent, 2006). Moreover, RA inhibited at 200 µM, the mycelia growth of *P. drechsleri* by 71.9 %, *P. megasperma* by 43.8 % and *P. parasitica* by 43.4 % (Bais *et al.*, 2002).

[44] Appendices IV.II., IV. III.

In our study, chlorogenic acid, hederagenin and rutin showed no inhibitory effect on *P. cinnamomi* mycelial growth and as far as we know, there are no references to their activity on *Phytophthora* spp.

It appears that none of the above commercially supplied natural compounds tested are responsible for the inhibitory activity of the *P. purpurea* extract on *P. cinnamomi* mycelial growth, they had no activity comparable with the PRE or their derived fractions 4, 5 and 6.

Table 7.5. *Phytophthora cinnamomi* colony growth in the presence of 200 µM phenolic acids, hederagenin and rutin.

Commercially supplied compounds	Colony diameter* ± SD		Percentage of inhibition of *P. cinnamomi* (%)
Control	4.1 ± 0.2	f	-
Caffeic acid	3.6±0.1	d, e	10.6
Vanillic acid	**2.7±0.2**	**a, b**	33.3
Chlorogenic acid	4.1±0.2	f	0.0
Rosmarinic acid	4.1±0.2	f	0.4
Hederagenin	4.1±0.1	f	0.4
Rutin	4.1±0.1	f	0.0
Control 2 combinations	4.0±0.1	f	-
Caffeic+Vanillic acid	**2.6±0.1**	**a**	36.7
Caffeic+Chlorogenic acid	3.3±0.1	c	18.8
Caffeic+Rosmarinic acid	3.4±0.1	c, d	16.3
Vanillic+Chlorogenic acid	**2.7±0.1**	**a, b**	32.8
Vanilic+Rosmarinic acid	2.9±0.2	b	28.3
Chlorogenic+Rosmarinic acid	3.8±0.2	e, f	5.8
Control 3 combinations	3.9±0.1	f	-
Caffeic+Vanillic+Chlorogenic acid	**2.7±0.1**	**a, b**	31.9
Caffeic+Vanillic+Rosmarinic acid	**2.7±0.1**	**a, b**	31.2
Caffeic+Chlorogenic+Rosmarinic acid	3.5±0.2	c, d, e	10.6
Vannilic+Chlorogenic+Rosmarinic acid	2.9±0.1	b	26.2

*Statistical comparison was made among treatments. The means of six replicates ± the Standart Deviation (SD) followed by the same letter are not significantly different following Fisher's protected LSD (Least Significant Difference) test (Steel and Torrie, 1985) with $P<0.05$.

7.3.7. About hederagenin

Hederagenin is a triterpenoid, isolated from *Hedera helix* (common ivy). It is the aglycone moiety of numerous saponins found in *H. helix*. It is also one of three primary triterpenoids extracted from the seeds of *Chenopodium quinoa* (Madl *et al.*, 2006) and categorized by the US Environmental Protection Agency (EPA) as a biological pesticide.

We have first, tentatively attributed the m/z $[M+H]^+$ =473.4 to the compound eluting at 12 min in HPLC-PDA-ESI/MS/MS in positive mode (see 7.2.4) and assumed as hypothesis that it was hederagenin which has the same molecular weight (MW=472.3552). However, they

have distinct fragmentation patterns as shown in their MS^2 (Figure 7.10) and hederagenin is insoluble in water and in 10 % aqueous ethanol (v/v), whereas that compound is soluble in these solvents. Other triterpenoid saponins that have the same monoisotopic mass as hederagenin are corosolic acid isolated from the *Lagerstroemia speciosa* leaf extract, and maslinic acid isolated from *Olea europaea* fruit, both with the molecular formula $C_{30}H_{48}O_4$.

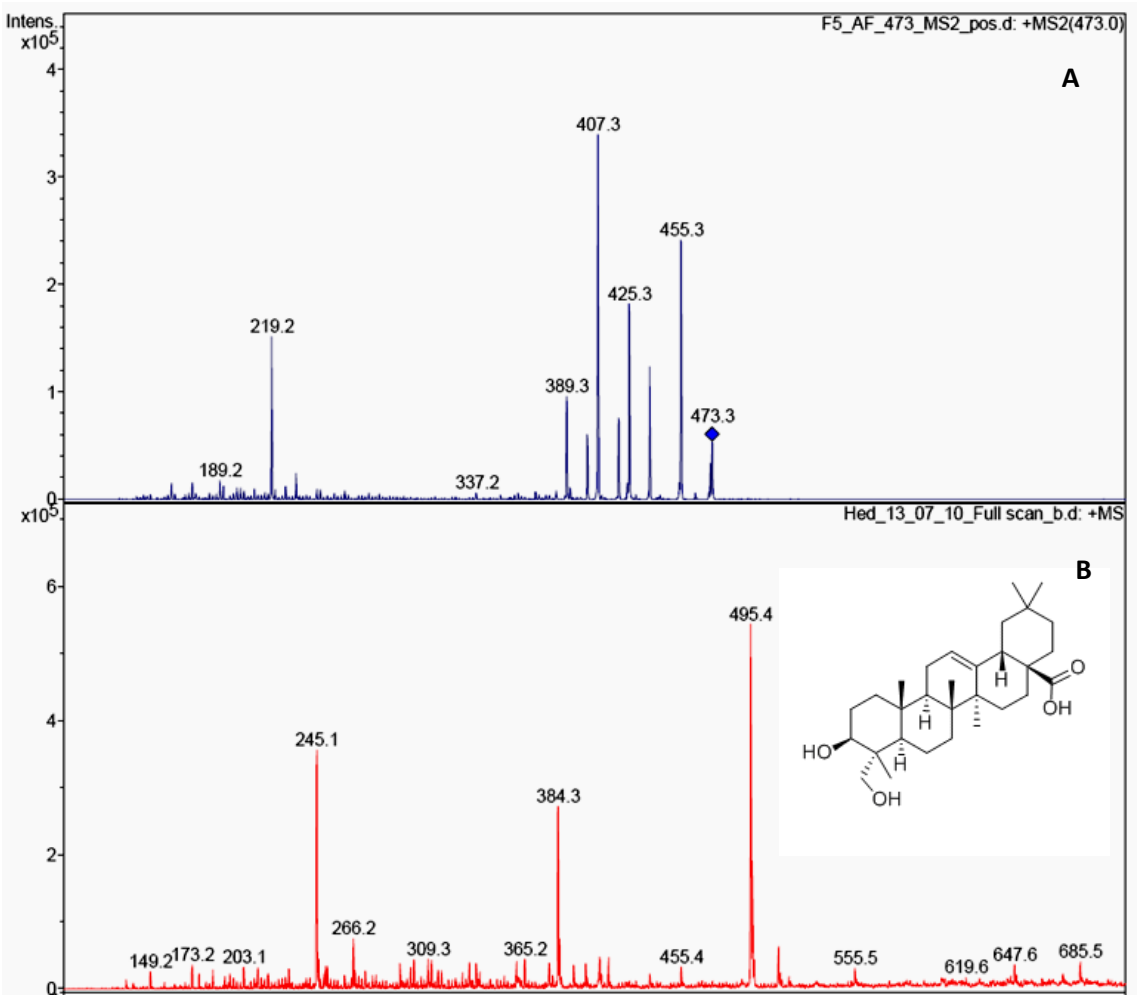


Figure 7.10. A. MS^2 mass spectra of $[M+H]^+$=473 ion (fraction 5) and of **B.** hederagenin at fullscan. For conditions see section 7.2.4. The intensity was measured in absorbance units (mAU).

7.3.8. Preparative isolation of the active compound by column chromatography

In order to structurally characterise the compound responsible for the inhibition of *P. cinnamomi* mycelial growth, isolation and purification was accomplished from PRE yielding the amount needed to perform spectroscopic, spectrometric and XRD analyses.

The yield was 16 mg from 3.2 g. In the former optimization by Campos (2012) 197 mg were obtained from 7.16 g PRE. The discrepancy is due to accidental losses in our work.

The *Phlomis purpurea* crude root extract (PRE) was separated by column chromatography on silica gel under the conditions described in M&M (section 7.2.6.). The chromatographic fractions were analysed by TLC (data not shown) and LC-MS (data not shown). The active compound was identified by chromatographic comparison with the product with retention time of 14 min in HPLC (corresponding to F5 that showed activity against *P. cinnamomi*). These fractions were pooled. The compound was obtained with a purity of 90 %, as a colourless amorphous powder that was further purified by recrystallization (see section 7.2.6 under column chromatography separation).

The bio-assay was performed after precipitation.

7.3.9. Structural characterization

The crystals were analysed by 1D and 2D nuclear magnetic resonance (NMR), electrospray ionization mass spectrometry (ESI MS/MS), infrared spectroscopy (IR) and X-ray diffraction (XRD).

ESI-MS indicated the molecular formula $C_{29}H_{46}O_6$ (m/z 513.3 ($[M+Na]^+$; calc. 513.3186).

The molecular ion 491 forms an adduct with sodium (Na) which is visible in the peak m/z $[M+Na]^+$ =513 (Figure 7.11) and in XRD.

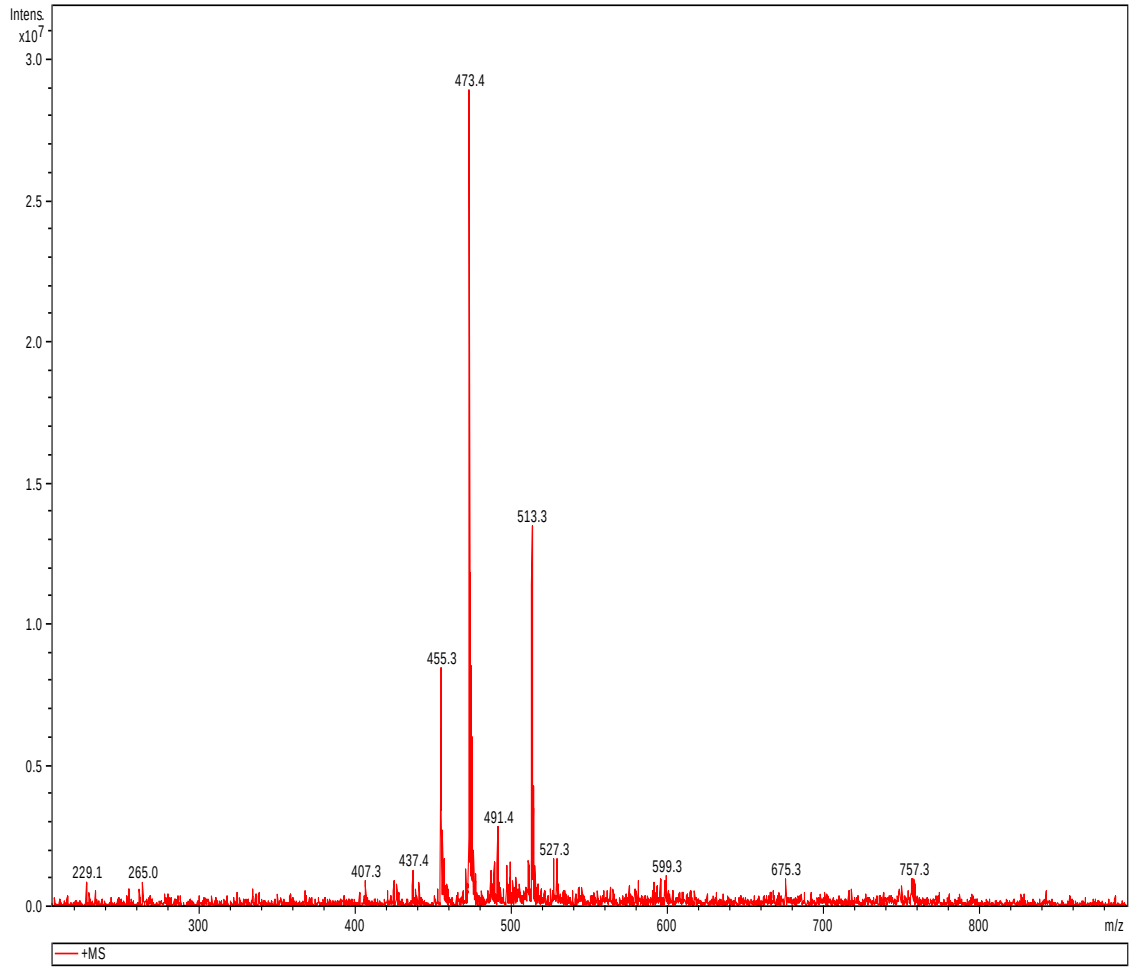


Figure 7.11. Full scan MS spectrum of m/z $[M+H]^+$=491.4. The molecular ion 491 forms an adduct with sodium (Na) which is visible in the peak m/z $[M+H]^+$=513. See section 7.2.4 for conditions.

Fragment m/z $[M+H]^+$ =491 is easily ionisable and loses 1 molecule of water originating the fragment m/z $[M+H]^+$ =473. MS^2 mass spectra of the fragmentation of ion m/z $[M+H]^+$ =473 (Figure 7.12) can be explained by a loss of water resulting in $[M+H]^+$ =455. The loss of a molecule of water, formaldehyde or formic acid from this ionic fragment yields ionic fragments at $[M+H]^+$ =437, 425 and 407, respectively. A loss of water from the latter yields an ionic fragment at m/z 389 $[M+H]^+$. Ion m/z 219 $[M+H]^+$ is also always present in all other mass spectra (data not shown) appearing to be a very stable ion.

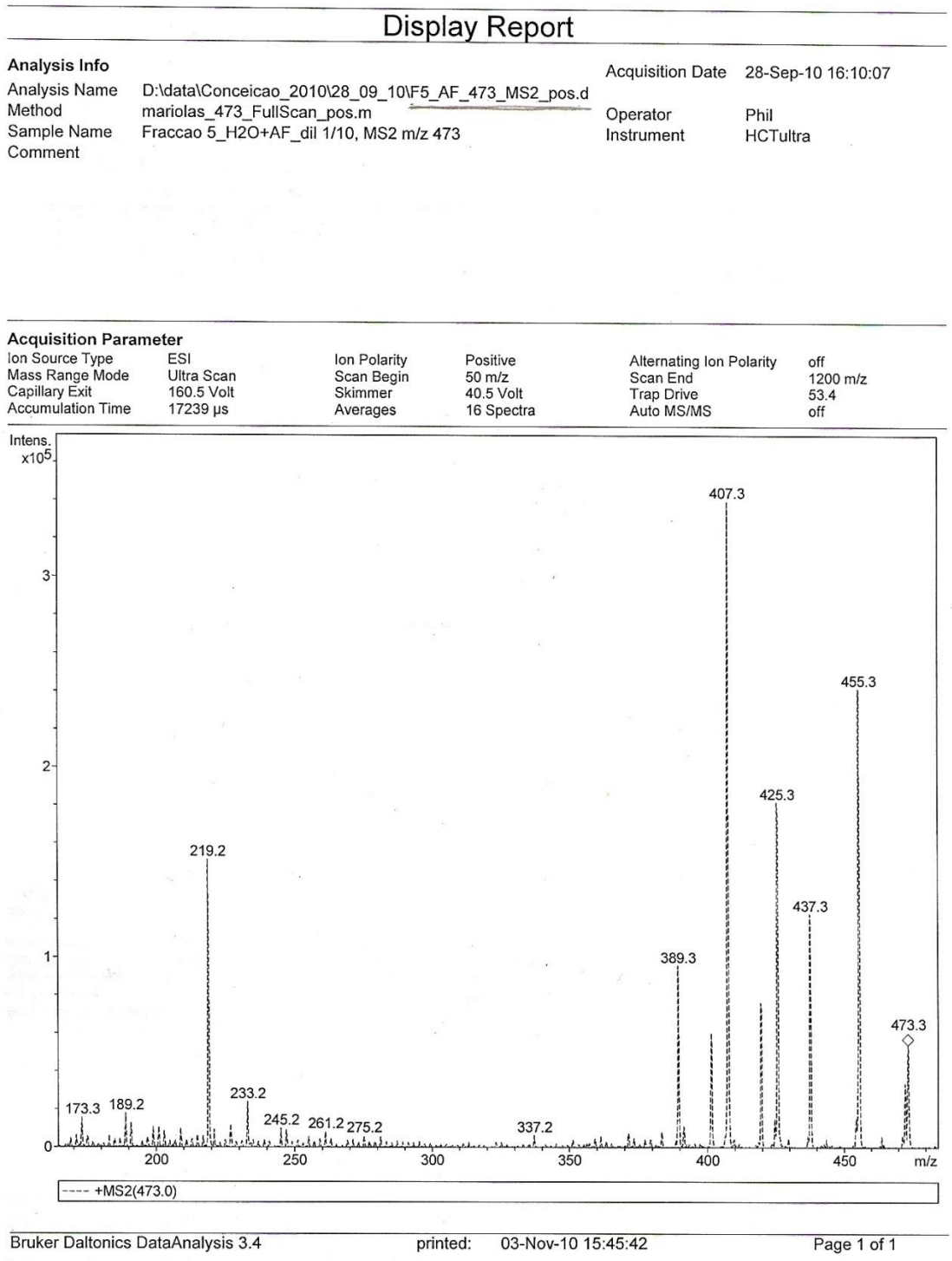
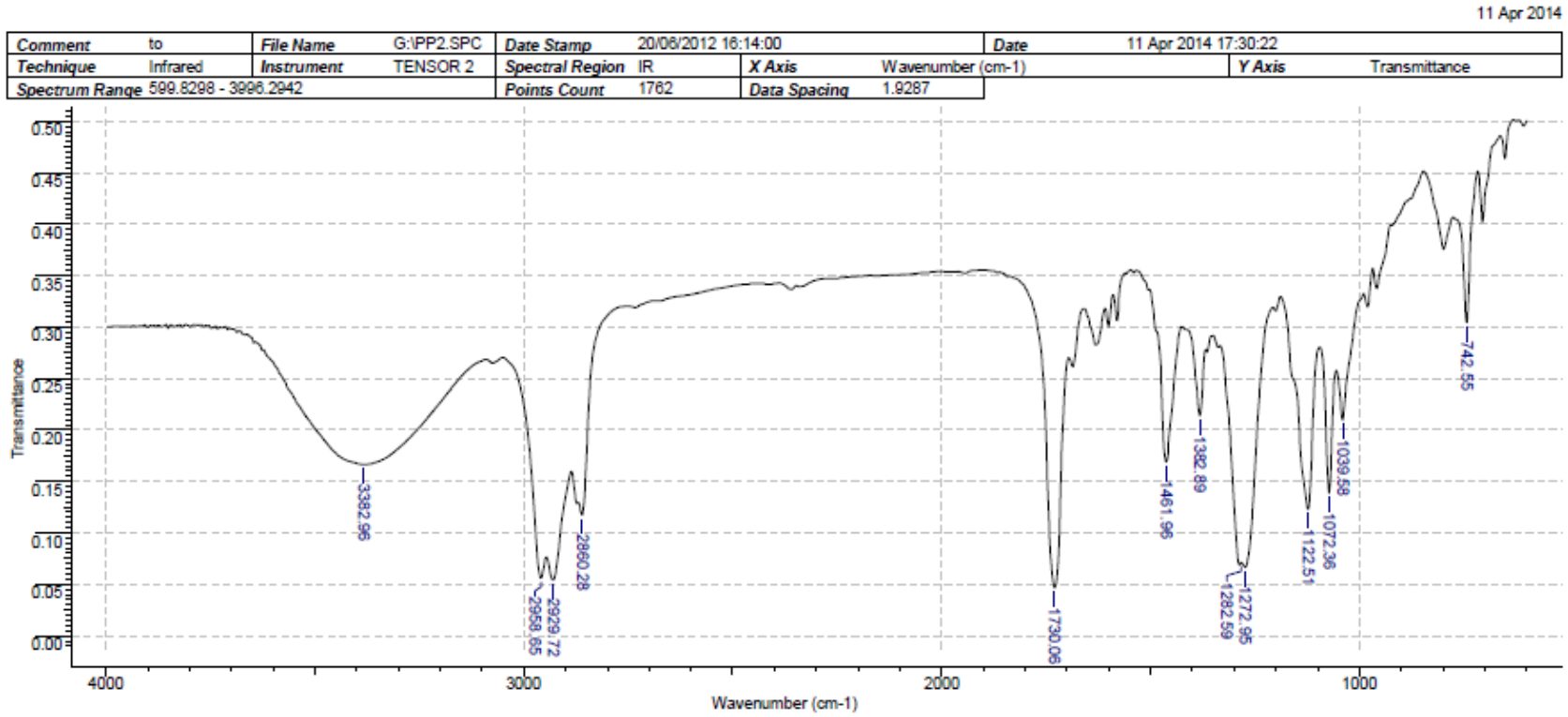


Figure 7.12. MS^2 of m/z =473.3 $[M+H]^+$ ion derived from the molecular ion m/z =491 $[M+H]^+$by loss of H_20.

The IR spectrum revealed the presence of hydroxyl (3383 cm^{-1}) and ketone (1730 cm^{-1}) groups (Figure 7.13)

Figure 7.13. Infrared spectra of phlomispurpentaolone.



No	cm-1	T	Intensity
1	742.55	0.305	M
2	1039.58	0.210	S
3	1072.36	0.139	S
4	1122.51	0.123	S
5	1272.95	0.067	VS
6	1282.59	0.071	VS
7	1382.89	0.214	S

No	cm-1	T	Intensity
8	1461.96	0.169	S
9	1730.06	0.047	VS
10	2860.28	0.117	S
11	2929.72	0.054	VS
12	2958.65	0.056	VS
13	3382.96	0.167	S

^{1}H NMR and ^{13}C (600 MHz, DMSO-d6) analyses were performed and included DEPT as well as correlation NMR spectrometry (COSY, TOCSY, HMBC and Multiplicity Edited HSQC).

These data will be soon submitted for publication.

Structural characterization was further elucidated by single-crystal X-ray diffraction after crystals suitable for X-ray structure analysis were obtained[45,46] (Figure 7.14).

A monoclinic crystal was obtained from a CH_3OH solvent system. Crystal data: $4(C_{29}H_{46}O_6)\cdot Na\cdot CH_4O\cdot 9.5(O)$ *Mr*=1100.83, monoclinic. Crystal size=0.4 × 0.05 × 0.05mm3. Cell parameters: *a*=51.762 (10)Å, *b*=8.7460 (17)Å, *c*=26.208 (5)Å, *V*=11778 (4)Å3, space group *C*2. Data collection was performed on a Micro diffractometer (MAATEL, MD2), the structure was resolved by direct methods (SHELXS-97), and the final *R* and *Rw* values were 0.092 and 0.264, respectively, for 9594 observed reflections.

This structure corresponds to a novel 28-noroleanane-derived spirocyclic triterpenoid (17*R*)-2*α*,3*α*,11*α*,23,24-pentahydroxy-19(18→17)-*abeo*-28-norolean-12-ene-18-one designated as (phlomispurpentaolone).

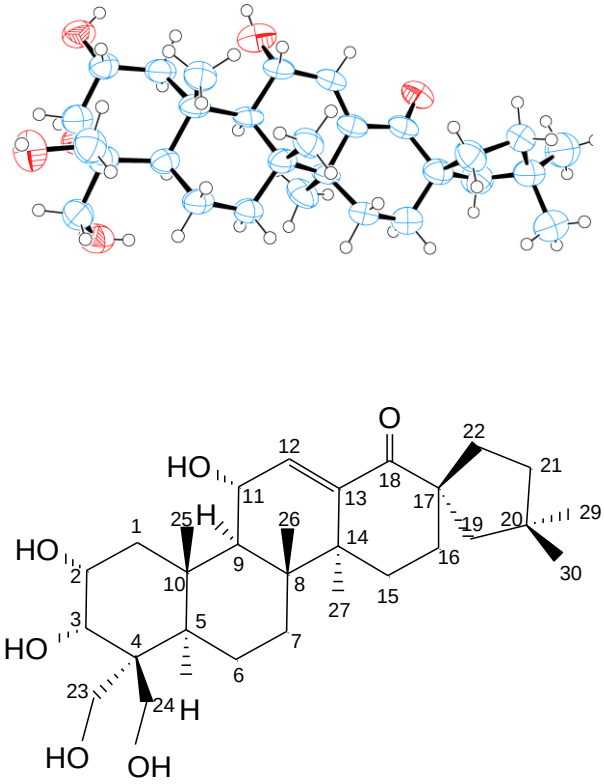


Figure 7.14. Molecular model and structure of (17*R*)-2*α*,3*α*,11*α*,23,24-pentahydroxy-19(18→17)-*abeo*-28-norolean-12-ene-18-one (phlomispurpentaolone).

[45] In Unidade de Diffraccion de RaiosX, RIAIDT, Santiago de Compostela
[46] Appendices IV.IV., IV.V, IV.VI.

7.3.10. Structure and activity of triterpenoids occurring in plants

Terpenes are a large and diverse class of organic compounds, produced by a variety of plants. Terpenes may be classified by the number of isoprene units (C_5H_8) in the molecule; a prefix in the name indicates the number of terpene units needed to assemble the molecule. Triterpenes consist of six isoprene units and have the molecular formula $C_{30}H_{48}$. Triterpenoids are triterpenes that may contain additional oxygen functionality or some rearrangements. However, the two terms are often used interchangeably. Some triterpenoids are steroidal in nature and are found as saponin glycosides which refer to the attachment of various sugar molecules to the triterpene unit. The glycoside moiety can be cleaved off leaving the free aglycone (triterpene).

Triterpenoids are compounds present in a wide range of plants. It has been estimated that more than 20,000 triterpenoids exist in nature (Liby *et al.*, 2007). Triterpenoids are biosynthesized in plants by the cyclization of squalene ($C_{30}H_{50}$), triterpene hydrocarbon, and a natural and vital part of the synthesis of all plant and animal sterols. They can further be subclassified into various groups including cucurbitanes, cycloartanes, dammaranes, euphanes, friedelanes, holostanes, hopanes, isomalabaricanes, lanostanes, limonoids, lupanes, oleananes, protostanes, sqalenes, taraxasteranes, tirucallanes, ursanes, steroids and miscellaneous compounds (Vincken *et al.*, 2007; Bishayee *et al.*, 2011; Mullaeur, 2011).

Triterpenoids are used in traditional medicine, and many of them or their synthetic derivatives are investigated as medicinal products for various diseases, including cancer (Haridas *et al.*, 2001; Lee *et al.*, 2006; Liby *et al.*, 2007; Bishayee *et al.*, 2011; Mullaeur, 2011). Triterpenoid saponins include, for instance, astragaloside (Figure 7.15A) isolated from *Astragalus membranaceus* (Ren *et al.*, 2013), bacoside A (Figure 7.15B) from *Bacopa monniera* (Janani *et al.*, 2010), eleutheroside (Figure 7.15C) from *Eleutherococcus senticosus* (Yu *et al.*, 2003), ginsenoside (Figure 7.15D) from *Panax ginseng* (Deb *et al.*, 2014) and withanolide (Figure 7.15E) from *Withania somnifera* (Subramanian *et al.*, 2014); all of these are well-known medicinal plants.

Pentacyclic triterpenoids have a wide array of biological activities and some of them may be useful even in medicines. These include the pentacyclic lupane-type triterpenoids which are represented by a diverse variety of bioactive natural products (Chaturvedi *et al.*, 2008). Lupeol (Figure 7.15F) is an example that occurs in medicinal plants and even in in various edible fruits such as olive, fig, mango, strawberry and red grapes (ibid). Betulinic acid (Figure 7.15G), another pentacyclic lupane-type triterpenoid found in many plant species, especially in the bark of *Betula alba* and from the bark of *Ziziphus mauritania* Lam., has been discovered through a drug screening program of the National Cancer Institute and is known to

possess diverse pharmacological properties including a remarkable cytotoxic effect against human melanoma cells (Mullaeur, 2011).

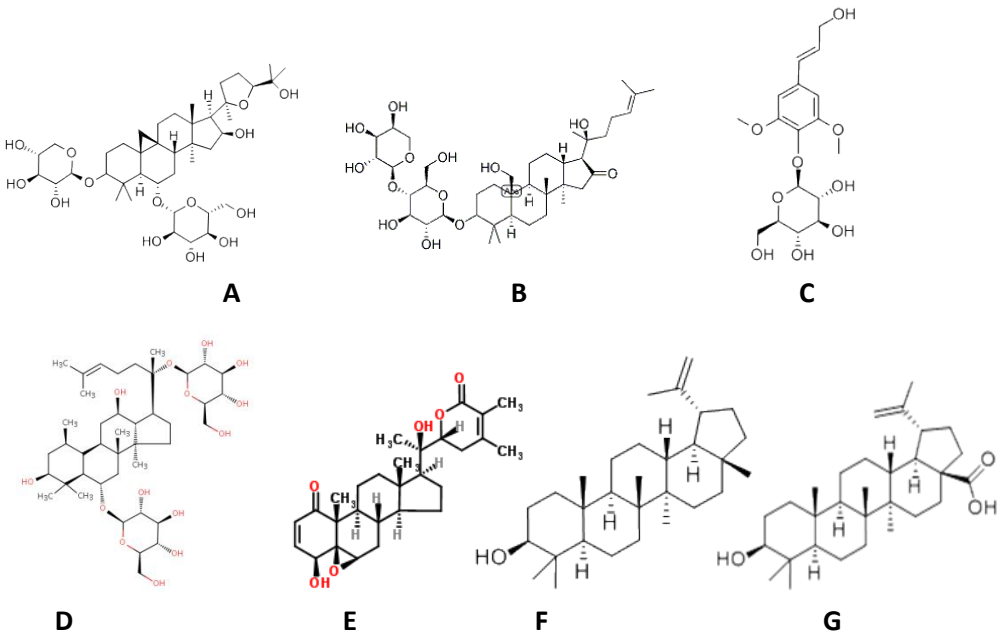


Figure 7.15. A. Astragoloside IV.
Source:http://www.chemicalbook.com/ProductChemicalPropertiesCB8323949_EN.htm;
B. Bacoside A.
Source:http://www.chemicalbook.com/ProductChemicalPropertiesCB9415557_EN.htm;
C. Eleutheroside B.
Source:http://www.chemicalbook.com/ProductChemicalPropertiesCB0217631_EN.htm.
D. Ginsenoside Rg1. Source: http://www.drugbank.ca/drugs/DB06750.
E. Withanolide. Source: http://www.chemspider.com/Chemical-Structure.141995.html.
F. Lupeol. http://www.chemicalbook.com/ProductChemicalPropertiesCB3429132_EN.htm.
G. Betulinic acid.
Source: http://www.chemicalbook.com/ProductChemicalPropertiesCB4667160_EN.htm.

Also oleananes are being investigated for their anti-tumor properties (Sun *et al.*, 2006). Low concentrations of maslinic acid (Figure 7.16A) are to be found in plants with medicinal properties but its concentration in the waxy skin of olives may be as high as 80 %, which triggers apoptosis in human colon-cancer cells (Reyes-Zurita *et al.*, 2013). Oleanolic acid (Figure 7.16B), among others less well studied (reviewed by Sun *et al*. 2006), exhibited potent activity against human leukemia and lymphoma cells, as well as anti-viral, anti-inflammatory, hepatoprotective, gastroprotective, anti-diabetes, hemolytic and antimicrobial activities.

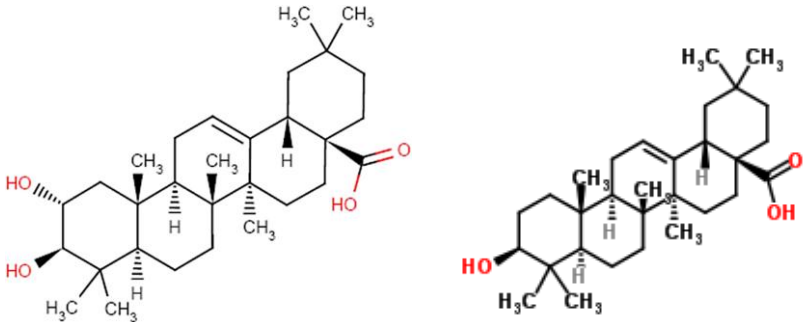


Figure 7.16. A. Maslinic acid [(2a, 3b)-2,3-dihydroxyolean-12-en-28-oic acid]. Source: Reyes-Zurita *et al.* 2013. **B.** Oleanoic acid [(2a, 3b)-2,3-dihydroxyolean-12-en-28-oic acid]. Source: http://www.chemspider.com/Chemical-Structure.10062.html.

The oleanane skeleton (5) can be fragmented to the 17, 22-seco skeleton (5a) or rearranged to a ring A nor-ring B homo skeleton (5b). Degradation of the oleanane skeleton leads to the 23-nor (5c) the 27-nor (5d), the 28-nor (5e) or the 30-nor skeletons (5f) (Figure 7.17)(reviewed by Vincken *et al.* 2007). Oleananes is the most common skeleton occurring in almost all plant orders (ibid). Oleananes can have attached several functional groups at different positions of the skeleton. In the Lamiales order, that includes the genera Phlomis, the following sugars can be present in the saccharide chains: apiose, arabinose, fucose, glucose, rhamnose and xylose.

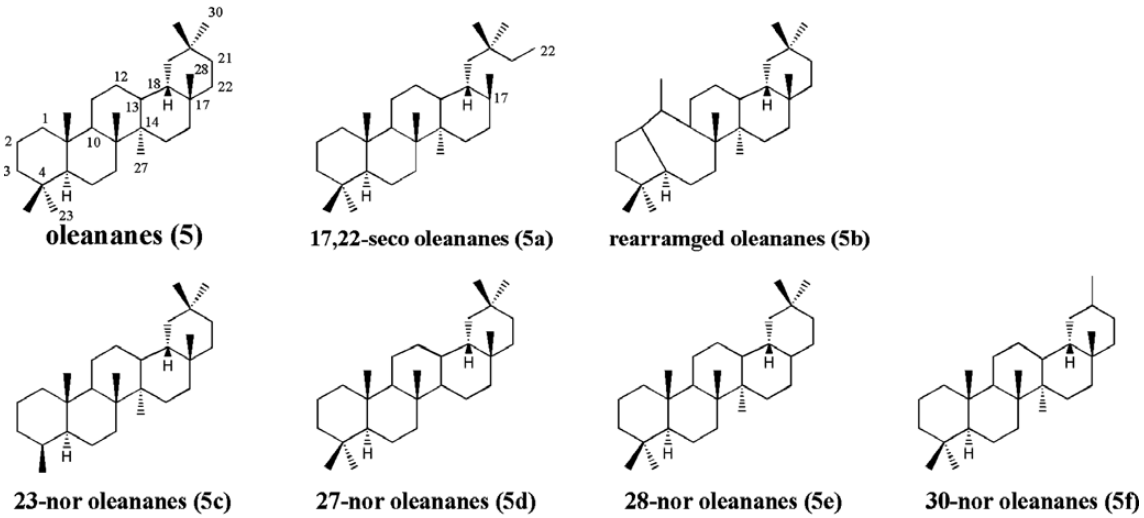


Figure 7.17. Structure of oleananes and their various derivatives. Source: Vincken *et al.* 2007.

The compound we have isolated from *Phlomis purpurea* (phlomispurpentaolone), is structural related to 28-noroleanane-derived spirocyclic triterpenoids isolated from

Phlomis umbrosa rhizomes (Liu *et al.*, 2006, 2008; Deng *et al.*, 2011). It has the same molecular formula as phlomisone but different structure (Figure 7.18). Besides anti-*P. cinnamomi* activity, phlomispurpentaolone might have other antimicrobial and/or medicinal activities that it would be interesting to evaluate.

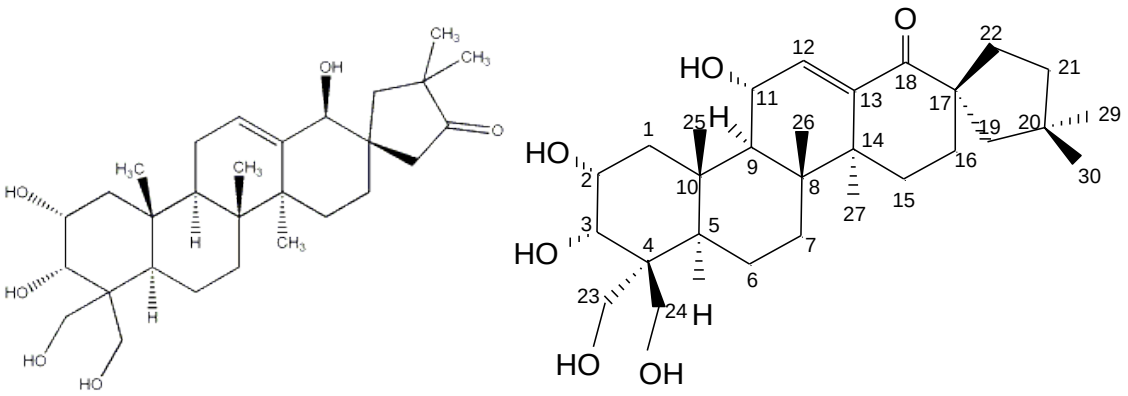


Figure 7.18. Structures of **A.** phlomisone and **B.** phlomispurpentaolone.

7.3.11. Activity of phlomispurpentaolone on *Phytophthora cinnamomi* mycelial growth

Phlomispurpentaolone (Figure 7.14), isolated from the preparative chromatographic column after recrystallization, inhibited at 0.1 $mgml^{-1}$ (204 µM), the mycelial growth of *P. cinnamomi* by 75.7 % (Table 7.6). This value is higher than the inhibition caused by any of the commercially supplied natural compounds tested at a similar molarity (Table 7.5.) and by PRE at all the concentrations tested, as expected (see section 7.3.2). However we could not compare it with F5, since F5 was first tested at 0.5 $mgml^{-1}$ and we could not repeat the bioassays at other concentration because there was not enough compound isolated by that time consuming analytical procedure.

Table 7.6. Colony growth of *Phytophthora cinnamomi* in presence of 0.1 $mgml^{-1}$ (204 µM) phlomispurpentaolone.

Sample	Colony diameter ± SD	Inhibition of *Phytophthora cinnamomi* (%)
Control	3.09± 0.3 a	
Phlomispurpentaolone	0.75± 0.3 b	75.7

*Statistical comparison was made between control and compound. The means of nine replicates ± the Standart Deviation (SD) followed by the same letter are not significantly different following Independent Samples T- Test with *P<0.05*.

Sánchez-Pérez *et al*. (2009) showed that stigmastan-3,5-diene (Figure 7.18) (100 ppm, *ca* 0.1 $mgml^{-1}$, 252 µM) isolated from *P. americana* root extract fraction, completely inhibited the mycelial growth of *P. cinnamomi*. However, the technique they used was slightly different from ours. The compound in 50 µl absolute ethanol was impregnated in filter paper discs, inside a Petri dish containing potato dextrose agar (PDA) culture media, with an inoculated agar plug on top of the disc. In these conditions, stigmastan-3,5-diene (Figure 7.19) appears to have a slight higher activity on *P. cinnamomi* mycelial growth than phlomispurpentaolone.

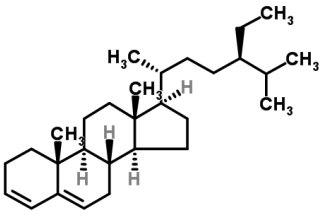


Figure 7.19. Stigmastan-3,5-diene. http://www.chemspider.com/Chemical-Structure.23253957.html.

Phytophthora cinnamomi has a life history along which several kind of structures (see Chapter 1) required for its survival and infection of susceptible hosts (Zentmyer, 1980) are produced. Mycelia of this oomycete give rise to chlamydospore and sporangia (Erwin & Ribeiro, 1996). These structures may geminate directly to form hyphae. The sporangia, under adequate temperature and moisture conditions, produce motile zoospores. Zoospores are the main infective propagules, although hyphae can also infect roots, being the most important inoculum source (ibid). However, zoospores lack cell wall during their motile phase and early stages of encystment, so are the weakest link, and susceptible to disruption by surface-active agents (surfactants), such as saponins. It has been reported in previous studies that *Avena sativa* extracts cause lysis of *P. cinnamomi* zoospores and it was suggested that avenacin(Figure 7.20) a triterpenoid saponin or a related saponin might be involved in the zoospore lysis (Deacon & Mitchell, 1985).

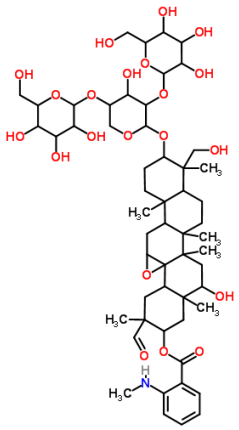


Figure 7.20. Avenacin A 1.
http://www.chemspider.com/Chemical-Structure.152437.html?rid=88ba98d0-4625-4edf-bc9d-d890eea6611b

Neves *et al*. (2014) showed that PRE at 10 mgml^{-1} completely inhibited sporangial production, zoospore and chlamydospore germination, whereas the inhibition of mycelial growth was 85 %. Although we have not examined whether zoospores were disrupted, we observed they could not germinate hence they were not viable. Extensive studies of chemical components extracted from *Phlomis* spp. have led to the identification of several compounds including triterpene saponins (Li *et al.*, 2010). However, these saponins have not been evaluated for their antimicrobial activity. We previously reported that *P. purpurea* leaf extracts, which also contain saponins, did not induce any inhibition of *P. cinnamomi* mycelial growth (Neves, 2007). Also, Zentmyer and Thompson (1968) showed that saponins presented only slight toxicity to *P. cinnamomi* mycelium, but at 2 mgml^{-1} inhibited sporangial production and zoospore germination. However, it cannot be excluded that the saponins or some of their derivatives, extracted from *P. purpurea* roots, are different from those present in the leaves.

Phlomispurpentaolone, like saponins, has both a hydrophobic and a hydrophilic domain. This suggests that the lipophilic domain can penetrate into the cell membrane of wall-less zoospores and disrupt these cells. This activity would also apply to mycelia, since this compound, at 0.1 $mgml^{-1}$, inhibited mycelial growth of *P. cinnamomi* by 75.7 %. It must be stressed that at 10 $mgml^{-1}$ (100 times more) the PRE inhibited mycelial growth of *P. cinnamomi* by 85 %. It is expectable that phlomispurpentaolone will inhibit zoospores at a lower concentration than 0.1 $mgml^{-1}$. The use of this compound, or eventually *P. purpurea* extracts can be extremely useful to control *P. cinnamomi*, especially in hydroponic systems.

The appendices can be found at **https://meocloud.pt/link/12101580-ac84-4f75-bcd6-6fad66fb9d80/Appendices_Chapter%207.docx/** (see CD in attachment).

7. 4. Acknowledgments

We are indebted to Prof. Dr. Mª Conceição Mateus who performed the LC-MS analysis for all the steps, from the crude extract to the purified compound. I would like to thank her for the useful discussions and personal support in the difficult moments. I would like to acknowledge Prof. Dr. Lurdes Cristiano and her team for the technical support. Thanks to Prof. Dr. Custódia Fonseca and Prof. Dr. Américo Lemos for the help with IR. To the technicians and assistants in the chemistry labs a huge thanks for everything. We acknowledge Dr. Gregory Walker, at Pfizer Inc. Groton CT (USA), and his colleague Dr. Alfin Vaz, for recording the pure compound NMR and LC-MS spectra, respectively.

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Chapter 8

Metabolite profiling of *Phlomis purpurea* challenged with *Phytophthora cinnamomi*

The introduction has been omitted, as a general introduction was presented in Chapter 6.

8.1. Abstract

In order to discover metabolites that are potentially bioactive against *P. cinnamomi*, two and half-month-old *P. purpurea* seedlings (n=10) were inoculated with zoospores at 6 time points (0, 6, 12, 24, 48 and 72 h). Controls and root exudates of the same plants at the same time points were also prepared. Five replicates were performed. The material from the plants at each time point was pooled for each independent replicate (11 pools x 5 replicates). Roots and leaves were extracted with MeOH. Lipids and slightly polar metabolites were separated using reversed phase chromatography (RPC) with HSS T3 C_{18}. The exudates were also collected and filter sterilised at each time point, immediately submersed in liquid nitrogen and kept at -80 °C.

P. purpurea metabolites produced constitutively and upon challenge with *P. cinnamomi* were quantified using a LC-MS system, according to established standard workflows. Root exudates were analysed by GC-MS after derivatization.

Data analysis targeted the already known anti-fungal compound, phlomispurpentaolone, and revealed that it is produced constitutively by *P. purpurea.* Data set will be explored for new potential compounds of interest.

8.2 Material and methods

8.2.1. Establishment of *Phlomis purpure*a hairy root cultures

Several trials were performed with the primary aim of producing *Phlomis purpurea* hairy roots to study the secondary metabolites produced by this resistant plant when challenged with *Phytophthora cinnamomi*.

First trial

Preparation of aseptic seedlings

Phlomis purpurea seeds were selected by placing them in water. Those that floated were excluded and only the ones that precipitated were selected. Selected seeds were surface sterilized with one of following conditions: commercial sodium hypochlorite 10 % or 20 % or 25 % or 50 % (v/v) in water (Zolala *et al.*, 2007) or *P. purpurea* root extracts at 10 mg ml^{-1}. *P. purpurea* seeds were rinsed twice in sterile distilled water (SDW), dried in sterile absorbent paper and plated on 90 mm Petri dishes containing MS/2 [half strength Murashige and Skoog

medium (Murashige & Skoog, 1962)]. A maximum of 12 seeds were transferred to each Petri dish. As they got easily contaminated by fungi and bacteria, the seeds had to be transferred to new MS/2 Petri dishes every week.

As the seedlings were contaminated it was decided to surface sterilize half of them with 100 % commercial sodium hypochlorite for 2 seconds and dry them in absorbent paper (A. C. Figueiredo, personal communication).

First attempt of transformation of *Phlomis purpurea* seedlings with *Agrobacterium rhizogenes*

The transformation procedure was performed as a modification described by (Santos *et al.*, 2002, 2005). Briefly, three-week-old seedlings (half were surface sterilized and half non-sterilized) were carefully wounded (root, stem and leaves) with a scalpel previously dipped in an overnight grown *A. rhizogenes* suspension (Yeast mannitol broth - YMB) strain A4 (pRiA4::70GUS) and A4 Biovar 2[47] (hereinafter Biovar) with OD_{600nm} = 1.69 and 1.86 respectively and diluted 1:10. Seedlings were dried in absorbent sterilized paper before being co-cultivated in Schenk and Hildebrandt (SH) solid culture media (Schenk & Hildebrandt, 1972). Three SH Petri dishes, for each strain and treatment, received 4 seedlings making a total of 48 seedlings in co-culture. After 48 h of co-culture with the bacterial suspension in the dark, the seedlings were transferred to medium containing 150 mgl^{-1} of cefotaxime and carbenicilin for *A. rhizogenes* elimination. The plants become covered by fungi and it was not possible to continue.

Second trial

Preparation of aseptic seedlings

Phlomis purpurea seeds were surface sterilized with 1 % Folpaxil (fungicide from Selectis®) in water, 2 % Neemkop [biological fungicide from *Azadirachta indica* (Koopert®)] in water, 7 % Sabokop [Sabokop is a fungicide based in soap with fatty acids (Sybiol®)] in water and 100 % sodium hypochlorite. Selected seeds (n=26) for each treatment were surface sterilized for 10 min and rinsed for 10 min in SDW, dried in sterile absorbent paper and transferred to slanted tubes containing MS/2. Seedlings were transferred to fresh MS/2 media every week for 4 weeks but at the end they were all contaminated with fungi and no attempt to transformation was made.

[47] Kindly provided by Professor Dr. Jorge Vivanco.

Third trial

Preparation of aseptic seedlings

Two more attempts were made: 1) *P. purpurea* seeds (n=91) were surface sterilized with 1 % Folpaxil in water for 10 min; 2) seeds (n=172) were surface sterilized with sulphuric acid 50 % in water for 1 min. Seeds were transferred to MS/2 Petri dishes. After 1 week all the germinated plants were contaminated with fungi.

Hymexazol appeared to be the best option to avoid exuberant fungal contamination. Thereafter, an indicative test was performed whereby seeds (n) were surface sterilized with 10 % hymexazol for 5 min (n=138) and for 10 min (n=139). The seeds were rinsed twice in sterilized absorbent paper. These seeds were plated on MS/2. Another group of seeds (n=111) were surface sterilized with 10 % hymexazol for 10 min, rinsed and dried as mentioned above, and plated on MS/2 containing 10 % hymexazol (40 µl). After 15 days, seedlings were surface sterilized with 10 % hymexazol for 5 min (n=33), for 10 min (n=23) and with 10 % hymexazol for 10 min followed by plating on MS/2 containing 10 % hymexazol (n=28), and were used for the transformation as described below.

Second experiment of transformation

It was decided to proceed using the less contaminated seedlings from 1) and 2). One-week-old seedlings (n=20) were carefully wounded (root, stem and leaves) with a scalpel previously dipped in an overnight grown *A. rhizogenes* suspension (YMB medium) strain Biovar (OD_{600nm} = 0.90). Seedlings were dried in absorbent sterilized paper before being co-cultivated in MS/2. After 72 h of co-culture with the bacterial suspension in the dark, the seedlings were transferred to MS/2 containing 150 mgl^{-1} of cefotaxime, of carbenicilin and of pentachloronitrobenzene [PCNB (0.4 gl^{-1})].

The fungi–free seeds that germinated (n=38), now 2-week-old, were carefully wounded in the meristematic tissue (between the stem and the root) that contains undifferentiated cells, with a scalpel previously dipped in an overnight grown *A. rhizogenes* suspension (YMB medium) strain Biovar (OD_{600nm} = 0.77). Seedlings were dried in absorbent sterilized paper before being co-cultivated in MS/2. After 72 h of co-culture with the bacterial suspension in the dark, the seedlings were transferred to MS/2 containing cefotaxime (150 mgl^{-1}), carbenicilin (150 mgl^{-1}), PCNB (0.4 gl^{-1}) and 10 % hymexazol (40 µl). For the first time, fungi were not visible macroscopically although they were visible under the microscope. Seedlings kept the vitality and were transferred, after a week, to fresh MS/2 containing cefotaxime (150 mgl^{-1}), carbenicilin (150 mgl^{-1}), PCNB (0.4 gl^{-1}) and 10 % hymexazol (40 µl).

Fourth trial

Preparation of aseptic seedlings

Phlomis purpurea seeds were surface sterilized with 10 % hymexazol for 5 min (n=24, Treatment A), for 10 min and with 10 % hymexazol for 10 min (n=24, Treatment B) followed by plating on MS/2 containing 10 % hymexazol (n=22, Treatment C). Then, they were transferred to MS/2 containing cefotaxime (300 mgl^{-1}) and 10 % hymexazol (40 µl) to avoid microbial contamination.

Third experiment of transformation

In this trial, the procedure was as described in the 3rd trial with the following alterations: seedlings from treatments A, B and C were submerged, after inoculation, in the *A. rhizogenes* suspension strain Biovar (OD_{600nm} = 0.80); two control (non-inoculated) seedlings were used to observe how non-transformed roots behave in MS/2. Seedlings in co-culture were incubated at 22 °C with natural photoperiod for 48 h, 72 h and 168 h (1 week).

After 10 days all the seedlings were transferred to fresh MS/2 containing cefotaxime (300 mgl^{-1}) and 10 % hymexazol (40 µl). After 5 days the majority of the seedlings were contaminated with bacteria, probably *A. rhizogenes* and only a few with fungi, and were transferred to fresh MS/2 containing cefotaxime (300 mgl^{-1}) and 10 % hymexazol (40 µl) to eliminate *A. rhizogenes.* After 10 more days all the roots of the *P. purpurea* seedlings were detached with a sterilized scalpel and inoculated in three 500 ml *Erlenmeyer* flasks (1 per treatment) containing 200 ml SH with 40 µl hymexazol (1:10). The cultures were incubated in an orbital shaker at 80 rpm at 24 °C.

Fifth trial

Preparation of aseptic seedlings

Phlomis purpurea seeds were surface sterilized with 10 % hymexazol for 5 min and immediately transferred to MS/2 containing cefotaxime (300 mgl^{-1}) and 10 % hymexazol (40 µl) to avoid microbial contamination.

Fourth experiment of transformation

In order to produce root cultures, 342 seedlings *ca* 1-month-old were infected with a 3-day-old culture *A. rhizogenes* (Biovar) or LBA 9402[48] (a different strain to exclude any problem with the Biovar) in YMB liquid media for 24 h. Three infection methods were used:

[48] Kindly provided by Professor Dr. Pedro Fevereiro

A) Plants were placed in a concentrated suspension of *A. rhizogenes* (OD_{600nm}≈ 1.0) for 30 min, at 22 °C, in the dark, at 80 rpm (n=187);

B) The root was cut 0.5 cm above the apex and put into contact with a colony (n=115);

C) The leaves were randomly wounded with the tip of a sterile hypodermic needle carrying one drop of the bacterial suspension (n=40). The seedlings were dried in sterile absorbent paper and then co-cultivated with the bacteria on MS/2 solid medium, with 30 gl^{-1} of sucrose and 100 µM acetoceringone, in a 16 h light/8 h dark photoperiod at 23 °C and 20 °C respectively, for 48 h. Roots that developed at the infection sites were transferred to Petri dishes containing solid MS basal medium supplemented with 500 mgL^{-1} ampicillin for the LBA strain or 250 mgl^{-1} carbenicillin and 250 mgl^{-1} cefotaxime for the Biovar strain, to eliminate the bacteria, 394 μll^{-1} hymexazol, to eliminate fungi, and 100 µM acetoceringone, and were incubated with the same photoperiod. When appropriate, according to root and bacteria development, 1 cm root tips were sub-cultured twice to eradicate the bacteria before transferring them to fresh medium in the absence of antibiotic. However, contamination developed with other bacteria and fungi, even in control plants, and it has been impossible to obtain sterile cultures. Roots that developed were transferred to liquid MS/2 medium without antibiotics, maintained in the dark at 24 °C in an orbital shaker (80 rpm) and subcultured every 15 days.

Sixth trial

Preparation of aseptic seedlings

Due to contamination of *P. purpurea* seeds, and consequent contamination of *P. purpurea* radicles using the above described procedures, three further approaches were set to overcome this problem.

Firstly, the seeds (n=10) were disinfected with ethanol at 70 % for 2 minutes, sodium hypochlorite at 6 % for 2 minutes (Santos *et al.*, 1998), hymexazol 10 % for 25 minutes (to avoid fungal contamination) and washed twice with SDW. To avoid extra contamination, seeds were not plated on MS nutritive media but rather germinated on moist sterile paper. Briefly, the seeds were covered with moist sterile paper and incubated at 4 °C for a week and at 22 °C until germinated. However, after one month only 20 % of the seeds had germinated and all became necrotic within two days.

A second approach was followed: the seeds (n=10) were soaked in SDW overnight and the treatment above repeated. After a month, 60 % of the seeds had germinated but all became necrotic within two days.

Third approach: the seeds (n=10) were disinfected with ethanol at 70 % for 1 min, sodium hypochlorite at 6 % for 1 min (Santos *et al.*, 1998), hymexazol 10 % for 1 min and washed twice with SDW. The seeds were soaked in SDW overnight.

Since no *P. purpurea* hairy roots could be produced, an alternative method using non axenic seedlings was used to study the metabolites produced upon challenged with *Phytophthora cinnamomi*.

8.2.2. Growth and maintenance of experimental plants

Phlomis purpurea seeds were collected from the field, at Moncarapacho, Algarve, Portugal. Seeds were selected and kept in the refrigerator at 4 °C before being used. The seeds to be germinated were surface sterilized with sodium hypochlorite 25 % for 25 min and rinsed twice in sterile distilled water (SDW). Seeds were moistened with water and covered with wet absorbent paper, inside Petri dishes, until germination occurred. When the radicles were 2-3 cm long they were transferred into cylindrical soft black plastic tubes (25 cm x 3 cm) containing vermiculite (see Chapter 5 – histology, section 5.2.1, Method 2).

8.2.3. *Phytophthora cinnamomi* growth and maintenance and zoospore production

The *Phytophthora cinnamomi* isolates PA37 and PA45 growth and maintenance are described in Chapter 2, section 2.2.2.

Zoospores were produced according to the procedure of Byrt and Grant (1979) (see Chapter 4, section 4.2.4.) With this method, 10^4–10^5 zoospores ml^{-1} were routinely produced. A zoospore suspension (200 µl) was plated onto V8A and incubated at 24.5 °C to check their viability.

8.2.4. Challenge of *Phlomis purpurea* with *Phytophthora cinnamomi* zoospores

Two and half-month-old seedlings were carefully removed from the tubes, washed to remove the substrate and were challenged with *P. cinnamomi* zoospores.

About 50 ml zoospore suspension containing (10^3 to 10^4 zoospores/ml) were used to challenge 20 *P. purpurea* roots at each of the following time points: 0, 6, 12, 24, 48 and 72 hours. Controls at the same time points were prepared with MSS. The plants were kept in glass tubes in the dark at 22 °C. At each time point the plants were harvested, divided into two groups of equal size, designed to metabolite profile and for transcriptomics analyses and immediately frozen in liquid nitrogen, to quench metabolic activity, and kept at -80 °C. The

experiment was repeated five times, giving a total of 55 plantlet samples. The transcriptional studies were done by a partner team.

8.2.5. Ultra performance liquid chromatography/mass spectrometry (UPLC/MS) analysis of *Phlomis purpurea* root and leaf extracts, and exudates

8.2.5.1. *Phlomis purpurea* root and leaf extracts

Optimization of the extraction protocol using different methods and solvents

All solvents used were MS-grade.

Extraction of organic compounds using methanol and methyl *tert*-butyl ether

Extraction of lipids and polar compounds from *P. purpurea* roots and leaves was according to the protocol by Matyash *et al.* (2008).

Briefly, 175 mg of roots and 75 mg of leaves of *P. purpurea* were crushed in liquid nitrogen with 3x500 µl methanol (MeOH) in a 1 ml Wheaton tissue grinder. As the shaker only takes 1.5 ml, the samples (roots or leaves) were divided into 5 tubes (0.5 ml/sample with methanol each) and 1 ml methy *tert*-butyl ether (MTBE) was added. Eppendorf tubes (1.5 ml) were shaken in a shaker (Thermomixer comfort) at 23 °C. Phase separation was induced by adding 0.25 ml water. Upon standing 10 min at room temperature, the sample was centrifuged at 1,000 g for 10 min. The upper (organic) phase was collected, and the lower phase was re-extracted with 2 ml solvent mixture, whose composition was equivalent to the expected composition of the upper phase [obtained by mixing MTBE/methanol/water (10:3:2.5, v/v/v) and collecting the upper phase]. Combined organic phases and aqueous phases were dried in a vacuum centrifuge.

The organic phase was resuspended in 50 µl acetonitrile 80 % in water with formic acid 0.1 %. The polar phase was resuspended in MeOH 50 % in water, with formic acid 0.1 %. This sample did not ionise well so it was added 5 µl acetonitrile with formic acid 0.1 %.

Extraction of organic compounds using MeOH

Phlomis purpurea roots and leaves (10 mg fresh weight) were macerated with 200 µl MeOH in a 1 ml Wheaton tissue grinder. The samples were transferred into a 1.5 ml Eppendorf tube and 100 µl MeOH from the grinder washing were added. They were centrifuged at 1,000 g for 10 min. The supernatant was recovered and the samples were dried overnight in a speed vac. The samples were resuspended in 200 µl MeOH 50 % in water.

Analysis of *Phlomis purpurea* metabolites by LC-MS injection

Based on the result of different compounds observed in the control and challenged samples at 72 h, MeOH was chosen as the extraction solvent and the positive mode was selected to run the samples. All samples (55 + 55−root+leafs) were extracted using the MeOH protocol above.

Column preparation

Silica tubing (200 µm inner diameter, 350 µm outer diameter, untreated) was cut by 30 cm. The external coat (14 to 16 cm) was burned using a lighter and the burnt black region cleaned with a special tissue with ETOH in the same direction till the glass was completely clean (checked under the microscope). The spraying tip was formed using a Sutter Instrument – Model 2000 that should be warmed up 1 hour before use. The column was placed into position, carefully tightened and Program 30 was selected to check that the column was correctly prepared. The 2 x 15 cm columns were washed with acetonitrile using helium at 30 Bar and were filled with BEH C_{18} or HSS T3 C_{18} (1.8 and 1.7 µm particle ID, respectively, both from Waters, Milford, USA) till the filling reached 5 cm using helium at 100 Bar. Ten columns of BEH C_{18} and 23 of HSS T3 C_{18} were prepared. The columns to be used were cut to a final length of 5 cm.

Sample run

An LC-MS system (LC−Waters nanoAquity coupled to MS−Waters Synapt G2 HDM equipped with a nanoESI source) was used with the following method: column type HSS T3 C_{18} (1.8 µm particle ID), Column length: 5 cm, Flow rate: 5 µLmin^{-1} decreasing to 4 µLmin^{-1} during the run. Run gradient duration: 30 min from 100 % A to 100 % B. Mobile phase A: water plus 5 mM ammonium acetate; mobile phase B: 90 % isopropanol, 10 % acetonitrile plus 5 mM ammonium acetate. Injected volume: 2 µL. The samples were diluted in 100 µL 80 % acetonitrile with 0.1 % formic acid. Leucine encephalin was used as a mass spectrometry standard. The mass spectra were recorded in the positive ion mode.

8.2.5.2. *Phlomis purpurea* exudates

The exudates were evaporated overnight at 30 °C (Eppendorf tube concentrator 5301). The pellets were resuspended in 50 % MeOH in water, centrifuged for 3 min at 3,000 rpm and the supernatant collected. About 400 µl were diluted 1:10 in 50 % MeOH in water with 0.1 % formic acid. The plant exudates were too much concentrated in mineral salt solution

(MSS). As the salts interfered with the analysis a C_{18} column (Waters Sep-Pak) was used to desalt the samples but the compounds were retained in the column. It was not possible to run LC-MS, the salt concentrations impaired chromatographic retention and electrospray ionization, despite several attempts to pre-process the samples by solid phase extraction, desalting or dilution. In a second approach the same exudate samples were analysed after derivatization as described by Lisec *et al*. (2006) by GC-MS (Agilent GC 7890A coupled to a Waters Micromass GCT Premier). Briefly, 100 µL dried down exudate was derivatized first with 40 µL methoxyamin hydrochloride solution in pyridine (20mg/ml) then with 70 µL N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA). Of the final solution containing the derivatized organic compounds 1 µL was injected on a baffled glass liner and transferred within 10 seconds by rapid heating to the analytical capillary GC column (Restek MS5 Guard, 30 m x 0.25 mm x 0.1 µm). As the salts are only slightly soluble in the derivatization solvent and not volatile, only organic compounds were transferred from the injection liner to the analytical column and the MS. Due to time constrains, exudates were analysed by GC-MS only in control and challenged samples at 72 hpi, as well as for MSS and zoospores suspension. An evaluation of the data was done using Progenesis QI software (NonlinearDynamics, Manchester, UK) and results were exported to an excel file.

8.2.6. Statistical analysis

R statistical free software was used to carry out multivariate analyses, namely principal components analysis (PCA).

Analyses of the results were performed using the software package “PASW statistics 18” (IBM software, 2009 SPSS Inc. Hong Kong). The dependent variable was the intensity of the peak, with two simultaneous independent variables, condition and time point. Data were analysed by univariate analysis. The mean values were compared with those from controls by Fisher’s protected least significant difference (LSD) test at $P \leq 0.05$ (Steel & Torrie, 1985). The non-parametric test “Mann-Whitney U-test” was applied to samples presenting a non-normal distribution.

8.3. Results and discussion

8.3.1. Germination rate and transformation of *Phlomis purpurea* seedlings to produce hairy roots

Hairy roots constitute a good system for the study of metabolic pathways that lead to the production of primary and secondary metabolites and of their behavior under stress-inducing environments namely biotic and abiotic factors.

We intended to transform *P. purpurea* with *Agrobacterium rhizogenes* in order to establish the metabolite profiling of root exudates from the hairy roots (mediated by *A. rhizogenes*) challenged with *P. cinnamomi.* Comparison with information obtained from the compound(s) that have activity anti-*P. cinnamomi* (Chapter 7) would allow the identification of antimicrobial compounds that are produced and eventually released into the surrounding rhizosphere upon microbe challenge.

In a preliminary test seeds (n=207) that floated, independently of the surface sterilization product, did not germinate at all.

Several trials were performed to have *P. purpurea* seedlings axenically and to be able to transform them with *A. rhizogenes* in order to produce hairy roots (Tables 8.1, 8.2 and 8.3).

Table 8.1. Trials to obtain axenic *Phlomis purpurea* seedlings for transformation with *Agrobacterium rhizogenes*.

Trial	N ° seeds	Treatment	Duration (min)	Percentage (%) germination	Inoculation and Transformation outcome
First	66	A: 10 % sodium hypochlorite in water	10	37.9	68 seedlings: 21 from treatment B; 25 from treatment C and 22 from treatment D were submitted to transformation with *Agrobacterium rhizogenes*. No transformation was achieved.
	105	B: 20 % sodium hypochlorite in water	20	54.2	
	94	C: 25 % sodium hypochlorite in water	25	53.2	
	99	*D:* *P. purpurea* root extract 10 $mgml^{-1}$	25	42.4	
Second	26	A: 1 % Folpaxil[b] in water	10	38.4	10 seedlings from treatment A; 8 from treatment B; 5 from treatment C and 7 from treatment D were submitted to transformation with *Agrobacterium rhizogenes*. No transformation was achieved.
	26	B: 2 % Neemkop[c] in water	10	30.7	
	26	C: 7 % Sabokop[d] in water	10	19.2	
	26	D: 100 % sodium hypochlorite	10	26.9	
Third	91	A: 1 % Folpaxil in water	10	38.5	35 seedlings from treatment A; 85 from treatment B; 33 from treatment C; 23 from treatment D and 28 from treatment D^{+} were submitted to transformation with *Agrobacterium rhizogenes*.
	172	B: sulphuric acid 50 % in water	1	49.4	
	138	C: 10 % hymexazol	5		
	139	D: 10 % hymexazol	10		
	111	D^{+}: 10 % hymexazol	10[a]		

[a]Further plated in culture media containing hymexazol; [b]Folpaxil is a fungicide from Selectis; [c]Neemkop is a biological fungicide from *Azadirachta indica* (Koopert®) and [d]Sabokop is a fungicide based in soap with fatty acids (Sybiol®)

In the first trial, although seeds were disinfected they were contaminated with fungi that immediately infected them after germination. However, seeds surface sterilized with *P. purpurea* root extract at 10 $mgml^{-1}$ were the ones with less fungal contamination but also presented reduced vitality and necrotic roots (Figure 8.1).

In the second trial, all the germinated seeds with (A), as an attempt to eliminate fungi but the roots became necrotic soon afterwards. Seedlings were transferred after 3 days to new MS/2 in an attempt to recover them. Seedlings were transferred to fresh MS/2 media every week for 4 weeks but at the end they were all contaminated with fungi.

In the third trial, after 1 week all the germinated plants were contaminated with fungi and an attempt to eliminate fungi, during transformation was made with pentachloronitrobenzene (PCNB). Even with PCNB plants were contaminated with fungi and no transformation was achieved. Another transformation attempt was made, with roots disinfected in hymexazol and adding hymexazol to the antibiotics in the transformation protocol, to avoid fungal contamination (Figure 8.2). The new roots that grew in the meristematic zone became necrotic. All the seedlings loss vitality after a few days and the contamination engulfed them.

Figure 8.1. *Phlomis purpurea* seedling (ca. 3-week-old) co-cultivated with *Agrobacterium rhizogenes* (strain Biovar) in Schenk and Hildebrandt (SH) solid culture media, showing necrotic leaves and radicles.

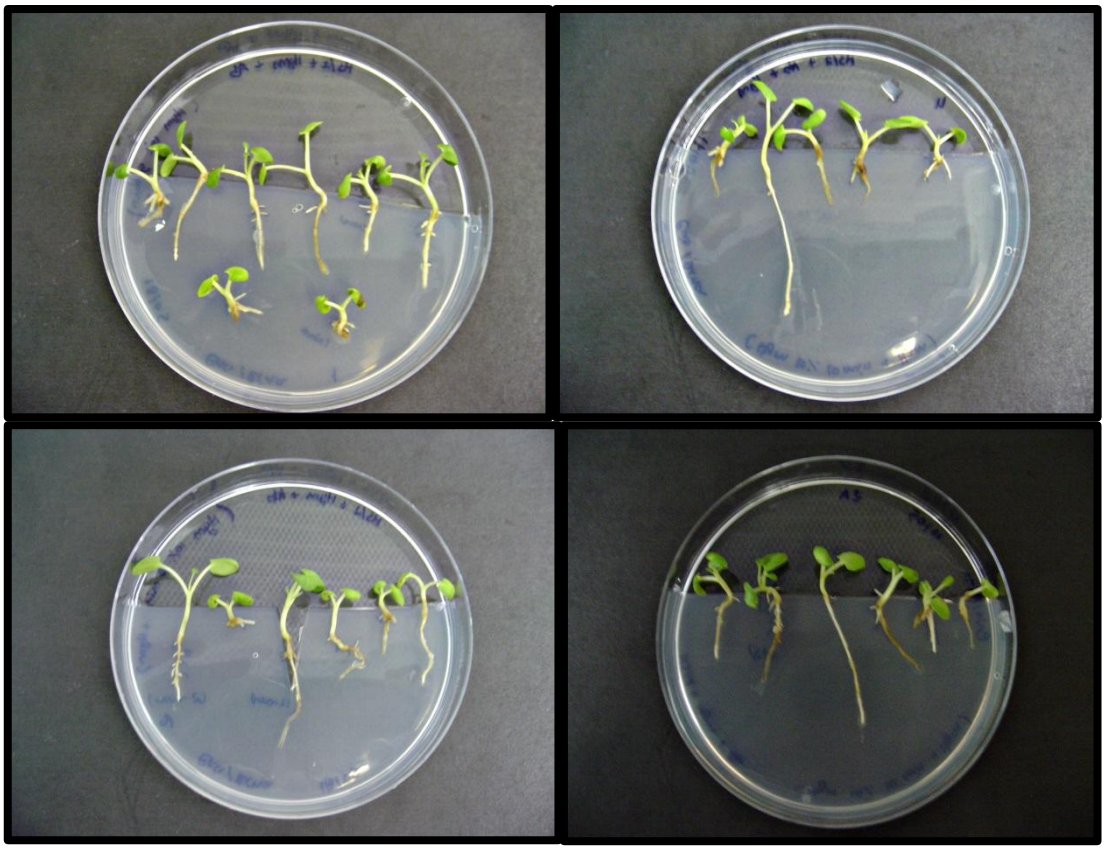

Figure 8.2. *Phlomis purpurea* seedlings (1 to 3-week-old) co-cultivated with *Agrobacterium rhizogenes* (strain Biovar) in half strength Murashige and Skoog medium (MS/2) containing cefotaxime (150 mgl^{-1}), carbenicilin (150 mgl^{-1}), PCNB (0.4 gl^{-1}) and 10 % hymexazol (40 µl).

The fourth trial differed from the previous one mainly by the duration of the co-culture (Table 8.2.).

Table 8.2. Number of *Phlomis purpurea* seedlings that developed roots after being surface sterilized with 10 % hymexazol for 5 min (A), with 10 % hymexazol for 10 min (B) and with 10 % hymexazol for 10 min followed by plating on MS/2 containing 10 % hymexazol (C) for different times of co-culture (48 h, 72 h and 168 h).

Time/Treatment	**48 h**			**72 h**			**168 h**		
	A	**B**	**C**	**A**	**B**	**C**	**A**	**B**	**C**
Seedlings with roots	7	4	3	3	5	5	6	6	4
Seedlings without roots	1	1	3	4	0	7	3	5	0
Dead seedlings	0	1	0	0	0	0	0	0	0
Total n ° seedlings	8	6	6	7	5	12	9	11	4

This was the first time hypothetical hairy roots were transferred to Erlenmeyer flasks containing liquid media. However, roots were infected with bacteria and became necrotic.

The fifth trial differed from the previous one by the nature of the *A. rhizogenes* strain used-LBA 9402 instead of Biovar. Three infection methods were used (see section 8.2.1). The hypothetical hairy roots transferred to Erlenmeyer flasks containing liquid media did not grow, most probably because they were not transgenic. The plants submitted to treatment A (placed in a concentrated suspension of *A. rhizogenes*) gave the best yield of grown roots. From treatment C in which the leaves were randomly wounded with the tip of a sterile hypodermic needle carrying one drop of the bacterial suspension, no root growth was observed.

The nutritive media MS/2 seems to be a source of nutrients not only for the plants but also for microorganisms preventing the formation of axenic *P. purpurea* seedlings. Therefore, in the sixth trial, seeds were covered with moist sterile paper and incubated at 4 °C for a week and at 22 °C for germination. Using the third approach, *P. purpurea* seedlings only 20 % of the seeds had germinated but remained healthy after a month. So, we will use this approach in the future.

To exclude any problem related to the ability of the *A. rhizogenes* LBA 9402 strain to infect plants, this was used to produce hairy roots of *Nicotiana benthamiana* by a previous settled procedure (N. Marques and S. Formiga, Universidade do Algarve). Hairy roots developed in the leaves of *N. benthamiana* attesting the quality of the strain (Figure 8.3).

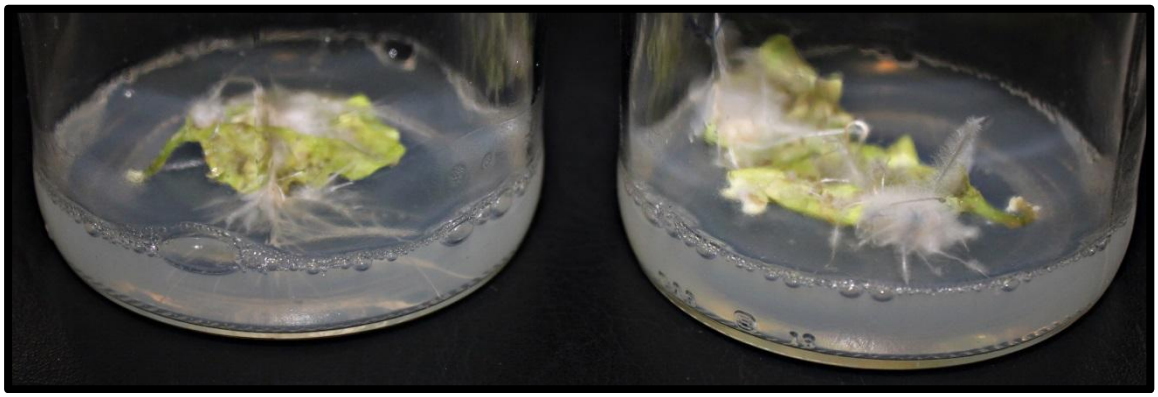

Figure 8.3. Hairy roots of *Nicotiana benthamiana*, transformed with *Agrobacterium rhizogenes* (LBA 9402) in Murashige and Skoog medium (MS) containing 250 mg ml^{-1} cefotaxime, after *ca* 8 weeks.

Three essential biological requirements to produce transgenic plants are: the availability of cells competent for plant regeneration, a method to introduce DNA into these cells and a way to select and regenerate transformed plants (Birch, 1997).

Using *Agrobacterium* spp it is possible to introduce DNA into virtually any plant cell type, however, only a small proportion of target cells typically receive the DNA and from those only a small proportion survive the treatment and stably integrate introduced DNA (ibid).

Phlomis purpurea seems to be a recalcitrant species and in our work no transformation was achieved, probably because any essential requirement failed or the number of explants was not enough, although optimization was tried. Other techniques can be used such as electroporation or bombardment with DNA coated micro-projectiles.

8.3.2. Characterization of metabolites in seedling root and leaf extracts and exudates

The methanol extracts of 55 root and 55 leaf samples were analysed by LC-MS according to established standard workflows (see section 8.2.5.1., sample run). Data analysis for the root and leaf extracts were pre-processed by MarkerLynx and the custom made R software package cosmiq based on the R packages xcms and MassSpecWavelet (cosmiq is available from the Functional Genomic Center Zurich FGCZ, ETHZ and University of Zürich, Switzerland). Briefly, pre-processing by MarkerLynx searches for sample related mass spectral signals on the base of single LC-MS runs, whereas cosmiq uses a combined master spectra from all samples and all runs. Using cosmiq, low abundant signals which are missed by MarkerLynx were detected with high confidence.

For both root and leaf, separate data corresponding to m/z masses from 50 to 1999, and intensities present in all the spectra were transferred to excel resulting in a data matrix of (55 root samples + 5 solvent control) x 8727 m/z values, which was normalized before applying principal component analysis (PCA).

Root extracts

Principal component analysis (PCA) is a method of identifying the pattern of dataset by reducing the information in many variables to a number of “new” variables (Multidimensional scaling), the principal components. A 2-D chart plots the projections of original variables on a reduced two dimensions by first two principal components (PC1 and PC2). The PCA scores plot for all root samples showed the data to be essentially not grouped/ungrouped for control and challenged samples for the five replicates (Figure 8.4). However, for one of the five replicates, the first to be prepared, the majority of samples (both control and challenged and even the blank) grouped in the upper right quadrant (Figure 8.4). It is possible that less careful manipulation could cause microscopic lesions on the roots, both controls and challenged

which seemed to respond more intensively. This corresponds to the first replicate to be prepared and it is not excluded that the manipulations were less accurate. This is only an indication for an experimental artifact. Therefore, it was decided to exclude this replica and repeat the PCA with four replicates.

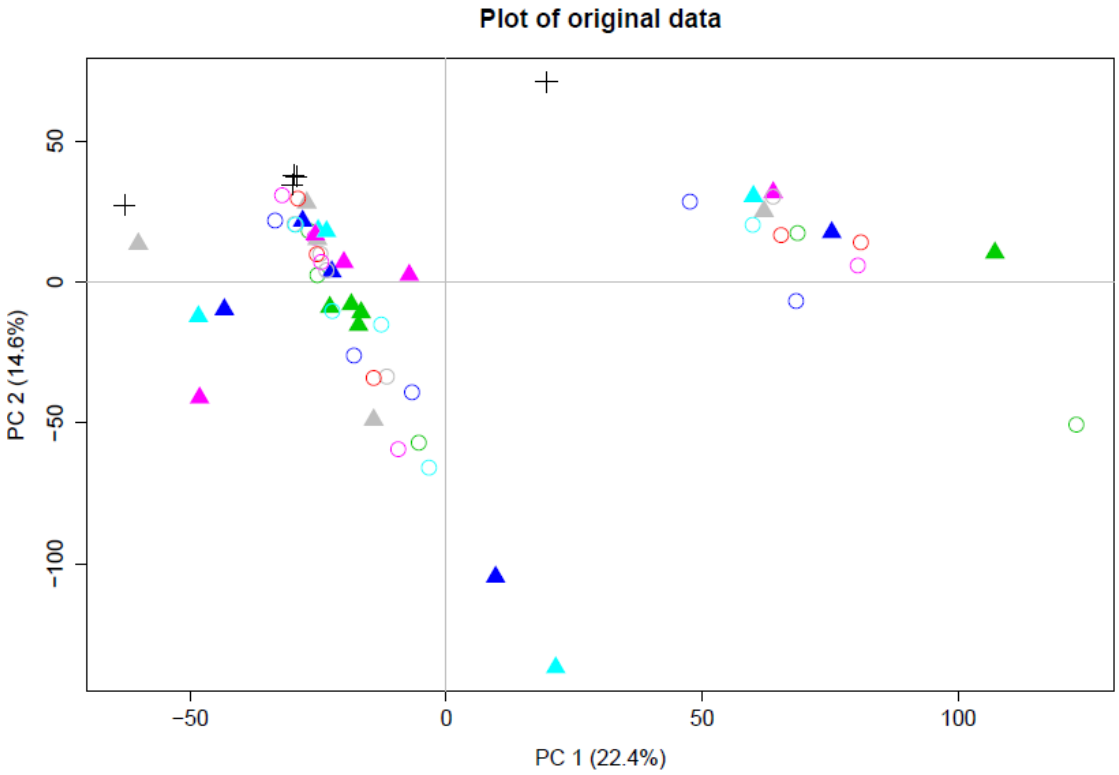

Figure 8.4. Principal component analysis (PCA) score plot for all the root samples (5 replicates). Blank (solvent) (+), control (o), inoculated with *Phytophthora cinnamomi* (Δ); blank (black); 0 hours post inoculation (hpi) (red); 6 hpi (green); 12 hpi (blue); 24 hpi (cyan); 48 hpi (magenta); 72 hpi (gray). PC1=First principal component; PC2= Second principal component calculated from normalized peak intensity data. Each percentage is the relative percentage of variance explained by PCs.

Another tool is OPLS-DA, which is a well-established regression and prediction method that finds information in the X data that is related to known information in the Y data (information about the extract). This discriminant analysis (DA) is useful in classification studies (predictions) and biomarker identification. The group differences between control and challenged *P. purpurea* in the root methanol extracts for the five replicates, using OPLS-DA show four possible biomarkers (m/z 149.0112; m/z 173.0692; m/z 217.0903 and m/z 556.2778) (Figure 8.5).

Group Differences between control and challenged samples in root methanol extracts using OPLS-DA

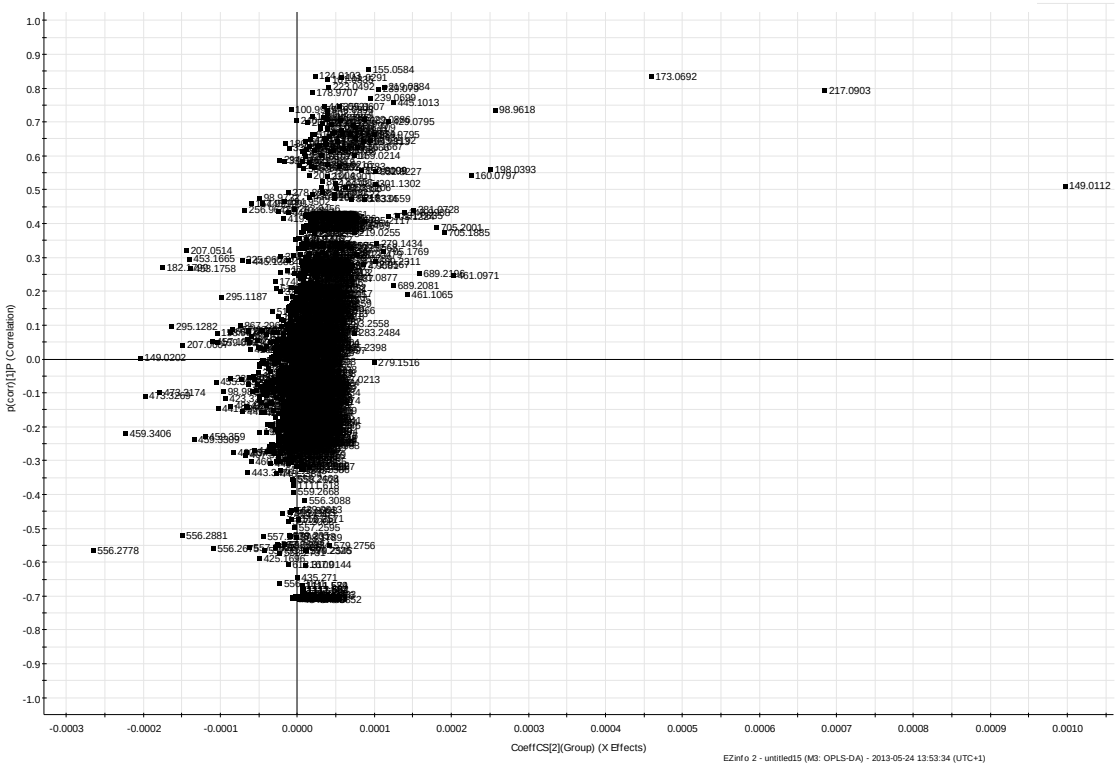

Figure 8.5. Group differences between control and challenged *P. purpurea* in the root methanol extracts for the 5 replicates, using OPLS-DA showing four possible biomarkers (m/z 149.0112; m/z 173.0692; m/z 217.0903 and m/z 556.2778).

The m/z 149.0112 is not present in the majority of samples and time-points and is only present in high intensity (up to 1050424) in one of the repetitions (R1). The m/z 173.0692 and m/z 217.0903 are present in the majority of samples; however only in one of the repetitions (R2) the intensities are very high (up to 1336782 and 1569758, respectively). The m/z 556.2778 is not present in the majority of samples and time-points and is only present in high intensity (from 211360 to 99459699) in one of the repetitions (R2). This occurred in both controls and challenged plants and therefore cannot be used as biomarkers.

The PCA was repeated with four replicates (Figure 8.6) and showed more uniform ungrouped samples, with control and challenged samples mixed, although the blank (solvent) can form a group almost isolated from the samples. The PCA showed three of the controls completely isolated (lower right quadrant). Removing these three controls other outliers appeared (data not shown) and therefore these four replicates were considered for analysis. In this case 21.5 % of the total variance from the multivariate analysis is explained by PC1, whereas 15.7 % is explained by PC2.

The identification and characterization of the thousands of metabolites represents a major challenge in metabolomics (Kopka *et al.*, 2005). The identification requires large-scale processing of pure standard substances to generate customized spectral libraries that can be used for the identification of unknown metabolic components from spectral data (ibid). The LC-MS system (see 8.2.5.1) we have used does not have any database for secondary metabolites and we could not find, up to now, any public database for data generated by LC-MS. Known metabolites present in other *Phlomis* spp were searched in *Phlomis purpurea* metabolite data but none was found[49]. Therefore, statistical analysis was performed using the m/z of phlomispurpentaolone (m/z= 491.4 $[M+H]^+$).

The aim was to find out whether this metabolite is being produced constitutively or is induced after challenged with *P. cinnamomi* zoospores. In fact, root samples used to prepare the PRE were collected from nature (Chapter7) and there is a chance for *P. cinnamomi* to be present in the soil where the roots were collected. Here, the roots were obtained from germinated seedlings and therefore, we could be able to conclude whether phlomispurpentaolone is a phytoanticipin (constitutive) or a phytoalexin (induced) (VanEtten *et al.*, 1994).

[49] Appendix V.I.

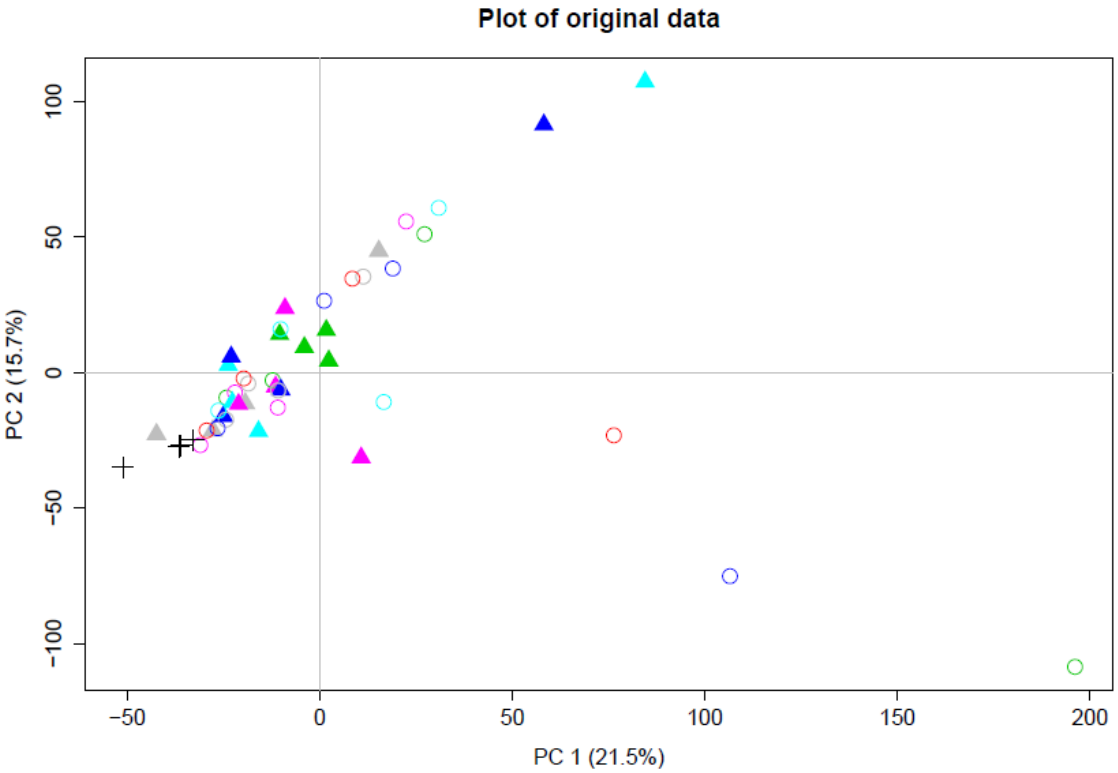

Figure 8.6. Principal component analysis (PCA) score plot for all the root samples (4 replicates). Blank (solvent) (+), control (o), inoculated with *Phytophthora cinnamomi* (Δ); blank (black); 0 hours post inoculation (hpi) (red); 6 hpi (green); 12 hpi (blue); 24 hpi (cyan); 48 hpi (magenta); 72 hpi (gray). PC1=First principal component; PC2= Second principal component calculated from normalized peak intensity data. Each percentage is the relative percentage of variance explained by PCs.

Before applying statistical analysis, the solvent and MSS data were subtracted from the samples. A descending index with the compounds present in all the samples at all-time points was prepared to have the information about the metabolites that are present constitutively. None was present at all time-points. Only, hypothetical five metabolites are present constitutively in the roots in nine out of ten time-points[50] (m/z 558.478, m/z 580.5597, m/z 597.5079, m/z 598.511, m/z 677.6623, all in positive ion mode). This short list may be even more reduced, since some of these ions are likely related. The metabolite m/z 580.5597 is the $[M+H]^+$ ion, 597.5079 is the adduct $[M+NH_4]^+$, 598.511 is the same adduct ion with one ^{13}C isotope (E. Laczko, personal communication). Therefore m/z 557.48, m/z 579.55 and m/z 676.65 (± 100 ppm) were searched for assignment at plant metabolic pathway database http://www.plantcyc.org/ and at MassBank database http://www.massbank.eu/MassBank/QuickSearch.html and no hits were found. At Pubchem

[50] Appendix V.VII.

compound database http://www.ncbi.nlm.nih.gov/pccompound, several hits for each compound are shown in appendix V.VIII.

Another descending index with the compounds present in all the samples at each time point was prepared. After 6 h, 79 hypothetic metabolites were present in all samples[51]; after 12 h, 98[52]; after 24 h, 240[53]; after 48 h, 289[54] and after 72 h, 27 metabolites[55]. After statistical analysis of the metabolite m/z=491.4 (phlomispurpentaolone) present in the *P. purpurea* control samples and samples challenged with *P. cinnamomi*, it was verified that it is produced constitutively because there are no statistically significant differences between controls and challenged *P. purpurea* samples, in all samples at all-time points[56] (Table 8.3, Figure 8.7).

Table 8.3. Quantification of the metabolite phlomispurpentaolone (m/z 491.4 $[M+H]^+$) present in the extract *Phlomis purpurea* control samples and samples challenged with *Phytophthora cinnamomi*.

Compound m/z	Exact m/z	Average peak intensity †		Percentage of presence◊
		Control	Challenged	
491.445[a]	491.337265	1883.40[a]±1713,50	1286,45[a]±752,25	95 %
491.424[a]	491.337265	34059.95[a]±26833.17	37703.10[a]±50465.79	87.5 %

Statistical comparison made between the condition (control and challenged) for each metabolite. Figures followed by the same letters, horizontally, are not significantly different at P≤0.05.
† Average peak intensity (± standard deviation).
◊Percentage of presence in all the samples at all time points.
Metabolites m/z 491.445 and 491.424 $[M+H]^+$ are most likely the same compound. The split into two masses is most likely an artefact of the pre-processing.
Exact mass calculator based on Phlomispurpentaolone ($C_{29}H_{46}O_6$) (m/z 490.329440) at http://www.sisweb.com/referenc/tools/exactmass.htm

Although there are no significant differences between the average peak intensity at all time-points of control and challenged at the m/z mentioned above, there are some punctual differences. For instance, in m/z=491.424 (Figure 8.7) at 24 hpi, the challenged samples had an abrupt drop while the controls reacted inversely. This might be due to water stress.

[51] Appendix V.II.
[52] Appendix V.III.
[53] Appendix V.IV.
[54] Appendix V.V.
[55] Appendix V.VI.
[56] Appendix V.IX

Physiological responses of plants to the environment involve changes not only at the transcriptional level but also in post-translational protein modifications and metabolite alteration (Verslues *et al.*, 2006). In the natural environment, adverse situations are always a combination of several stress factors and it is difficult to determine which stress factor(s) is(are) the cause of the elicitation of a particular physiological response. Using controlled conditions it is expected that only the elicitor is responsible for the physiological responses. However, flooding, per se, is a stress factor that cannot be neglected. During flooding, a fast depletion of O_2 occurs due to the low diffusion rate of this gas in water together with the consumption made by plants roots. This O_2 depletion can occur in less than 24 h, depending on the root/microbiota biomass present in soil (Arbona *et al.*, 2013). In 2-day-old soybean, 73 flooding responsive metabolites were identified using capillary electrophoresis-mass spectrometry (Komatsu *et al.*, 2014). It is possible that a similar amount of metabolites are present in the *P. purpurea* data. One of them is lactic acid ($C_3H_6O_3$), exact mass 90.031695 Da (Scientific Instrument Services). A metabolite m/z 91.1087 $[M+H]^+$, may be lactic acid and is present constitutively in three out of four replicates, with an increased peak intensity in challenged seedlings compared with controls. Although the increase was not time dependent, one of the replicates presented an increase in challenged samples of *ca* 7 and 5 times at 48 h and 72 h, respectively; another replicate presented an increase *ca* 4, 3 and 2 times at 12, 24 and 48 hpi, respectively; the third replicate presented an increase in challenged samples of *ca*. 4 and 38 and 2.5 times at 12, 24 and 48 hpi, respectively.

Unfortunately, we were not able to assign these metabolites, apart from m/z 491.4 (see chapter 7), to a determined compound due to the lack of comprehensive public databases to analyse secondary metabolites. This problem has handicapped the identification of thousands of metabolites (Burton *et al.*, 2008). Therefore, there is an urgent need for annotation of data from multiple metabolomics technologies in public databases.

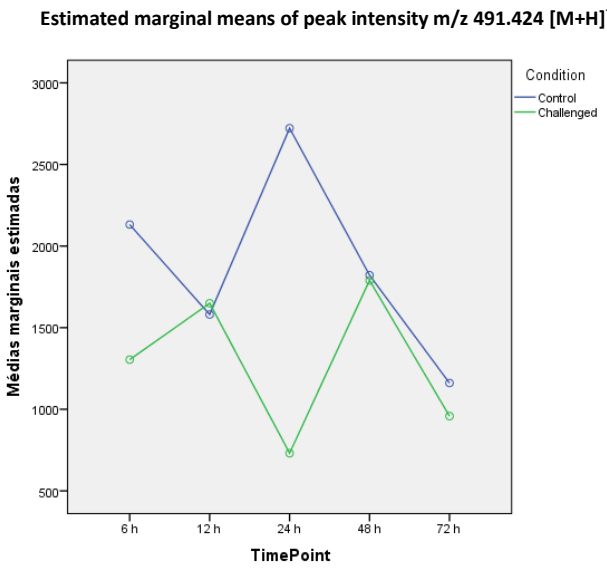

Figure 8.7. Estimated marginal means of peak intensity for metabolite m/z 491.4 $[M+H]^+$ from *Phlomis purpurea* root extract analysed by LC-MS. in control (blue) and challenged (green) samples.

Leaf (aerial) extract

A multivariate analysis (PCA) was performed to determine how/whether the samples group. Like in the root extract the samples were ungrouped with the exception of the solvent (methanol), labelled with a black "+" (Figure 8.8). Some control samples were dispersed. In this case 22.8 % of the total variance from the multivariate analysis is explained by PC1, whereas 16.3 % is explained by PC2.

Like in the root samples, there are not, in general, differences between the metabolites produced in control and challenged *P. purpurea* seedlings.

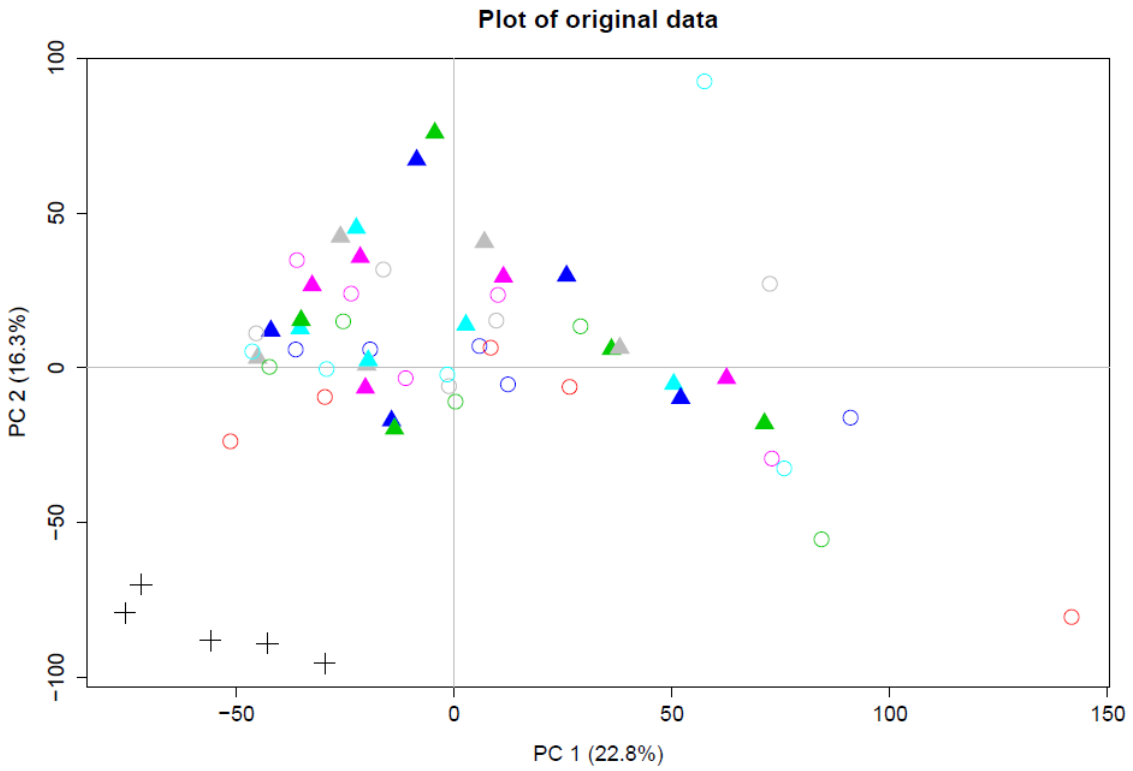

Figure 8.8. Principal component analysis (PCA) score plot for all the leaf samples (5 replicates). Blank (solvent) (+), control (o), inoculated with *Phytophthora cinnamomi* (Δ); blank (black); 0 hours post inoculation (hpi) (red); 6 hpi (green); 12 hpi (blue); 24 hpi (cyan); 48 hpi (magenta); 72 hpi (gray). PC1=First principal component; PC2= Second principal component calculated from normalized peak intensity data. Each percentage is the relative percentage of variance explained by PCs.

The group differences between control and challenged *P. purpurea* in the aerial (A) methanol extracts for the five replicates (Figure 8.9), using OPLS-DA shows two possible biomarkers (m/z 149.0112 and m/z 198.0393). Metabolite m/z 149.0112 is present constitutively, with low peak intensities in all the replicates. Metabolite m/z 198.0393 is present constitutively with high peak intensities (up to 3094089) although in replicates "A4" and "A1" the peak intensity is higher in the challenged samples (except 72 hpi in A1) and in replicate A2 is higher in control samples. According to http://www.massbank.eu/MassBank/jsp/Result.jsp m/z 149.01 , metabolite m/z 149.01±0.110 may be L-methionine (exact mass 149.05105; $C_5H_{11}NO_2S$), an aminoacid. Metabolite m/z 198.04 ±0.110 may be guaifenesin (exact mass 198.08921; $C_{10}H_{14}O_4$) which is an expectorant http://reference.medscape.com/drug/mucinex-organidin-nr-guaifenesin-343403 . The leaves of *Phlomis purpurea* are not known to have expectorant activity (Novais *et al.*, 2004) however, saponins are able to produce irritation on mucous membranes, namely bronchi and lungs, and should be then expected to have that activity.

Group Differences between control and challenged samples in aerial methanol extracts using OPLS-DA

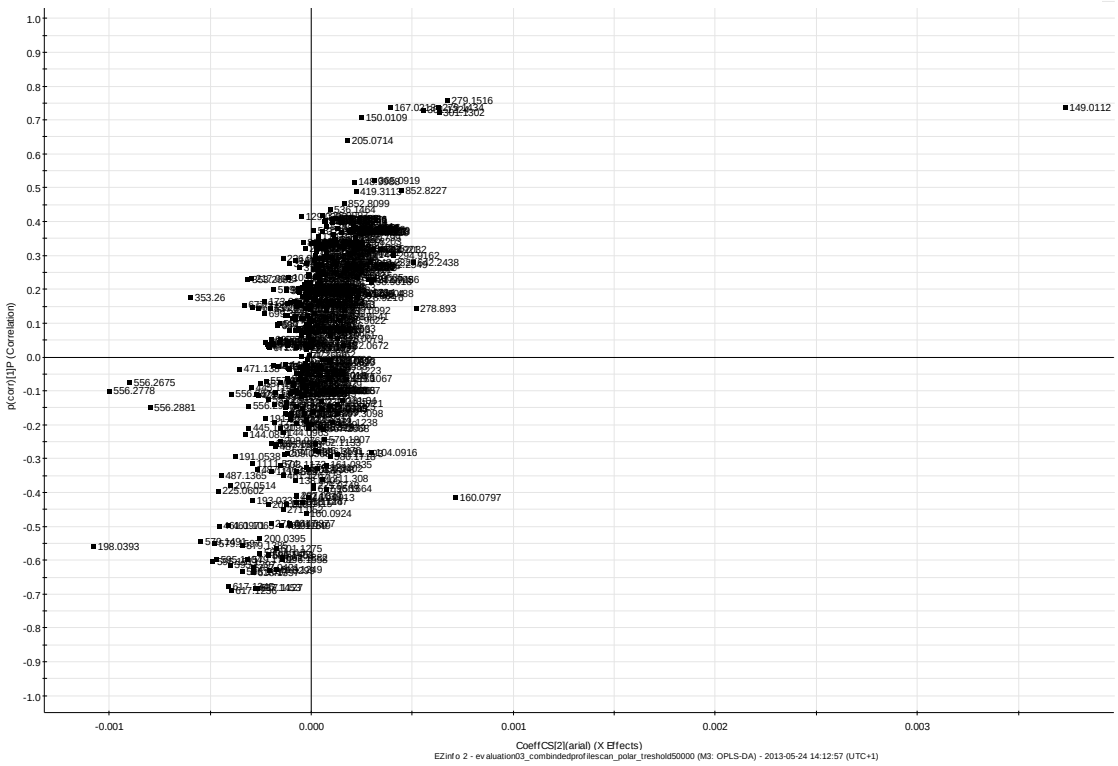

Figure 8.9. Group differences between control and challenged *P. purpurea* in the aerial methanol extracts for the 5 replicates, using OPLS-DA, shows two possible biomarkers (m/z 149.0112 and m/z 198.0393).

Exudates

As described before, exudate samples at 72 hpi were analysed by GC-MS after derivatization to enable a clean separation of the organic compounds from the salts[57]. The data generated will be analysed in the near future.

Integration of results

During the trials, there was no macroscopic visible damage on the roots of *P. purpurea* challenged with *P. cinnamomi*, even at 72 hpi and, from the analysis performed there were no significant differences in the root metabolism of *P. purpurea* root extracts. This strongly indicates that the anti-*P. cinnamomi* metabolites are produced constitutively and possibly exuded, although we cannot demonstrate these facts at the moment.

These results are in accordance with the previous findings, i.e. *P. purpurea* is not a host for *P. cinnamomi* and the root anatomy acts as a physical barrier to the entrance of pathogens.

[57] Appendix V.X.

These facts, along with the production of constitutive anti-*P. cinnamomi* metabolites may explain why *P. purpurea* manages to avoid infection. Moreover, preliminary studies showed that *P. cinnamomi* zoospores are not attracted to *P. purpurea* roots (data not shown). Half a century ago, Zentmyer (1961) reported that zoospores of *P. cinnamomi* were less attracted to roots of resistant than to roots of susceptible avocado cultivars.

Deacon and Mitchell reported that *Avena sativa* extracts causes the lysis of *P. cinnamomi* zoospores and suggested that avenacin or a related saponin might be involved in the zoospore lysis (Deacon & Mitchell, 1985). Avenacin is a triterpenoid saponin ($C_{55}H_{83}NO_{21}$) which is a mixture of four major compounds (Avenacins A1, A2, B1 and B2) comprising of glucoconjugates containing β-linked glucosyl residues (Figure 8.10). This compound has an exact mass of 1093.545764. A metabolite (m/z 1094.6640 $[M+H]^+$) is present constitutively in one of the replicates (R3) of *P. purpurea* root metabolite database, although it is not present at all-time points for the other replicates.

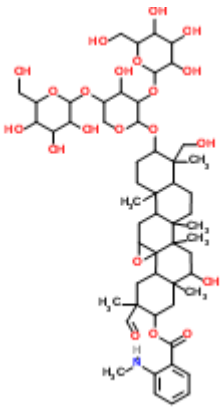

Figure 8.10. Structure of avenavin. Source: Chemspider at http://www.chemspider.com/Chemical-Structure.152437.html?rid=beee8b86-6c6b-4535-b40d-9335c04ef62e

Raftoyannis and Dick (2006) showed that zoospores of ten *Pythium* species and of two *Phytophthora* species (10^4 zoospores ml^{-1}) did not encyst on the root elongation zone and less than 8 % encysted in the root hair zone of *A. sativa,* after 1 h demonstrating that there was no attraction of zoospores by the roots of *A. sativa*.

Moreover, *A. sativa* root tips emit auto-fluorescence caused by the presence of avenacin (Deacon, 2006). Auto-fluorescence is the natural emission of fluorescent compounds present in different cellular compartments (Roshchina, 2012). The presence of secondary metabolites produced by plants such as terpenes, alkaloids or phenols can emit fluorescence (ibid).

Interestingly, *P. purpurea* also emits auto-fluorescence (Figure 8.11) caused probably by phlomispurpentaolone or other secondary metabolites.

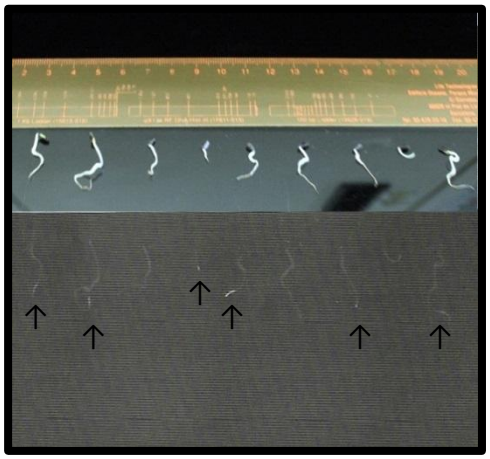

Figure 8.11. Ten-day-old *Phlomis purpurea* seedlings whose roots emit auto-fluorescence under UV lights. Fluorescence is black arrow-headed.

Recently, Dalio (2013), using a "zoospore trap" assay, showed that *P. nicotianae* zoospores were *ca* 6 times more attracted by root exudates of a susceptible Citrus than of the resistant cultivar. It seems that resistant plants do not have, or have in lesser amount, an attractor exudate.

8.3.3. Plants challenged with *Phytophthora cinnamomi*

Usually model plants, like *Arabidopsis thaliana, Medicago trunculata, Zea mays* or *Lupinus angustifolius* are used to study the metabolome and establish a metabolite profile. One of the great advantages of using *A. thaliana* or other model plants in the study of plant-pathogen interactions is the availability of a wide range of gene mutants and gene over-expressing lines. Even though, plants produce a remarkably high array of secondary metabolites, being most of them species specific. For instance, it is estimated that a single accession of *Arabidopsis* contains more than 5,000 metabolites, most of them yet uncharacterized (Arbona *et al.*, 2013).

There are numerous studies in plant-*P. cinnamomi* interactions. However, there are only a few studies based on the metabolites produced by plants upon challenge with *P. cinnamomi.* Studies in *Eucalyptus marginata* sprayed with phosphite and challenged with *P. cinnamomi* were conducted (Jackson *et al.*, 2000). Root segments were then analysed for activity of two selected host defence enzymes and for the concentration of soluble phenolics and phosphite. It was concluded that the effect of phosphite in controlling the pathogen is determined by the phosphite concentration. When phosphite concentrations within the roots are low, phosphite interacts with the pathogen at the site of ingress to stimulate host defence enzymes. At high phosphite concentrations, phosphite has a direct action on the pathogen to inhibit its growth

before it is able to establish an association with the host, and the host defences remain unchanged. In this study the research was orientated to known compounds.

Gunning *et al.* (2013) reported an increased synthesis of targeted phenylpropanoid secondary metabolites in the susceptible *Lupinus angustifolius* (lupin) after being challenged with *P. cinnamomi.* A correlation was found by PCA between root infection and the increase in concentration of the aglycones genistein, 2'hydroxygenisten and prenylated isoflavones and a decrease in malonylated genistein glucosides and 2'hydroxygenisten glucosides, although the defence response seems to be ineffective. The increase in the metabolites mentioned above is a well-recognised defence response from lupin species to biotic and abiotic stress, resulting in the production of anti-fungal metabolites (Tahara & Ibrahim, 1995). These metabolites (genistein 4', 7-*O*-diglucoside malonylated I, m/z 681.1650 $[M+H]^+$ and 2'-hydroxygenistein 7-*O*-glucoside, m/z 449.1067 $[M+H]^+$ were not found in the *P. purpurea* root extract database. However a metabolite m/z 449.3353 $[M+H]^+$ (shift 0.22) is present upregulated at 6 and 12 hpi in three out of four challenged samples, at 24 and 48 hpi in two out of four challenged samples and at 72 hpi in one out of four challenged samples. With this shift (220 mDa) and not being present at all the samples, this metabolite should not be considered to be 2'-hydroxygenistein 7-*O*-glucoside.

Metabolites vary widely from species to species and are dependent on the stressor. Several metabolites present in other plant species including some with antimicrobial properties (see Chapter 1, Table 1.1) described to be produced upon microbial challenge (see Chapter 6, Table 6.1), were searched in the *P. purpurea* metabolite list. However, none exactly match even within a shift of ± 100 mDa (data not shown).

Three natural compounds were identified in PRE: chlorogenic acid (m/z 355); caffeic acid (m/z 181) and vanillic acid (m/z 169), all in positive ion mode (Cravador *et al.*, 2010). These compounds were detected in the metabolite analysis of roots of control and challenged plants: chlorogenic acid (m/z 355.1029 $[M+H]^+$) within a shift of ± 110 mDa is present (m/z 355.0929 and m/z 355.1442) both in twenty three out of forty samples; and (m/z 355.2116) is present in thirty one out of forty samples[58]; caffeic acid (m/z 181.0501 $[M+H]^+$) within a shift of ± 110 mDa is present at m/z 181.0354 in twenty five out of forty samples, whereas m/z 181.0713 is present in twenty nine out of forty samples and both are significantly down regulated in challenged samples[59].

Several molecular masses differing by (-16, +22 and +36 mDa) from that of vanillic acid (m/z 169.0501 $[M+H]^+$) were detected. They correspond probably all to this compound within

[58] Appendix V.XI.
[59] Appendix V.XII.

errors of measurement. Together they appear to be present in (twenty six; twenty seven and in twenty out of forty samples, respectively) being the first mass significantly down regulated and the other two significantly up regulated in challenged samples[60].

Metabolites such as salicylic acid, jasmonic acid , ethylene and nitric oxide are known common metabolites in plant defence (Zhao *et al.*, 2005).

A metabolite (m/z 139.0242) is present in twenty nine out of forty samples and is significantly down regulated in challenged samples[61]. It appears to correspond to salicylic acid (m/z 139.0396 $[M+H]^+$) within a shift of ± 110 mDa. A metabolite with m/z 139.1062 is present constitutively in thirty five out of forty samples without significant differences between the control and the challenged samples.

Jasmonic acid (m/z 211.13342 $[M+H]^+$) within a shift of ± 110 mDa is present constitutively (m/z 211.1925; m/z 211.201 and m/z 211.1553) in thirty, twenty eight and twenty three out of forty samples respectively and there are no significant differences between control and challenged samples. However a metabolite at m/z 211.0463 is present in twenty six out of forty samples and is significantly up regulated in control samples[62].

Ethylene and nitric oxide are metabolites with an m/z less than 50 and therefore not included in the list of *P. purpurea* root metabolites.

Although these are provisory results, it appears as a general picture that challenged samples are not responding differently from control samples, which means that metabolites are being produced constitutively.

As far as we know there are no studies, up to date, on the metabolites of *Phlomis* spp. challenged with abiotic or abiotic stressors. The huge amount of data gathered during this work should be thoroughly exploited in the near future. Although most databases are commercial, there are some public databases that can complement each other and could be used in an attempt to assign the m/z to compounds:

Metlin https://xcmsonline.scripps.edu/; Chemspider http://www.chemspider.com/; Plant metabolomic pathway http://pmn.plantcyc.org/

The appendices can be found at **https://meocloud.pt/link/bebb019f-0fbd-45f6-99c7-fa469862379b/Appendices_Chapter%208.docx/** (see CD in attachment).

[60] Appendix V.XIII.
[61] Appendix V.XIV.
[62] Appendix V.XV

8.4. Acknowledgements

We would like to acknowledge Prof. Dr. Ana Cristina Figueiredo from "Faculdade de Ciências" – University of Lisbon for the private practical lesson on hairy roots and all suggestions trial after trial. We also would like to thank Prof. Dr. Jorge Vivanco from Department of Agriculture, Center for rhizosphere biology, Colorado State University for providing *A. rhizogenes* Biovar. Thanks must be given to Prof. Dr. Pedro Fevereiro for providing strains of A. rhizogenes (LBA 9402 and A4-70GUS). Related to this issue, we are thankful to the colleagues Prof. Natália Marques and Sandra Formiga, Algarve University, for trying our *A. rhizogenes* LBA 9402 on *Nicotiana benthamiana*.

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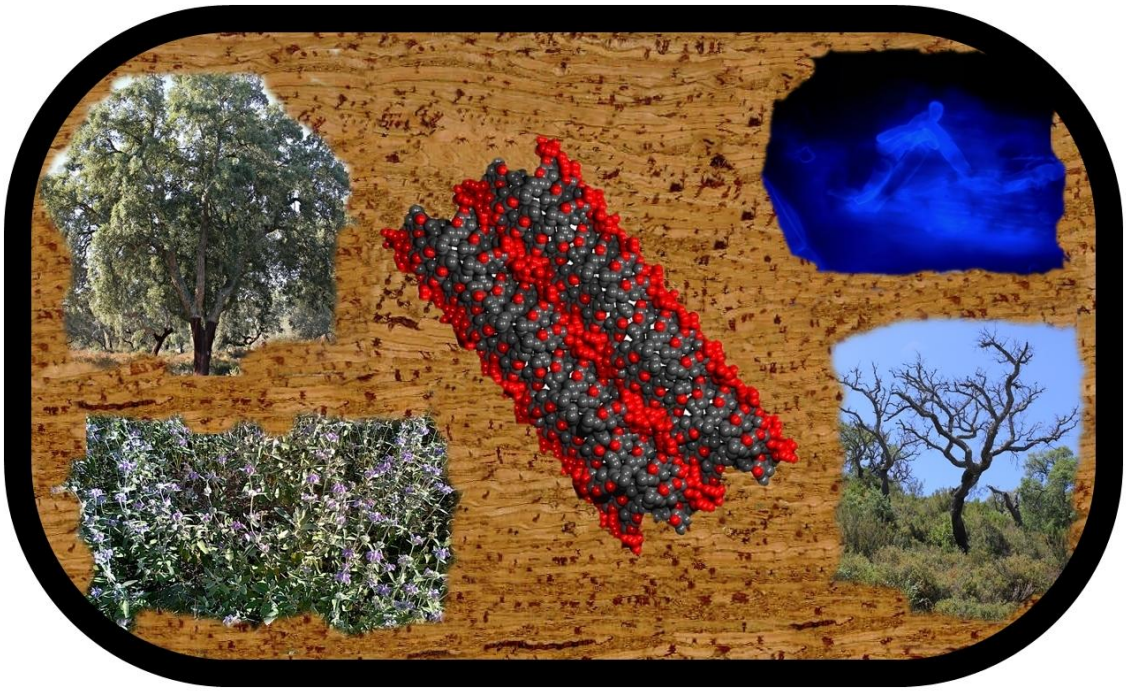
Chapter 9

General conclusions and future perspectives



9.1. General conclusions and future perspectives

Plants contain an enormous potential as source of useful molecules, many of which have been used for millennia. Nowadays, science confirms the importance of the traditional use of many plants and discovers new molecules and bioactivities. These molecules can be extracted or exuded from plants and are known as secondary metabolites.

Phlomis purpurea is a medicinal plant, whose leaves are used in traditional medicine (Novais et al. 2004). The potential of their roots was discovered when screening plant extracts for anti-*Phytophthora cinnamomi* activity (Neves 2007).

In this study, greenhouse (Chapters 2) and field trials (Chapter 3) confirmed that *P. purpurea* is not a host for *P. cinnamomi*, since the pathogen was never isolated from infested roots or naturally infested soil. *P. purpurea* reduces the percentage of host roots infected by the pathogen when planted together and consequently improves the health status of the susceptible hosts. These findings support the hypothesis that this plant exudes metabolite(s) that reduce soil inoculum potential. Moreover, our findings suggest that the age and the amount of *P. purpurea* roots influence the outcome, i.e. *P. purpurea* with a better developed root system has a better performance. This knowledge could be used to help the successful establishment of new cork oak plantations from seeds, in naturally infested fields, using an autochthonous herb and avoiding the use of soil fumigants.

Phlomis purpurea root extract (PRE) was also shown to significantly protect *Q. suber* and *Q. ilex* subsp. *rotundifolia* seedlings from infection by *P. cinnamomi* zoospores, possibly by having a direct effect on zoospores (Chapter 4). It would be interesting to see whether zoospores are lysed or immobilized by PRE and by what mechanism. PRE also seems to elicit a defence response in *Q. suber*, when in contact with the roots 24 h prior infestation. What are the mechanisms underlying a defence response eliciated by PRE is another question that needs to be elucidated.

An important finding arising from the histological studies (Chapter 5), was the observation that *P. purpurea* roots present a constitutive reinforcement of the exodermis preventing *P. cinnamomi* of penetrating them, and confirming that this plant species is a type I non-host. It would be interesting to determine the exact composition of the exodermis that cannot be degraded by the enzyme arsenal of *P. cinnamomi*.

This work was a contribution to the isolation and molecular characterization of a new metabolite, phlomispurpentaolone, produced by *P. purpurea* roots, with anti- *P. cinnamomi* mycelial growth activity (Chapter 7). Its activity against all the other life cycle structures of the

pathogen needs to be further assessed. Zoospores are the main infective structures and also the more vulnerable in the *P. cinnamomi* life cycle; it is expected that phlomispurpentaolone, like PRE, is able to eliminate them at small concentrations, and probably, also all the propagules, but this remains to be demonstrated. If there were not the case, other active principles need to be found and isolated from PRE.

Triterpenoids are chemically diverse and known to have many functions in plants and in human health and nutrition. Several triterpenoids are used as phytopharmaceutical agents including anticancer (Bristol-Myers Squibb Company 2011). The potential of phlomispurpentaolone, a novel nortriterpenoid, has to be thoroughly explored by determining a diverse array of activities from antimicrobial to anti-tumoral.

The transformation of *Phlomis purpurea* with *Agrobacterium rhizogenes* was not achieved (Chapter 8). New methodologies have to be implemented, and when successful, the yield of phlomispurpentaolone production determined and compared with the one obtained from wild roots.

From the *ca* 8000 metabolites detected by UPLC-MS in root extracts (Chapter 8), a subset is probably related to defence responses. Exudates at 72 hpi were also analysed by GC-MS and will be explored. Phlomispurpentaol is present constitutively in the extracts. This huge amount of data is a potential source of information and a big challenge to identify known and unveil new metabolites and determine their activities.

The integration of these results with the ones being acquired from transcriptomics will enable a deeper knowledge about the resistance mechanisms involved in the *P. purpurea* - *P. cinnamomi* interaction.

9.2. References

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Appendices

Appendix I - Cited in Chapter 2

The appendices can be found at **https://meocloud.pt/link/047230bb-31f7-43e0-8a04-5d7c3a20a6ae/Appendices_Chapter%202.docx/** (see CD in attachment).

Appendix I I- Cited in Chapter 3

The appendices can be found at **https://meocloud.pt/link/a1bcf83d-1669-4410-b338-b1c890ac3774/Appendices_Chapter%203.docx/** (see CD in attachment).

Appendix III - Cited in Chapter 4

The appendices can be found at **https://meocloud.pt/link/75be6d45-169f-4fac-8af6-13cba3729b73/Appendices_Chapter%204.docx/** (see CD in attachment).

Appendix IV - Cited in Chapter 7

The appendices can be found at **https://meocloud.pt/link/12101580-ac84-4f75-bcd6-6fad66fb9d80/Appendices_Chapter%207.docx/** (see CD in attachment).

Appendix V - Cited in Chapter 8

The appendices can be found at **https://meocloud.pt/link/bebb019f-0fbd-45f6-99c7-fa469862379b/Appendices_Chapter%208.docx/** (see CD in attachment).

Appendix VI

Table of figures

Appendix VII

Index of tables